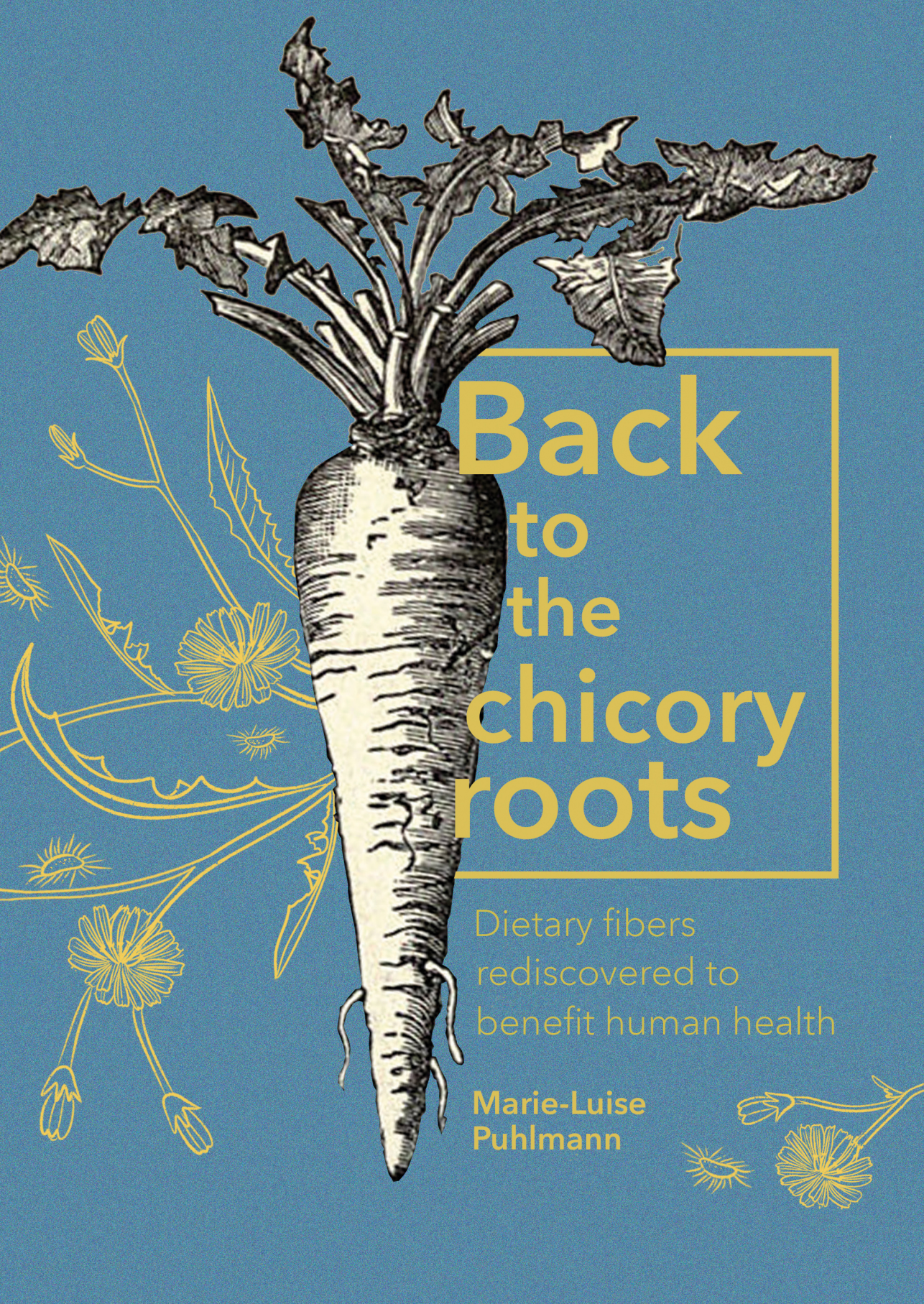

Back to the chicory roots

Dietary fibers rediscovered to benefit human health

Marie-Luise Puhlmann

Propositions

1. Distal colonic conversion of dietary fiber is a main driver of human health.
   (this thesis)

2. Neglecting the plant cell matrix ignores the essence of dietary fiber.
   (this thesis)

3. Technological advances have facilitated a rise in the quantity of research output and a fall in its quality.

4. The most misused discipline in science is statistics.

5. As a scientist, following trends in science is necessary, but only a unique style makes one memorable.

6. Researching the biological female body is key to addressing current unexplained interindividual variations.

7. To reverse the climate and metabolic health crisis, everyone must shift their mindset and change their dietary choices.

8. The food industry holds a central place in human health.

Propositions belonging to the thesis, entitled

Back to the Chicory Roots: Dietary Fibers Rediscovered to Benefit Human Health

Marie Luise Puhlmann
Wageningen, 9 October 2024

BACK TO THE CHICORY ROOTS: DIETARY FIBERS REDISCOVERED TO BENEFIT HUMAN HEALTH

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Back to the Chicory Roots: Dietary Fibers Rediscovered to Benefit Human Health

Marie-Luise Puhlmann

Thesis

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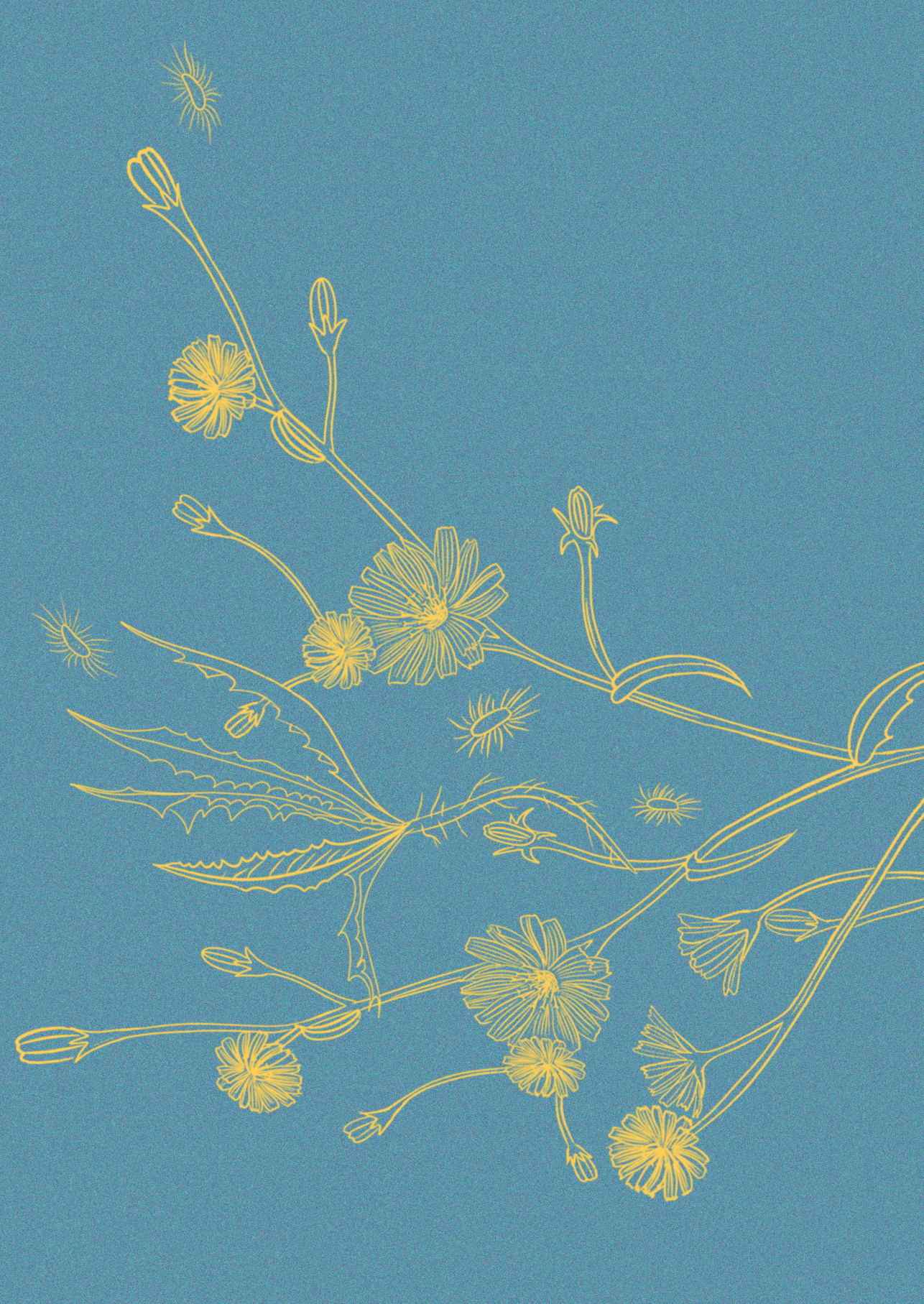

CHAPTER 1

General introduction

"But a definition and greater understanding of fibre was important for the book he and Trowell were to write because Burkitt realised that this was a story that went beyond bran to something that had powerful effects in the gut and more generally on metabolism and was removed or destroyed by the processing of modern foods."

Cummings, J., & Engineer, A. (2017). Denis Burkitt and the origins of the dietary fibre hypothesis. *Nutrition Research Reviews*, *31*, 1–15.

'BALLASTSTOFFE', DIETARY FIBER AND HUMAN HEALTH - A HISTORICAL PERSPECTIVE

The English term "dietary fiber" describes the well-known but hard-to-define fibrous components of our foodstuff. Most European languages have adopted specialized terms similar to English, such as "fibre alimentaire" in French, "voedingsvezels" in Dutch, and "kostfiber" in Swedish. However, in German, dietary fiber are called "Ballaststoffe". "Ballaststoffe" comes from the word "Ballast," which implies a degree of worthlessness or burden and, hence, could be translated into "burdensome, worthless compounds". This term is said to have been coined by the German physician Max Rubner, a nutrition pioneer of his time, living between 1854-1932. At this time, the digestibility of foodstuffs was extensively studied, though primarily from an economic perspective. The aim was to understand how much of the agricultural products used could be converted into food and how many nutrients and energy could be extracted by the human body (Rubner, 1883). Notably, in bread production, there was significant economic interest in the usefulness of bran, which was at that time discarded as animal feed. This practice resulted in economic losses, sparking a large interest in studying the impact of different milling techniques (resulting in various particle sizes) and the re-addition of bran to refined flour. At the time, bran and other agricultural products were thought to have very little to no nutritional benefit and were seen as "useless to the stomach," as they were simply considered as extra weight consumed and excreted. This dual perspective, both economic and nutritional, formed the basis of the term "Ballaststoffe" (Rubner, 1883).

DIETARY FIBERS AT THE BEGINNING OF THE 20TH CENTURY: 'USELESS COMPOUNDS'

Max Rubner performed fascinating, ethically debatable, experiments with various foodstuffs, among which both animal- and plant-based foods. One experiment by Rubner included feeding a person only peas in high quantities (one kilogram or 650 g per day) with a liter of beer and milk for four days. The research subject subsequently complained about the amount of gas experienced inside the gut, which Rubner, with fascination, marked as being surprising as the excreted peas did not contain bubbles (Rubner, 1880). Using such small human trials, he laid an important foundation for our current knowledge on digestion and conversion of foodstuff in the human gut (Chambers, 1952). At the time, feces were extensively used to study nutrient absorption. His work and that of others involved detailed descriptions of fecal collection strategies, nitrogen content, pH measurements, and microscopic assessments of food remains (Robinson, 1922; Rubner, 1879, 1883, 1933). In this way, Rubner also studied how bran and different wheat particle sizes affected nutrient absorption, bowel function, and general well-being (Rubner, 1883), compiling his observations in his extensive work "*Ueber den Werth der Weizenkleie für die Ernährung des Menschen*" (in English: "*On the value of wheat bran for human nutrition*"). There, he noted that using increasingly coarser flour containing more bran led to softer feces of larger volume due to gas formation and a stronger smell of butyric acid. The latter, he mentioned, was a product

of fermentation from dextrose or lactic acid, and the fermentation process was driven by the type of food consumed (Rubner, 1883).

Already back then, Max Rubner elaborated on the remainder of the fibrous "plant food structures", how those must be made up of different chemical compounds, and that they were used by bacteria. It was known that bacteria were present in feces, and he postulated that their presence was potentially impacted by the type of plant food structures consumed in the diet (Rubner, 1933). However, these "plant food structures" were poorly understood back then. It was known that they were primarily composed of cellulose, and different types of celluloses, meaning other plant food constituents, were hypothesized to be present but unknown. The term "dietary fiber" was not coined yet in the English scientific language, and also Rubner's term "Ballaststoffe" was not yet commonly used. Instead, these plant food remainders were referred to as "cell membranes", "plant skeletons", and similar expressions. Moreover, their importance to human health beyond bowel function was not yet recognized. This lack of recognition was likely due to food processing being centered on achieving sufficient nutrient intake rather than considering these compounds, which were seen as "mere burdens to the gut", beyond their economic benefits. Considering that Max Rubner made all these observations over 140 years ago and based these insights on literature dating back to the 17th century, his work is impressive in the light of present-day knowledge.

SHIFT IN THE PERSPECTIVE ON DIETARY FIBERS IN THE MID-20TH CENTURY: ESSENTIAL COMPOUNDS FOR HUMAN HEALTH

The view on dietary fibers slowly changed halfway through the 20th century with the work of several physicians, of which the most known is Denis Burkitt, a surgeon living from 1911-1993 and later known as "the fiber man" (Cummings & Engineer, 2017). Through exchange with his colleagues, Burkitt noticed a striking connection between the occurrence of non-communicable diseases—including colon cancer, diverticulitis, constipation, and diabetes—and their geographic location. In high-income countries, these diseases occurred far more frequently than in low-income countries. Moreover, within high-income countries, they were most prevalent in economically developed regions (Cummings & Engineer, 2017; O'Keefe, 2019). These economically more developed areas shared a common trait: a diet high in sugar but low in dietary fibers. Together with his colleagues, Burkitt defined these diseases as not being caused by the overconsumption of fats and sugar, as was popularly proposed at that time, but by the absence of fibers. This meant they were, in fact, diseases caused by fiber deficiencies (Cummings & Engineer, 2017; O'Keefe, 2019; Southgate, 1992).

However, at that time, meaning nearly 90 years after the extensive elaboration of Max Rubner on wheat bran, there was still limited understanding of what fibers were, no unified terminology existed, and these compounds were still linked to a connotation of "uselessness". It was thanks to Trowell that the term "dietary fiber" finally gained ground in 1972. He defined dietary fibers as "*The skeletal remains of plant cells that are resistant to digestion by enzymes of man* […]." (Trowell, 1972). The idea that insufficient

intake of these “skeletal remains of plant cells” or “cellular skeletons of plant material” (Cummings, 2001) was at the root cause of many human diseases and essential to human health slowly transformed dietary fiber research.

BLOOMING DIETARY FIBER RESEARCH IN THE LATE 20TH CENTURY: DIETARY FIBERS, HUMAN METABOLISM, AND THE GUT MICROBIOTA

The hypothesis developed by Burkitt and Trowell's fiber definition were successfully taken up by the next generation of fiber researchers. During the 1960s to 1980's physicians and gastroenterologists such as Heaton (1936-2013), Cummings, and O'Keefe continued the work of their predecessors. Through extensive studies involving human subjects and *in vitro* assessments, dietary fibers were elevated from an “underestimated component" to a crucial element for human health. Relationships between transit time, fecal water, fecal weight, bacterial content, and dietary fibers from different plant sources and their relation to other diseases were investigated (Brodribb & Groves, 1978; Cummings et al., 1978). Processing techniques were further explored, revealing the benefits of uncooked and coarse wheat bran on bowel function compared to more processed forms (Wyman et al., 1976). In addition to influencing bowel function and gastrointestinal diseases, dietary fibers became recognized for their impact on cholesterol and lipid metabolism (Stasse-Wolthuis, Hautvast, et al., 1979; Stasse-Wolthuis, Katan, et al., 1979). Analytical methods to measure fibers (soluble versus insoluble) were established, along with the identification of its various components and their conversion in the human gut. Finally, human intervention trials repeatedly acknowledged considerable differences in the response to dietary fibers among subjects (Cummings, 2001; Holloway et al., 1978, 1980).

With the observation that dietary fiber can be converted to a certain extent in the human gut, extensive research began to assess the gut bacterial breakdown of plant cell wall material from bran and other food components (Dekker & Palmer, 1981; Schel et al., 1980; Stevens et al., 1988). This research also revealed variations in bacterial capacity to break down fibers. Attributed notably to Cummings' work, it was understood that gut bacteria produce short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, as fermentation end products from dietary fiber breakdown. These SCFAs were found to be absorbed from the colon into the systemic circulation, pointing towards potential modes of action through which fibers impacted human health beyond local colonic effects (Cummings, 1984; Cummings et al., 1987). Collectively, these findings paved the way for further investigations into how gut bacteria metabolized s and its broader implications for human health.

MODERN FIBER RESEARCH IN THE EARLY 21ST CENTURY: EMERGING MOLECULAR INSIGHTS WITH A REDUCTIONIST VIEW

Since the 1990s, the field of dietary fiber research and its impact on the human gut and overall health has undergone significant changes. Improved analytical techniques have facilitated a deeper understanding of the types of dietary fibers and their molecular

composition. Additionally, with the advent of molecular biological methods, including next-generation sequencing techniques, detailed investigations of the gut bacteria involved in fiber breakdown have been made possible. Finally, the adoption of new data analysis software, personal computers, and the Internet has substantially increased research output and expanded the volume of available literature on the effects of dietary fibers on the human body through the gut microbiota. Our increasing molecular insights have shifted our understanding of dietary fibers from being viewed as plant cell material to specific molecular structures—an increasingly reductionist view. In Figure 1, I have summarized the historical development of fiber research.

Leveraging over 100 years of research and the recent surge in analytical possibilities, the compelling question remains: How far has fiber research advanced by 2024?

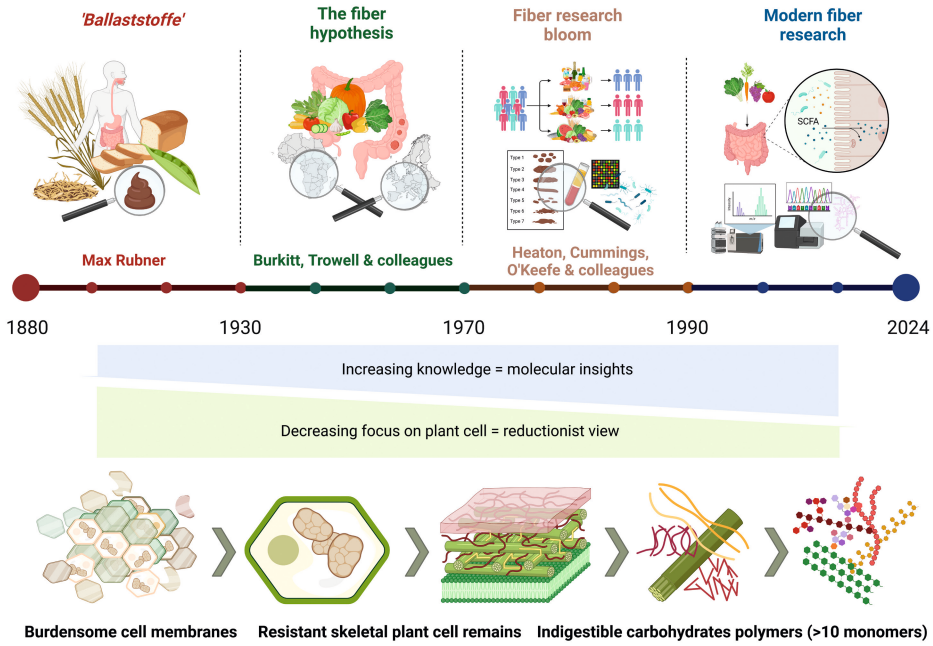

Figure 1. Fiber research of the last 150 years. Since the work of Max Rubner in the late 19th and early 20th centuries, dietary fibers have evolved from being considered "worthless, burdensome" compounds ("Ballaststoffe") to being recognized during the 20th century as "resistant skeletal plant remains" fundamental to human health. Following a surge of fiber research related to human health at the end of the 20th century, we have been able to use new technology to elucidate fibers' molecular composition and understand their effects on human health. Today, at the beginning of the 21st century, with increasing knowledge, our view on dietary fibers has become increasingly reductionist, focusing on the molecular makeup of these indigestible carbohydrate polymers and neglecting the three-dimensional structure of fibers in our foodstuffs. Created with Biorender.com

DIETARY FIBER RESEARCH IN 2024: "INDIGESTIBLE" DIETARY FIBERS AND THE GUT MICROBIOTA

Today, in 2024, we have a commonly accepted and more detailed definition of dietary fibers (Figure 1 and Figure 2). According to the Codex Alimentarius - a set of international food standards jointly published by the FAO and WHO – dietary fiber is defined as: *"carbohydrate polymers with ten or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans […]"* (Joint FAO/WHO Food Standards Programme, 2021). Additionally, today we have an accepted Atwater energy conversion factor for dietary fibers, amounting to 2 kcal/g of metabolizable energy for the human body (FAO, 2003). This means that dietary fibers are now accepted and recognized food compounds that are, today in the first place, defined by one property: "indigestibility". Consequently, dietary fibers are also often called non-digestible carbohydrates.

Based on increasing analytical insights, dietary fibers have been further classified using various systems. The most straightforward categorization includes fiber types based on food sources, commonly divided into cereal fibers and fruit/vegetable fibers. However, the distinction most commonly used among nutrition scientists is between "soluble" and "insoluble" fibers. This differentiation originates from the analytical processes used (whether fibers are water-extractable or not) in determining fiber content in foods, but it has been erroneously associated with the solubility of fibers in the gastrointestinal tract (McRorie & McKeown, 2017).

Other common categorizations refer to differentiation based on chemical composition. For instance, fibers have been divided into non-carbohydrate compounds, such as lignin (complex aromatic alcohol structures), and carbohydrate compounds with a sugar backbone, including cellulose, hemicellulose, pectins, gums, and resistant starches (Dhingra et al., 2012). Similar categorizations may also include differentiation based on polymer length, categorized as poly- or oligosaccharides, and by degree of polymerization (chain length), and linear or branched molecular organizations. These categorizations can further expand to include functional properties related to these chemical structures (physicochemical properties), such as solubility, viscosity, water-holding capacity, gel-forming ability, or bulking (McRorie & McKeown, 2017). As of 2024, our understanding of dietary fibers has primarily been shaped by analytical insights from the past 30 years, focusing on the characteristics of indigestibility in defining dietary fibers.

THE GUT MICROBIOTA – THE 'DIGESTER' OF DIETARY FIBERS IN THE HUMAN BODY

The key defining feature of fibers' indigestibility lies in the inability of human endogenous enzymes (referred to as Trowell's "enzymes of man") in the small intestine to break the chemical bonds between the monomers in dietary fibers (Trowell, 1972). However, bacteria inhabiting the human large intestine possess the enzymatic machinery necessary for fiber breakdown (Flint et al., 2008).

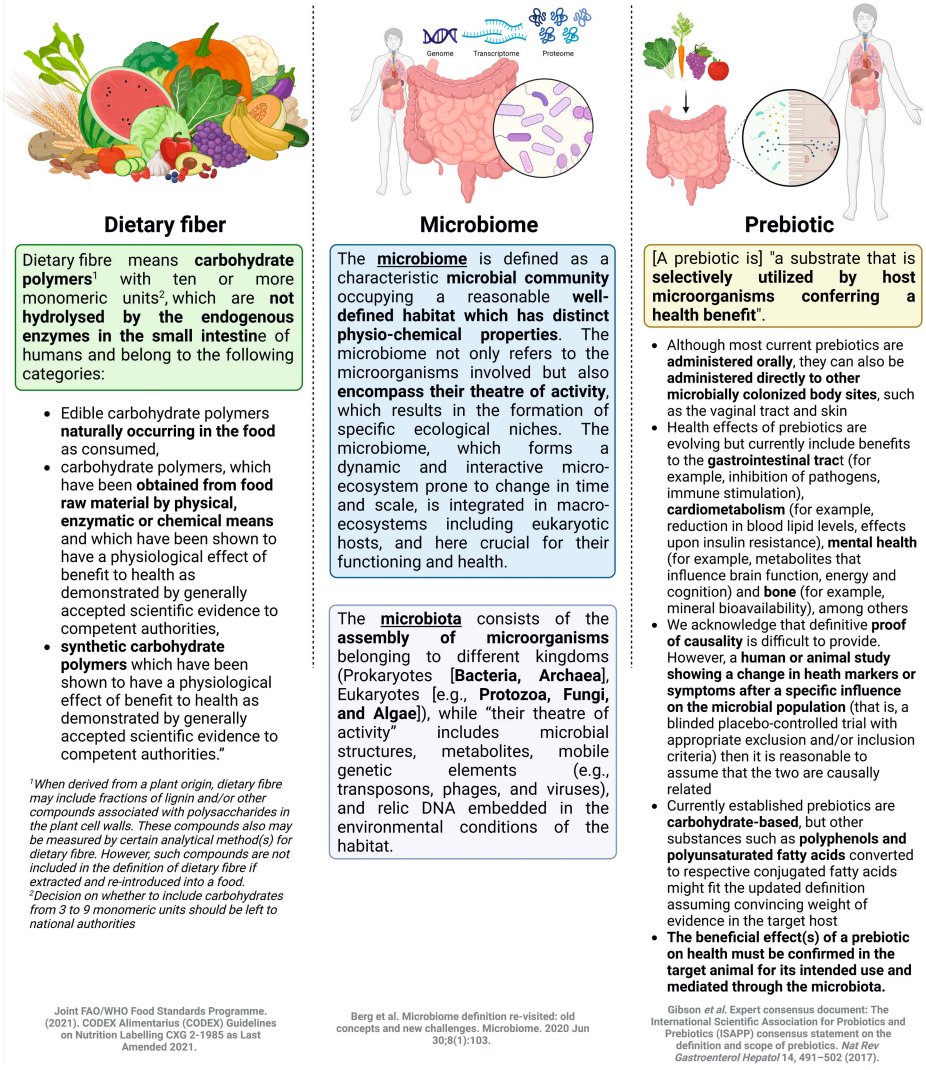

Figure 2. Important definitions in relation to dietary fiber research in 2024. Today, we have a widely accepted and implemented definition for dietary fibers, which is based on their indigestibility by human endogenous enzymes. Fiber can be broken down by primarily anaerobic gut bacteria, which along with other microorganisms, form the gut microbiota. The term gut microbiota is now often referred to as 'gut microbiome', although these terms encompass different ecological and biological aspects. The concept of a prebiotic refers to specific types of fibers defined by their effects on human health through the gut microbiota/microbiome. Created with Biorender.com

The presence of bacteria in feces and the large intestine has been well-known since before Rubner's time. Today, technological advancements have revealed that these microorganisms exist in large numbers and are not the sole inhabitants of the human gut; they coexist with viruses, fungi, archaea, and protozoa (Berg et al., 2020). This collective assembly of microorganisms is known as the "gut microbiota" (Figure

2). In recent years, the term "gut microbiome" has also gained prominence beyond microbiologists. Although the precise definition of "microbiome" was once a subject of scientific debate, a consensus has now been reached (Berg et al., 2020). The terms "microbiota" and "microbiome" are often used interchangeably but denote slightly different concepts. The "microbiome" refers not only to the assembly of microorganisms but to the "[…] *microbial community occupying a reasonable well-defined habitat* […]" and "[…] *encompass their theatre of activity, which results in the formation of specific ecological niches*."(Berg et al., 2020).

Inside the large intestine, the "theatre of activity" of mainly anaerobic bacteria enables the conversion of dietary fibers into structures usable by us, the host (Figure 3A). To achieve this, gut bacteria work together in an intricately orchestrated way. So-called primary degraders are bacteria that break down fibers into smaller molecular structures, which secondary degraders then utilize through a process known as cross-feeding (Flint et al., 2008; Flint, Scott, Louis, et al., 2012; Flint et al., 2017). At the same time, gut bacteria use dietary fibers as a substrate for growth and energy. The end product of fiber breakdown and metabolism by the gut bacteria are SCFAs, with acetate being the most abundant. Other intermediate metabolites, including organic acids such as lactate or succinate, are also produced along with gases like hydrogen or carbon dioxide (Flint, Scott, Duncan, et al., 2012; Flint, Scott, Louis, et al., 2012). Many gut bacteria can fulfill similar functional roles in the human gut, which is also referred to as functional redundancy (Louca et al., 2018; Reichardt et al., 2017). However, certain processes are reserved for a set of specialized bacteria. This includes the formation of the SCFAs propionate and butyrate (Louis & Flint, 2017) (Figure 3A). The SCFAs acetate, propionate, and butyrate are the most well-studied. These compounds can enter the bloodstream if not metabolized by the intestinal cells or liver (Figure 3B), as already elucidated by the work of Cummings and colleagues (Cummings et al., 1987; McNeil et al., 1978).

The latest advancements in DNA sequencing and other analytical technologies have allowed us to uncover that the composition of gut microbiota in each person is as unique as their fingerprint (Figure 3C) as first reported by Zoetendal et al. (Zoetendal et al., 1998) and confirmed later by others (Huttenhower et al., 2012). This unique composition remains relatively stable over time, but can be significantly altered by dietary factors such as fibers (David et al., 2014; Johnson et al., 2019; Rajilić-Stojanović et al., 2013). Variations in gut bacteria and their functions suggest that different individuals may respond differently to dietary fibers. By studying the impact of individual gut microbiota "signatures", we can gain insights into why different individuals have varied responses to dietary fibers, as noted by Cummings and his colleagues in the 1970s and 1980s (Cummings, 2001; Holloway et al., 1978, 1980). Overall, it's now recognized that "[the gut] *microbiome, which forms a dynamic and interactive micro-ecosystem prone to change in time and scale, is integrated in macro-ecosystems including eukaryotic hosts, and here crucial for their functioning and health*" (Berg et al., 2020).

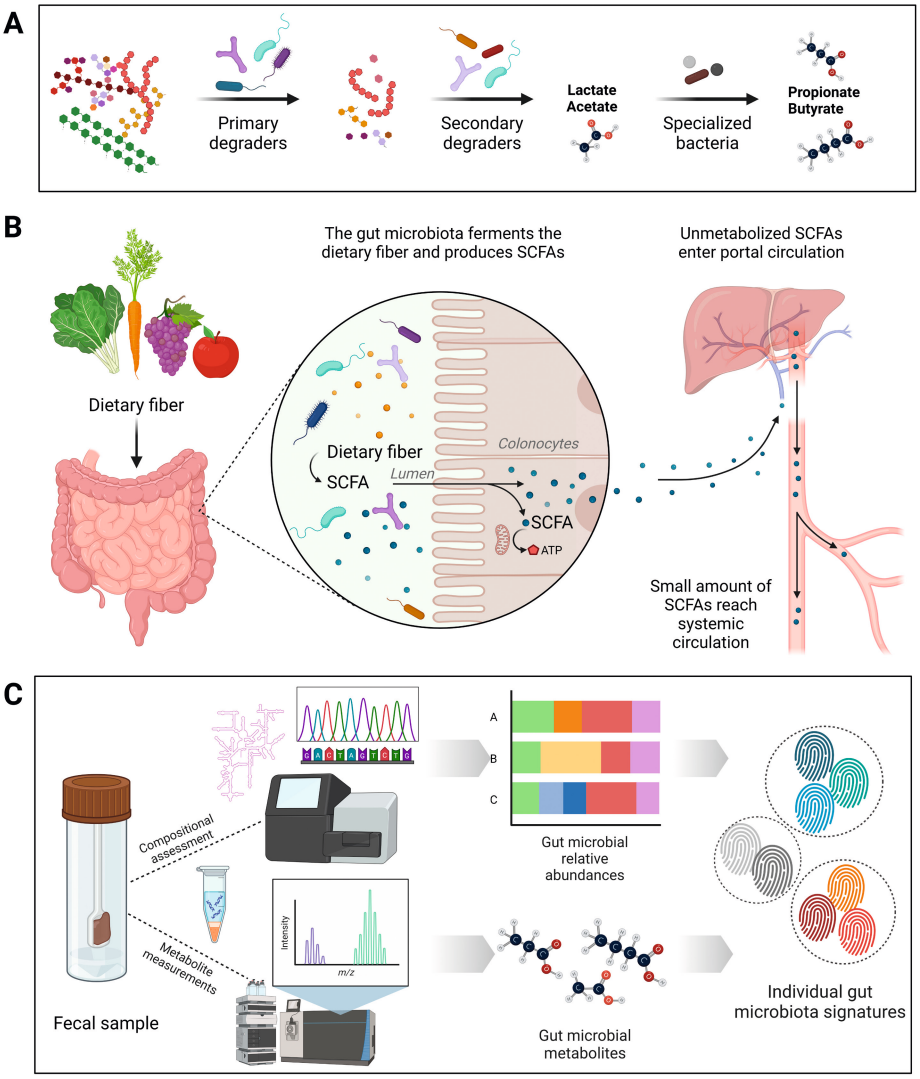

Figure 3. "Digestion" of dietary fibers by the human gut microbiota and uptake of fiber fermentation products, and the assessment of underlying mechanisms. (A) Dietary fibers are broken down by primary and secondary degraders into intermediate organic acids and short-chain fatty acids (SCFAs), with acetate being the most abundant. Through cross-feeding, specialized bacteria can further convert these fermentation products into the less common SCFAs, propionate, and butyrate. (B) If not eliminated by the liver, SCFAs can enter the bloodstream and reach the systemic circulation, which explains part of the dietary fibers' positive effect on human health. (C) With modern DNA sequencing and other analytical technologies, we have been able to establish that gut microbiota composition and function are highly personal, potentially explaining inter-individual differences observed in human dietary fiber intervention trials. Created with Biorender.com

THE GUT MICROBIOTA AND PREBIOTIC FIBERS – A MODULATOR OF HUMAN HEALTH

Not all dietary fibers can be "digested" by the human gut microbiota, nor have all dietary fibers been linked to specific health outcomes through a modulatory effect on the gut microbiota. Research focusing on fructose-based dietary fibers, such as fructooligosaccharides and inulin-type fructans, as well as galactose-based polymers, has revealed that certain fibers can selectively stimulate the growth of specific gut bacteria (Gibson et al., 2017). For instance, inulin-type fructans have been observed to notably increase bifidobacteria levels, known as the bifidogenic effect, which has been associated with beneficial effects on human health. Fibers that serve as "*a substrate selectively utilized by host microorganisms conferring a health benefit*" (Gibson et al., 2017) are designated as "prebiotics" (Figure 2). However, it is important to note that the bifidogenic effect alone does not suffice to claim a prebiotic effect, although it is still often erroneously assumed. The designation of a substance as a prebiotic requires demonstrating its beneficial effect on the host (human or animal), meaning on a health marker of symptom (Figure 2). In human studies, such effects must be validated using rigorous study designs, such as randomized placebo-controlled trials, to establish causal relationships between a new fiber product, its selective utilization by gut bacteria, and its beneficial impact on human health.

WHY IS FIBER RESEARCH STILL RELEVANT FOR HUMAN HEALTH IN 2024?

Given the advancements in dietary fiber research and the insights into its structure and impact on human health facilitated by gut microbiota, the relevance of further investigating dietary fibers in 2024 may come under scrutiny. Despite its omnipresence in foodstuffs, consuming sufficient amounts of dietary fibers has become increasingly challenging. With the development of food manufacturing practices, the production and consumption of refined food products, which provide little fibers in our Western diets, have continuously been on the rise (Stephen et al., 2017). The average fiber intake in the Dutch population is approximately 20 g per day (van Rossum et al., 2011), falling short of the recommended daily intake of 40 g for men and 30 g for women (Health Council of the Netherlands, 2006). This "fiber gap" (Jones, 2014) of 10 to 20 g parallels an unprecedented increase in overweight, obesity, and metabolic diseases, which is expected to escalate further. Essentially, little seems to have changed since Burkitt and colleagues established, over 50 years ago, that many non-communicable diseases stem from fiber deficiencies (Burkitt, 1971; Cummings & Engineer, 2017; O'Keefe, 2019).

A pivotal study demonstrating the ongoing relevance of fiber deficiency in modern society was conducted by O'Keefe in 2015 (O'Keefe et al., 2015). In a two-week experiment, African-Americans and rural South Africans swapped diets to assess the short-term impact on the gut microbiome and cancer risk biomarkers. Despite the brief duration of the experiment, adoption of the African-American diet, rich in refined food products and low in fibers, led to significant mucosal changes indicative of increased cancer risk and detrimental bacterial metabolites.

Recent evidence consistently shows that a high dietary fiber intake can reduce, among others, all-cause mortality, risk of type 2 diabetes (T2D), and colorectal cancer. Intakes exceeding 25 g per day have been associated with effective risk reduction (Reynolds et al., 2019). Interestingly, it has been suggested that Burkitt believed a daily fiber intake of 50 g is necessary to reduce the risk of colon cancer (O'Keefe, 2019). In the diet swap study, rural Africans consumed more than 60 g of fibers per day, while African-Americans, adopting the rural African diet, achieved a fiber intake of 50 g per day. In contrast, the fiber intake on the African-American diet was five times lower, highlighting the importance of dietary fibers in our diets (O'Keefe et al., 2015). Considering the ongoing trend of Westernization, where economically less developed regions are adopting diets high in refined foods, and with rapid technological advancements, the relevance of dietary fiber research in 2024 has never been more urgent.

MOVING FORWARD IN THE 21ST CENTURY: BACK TO THE ORIGINAL DIETARY FIBER DEFINITION

Dietary fibers are clearly no longer seen as burdensome, worthless food compounds. At the same time, our perspective on dietary fiber has fundamentally shifted away from viewing them as "skeletal remains of plants" to recognizing them as intricate molecular structures. The concept of dietary fibers as plant cell remnants prevailed until the 1990s (Cummings, 2001; Heaton, 1994; Selvendran, 1984; Southgate, 1992). However, over the past 30 years, our increasingly reductionist approach has shifted focus toward the specific molecular composition of individual fibers and their interactions with gut bacterial species (Cantu-Jungles et al., 2021; Cantu-Jungles & Hamaker, 2020), rather than viewing fibers within the context of whole foods. Trowell, who officially introduced the term "dietary fiber", already distinguished between dietary fibers found in whole plant foods and those derived from them (Trowell et al., 1978). This PhD thesis will revisit this distinction and explore why it remains relevant in the current context.

To start, I want to introduce a perspective on dietary fibers from another scientific discipline. Unlike nutrition and food sciences, plant scientists classify plant polysaccharides into structural and non-structural carbohydrates (Hartmann & Trumbore, 2016). This classification is not based on chemical composition but rather on the spatial, three-dimensional organization of polysaccharides.

The generally accepted model of the structural makeup of plant cells is illustrated in Figure 4 (Albersheim et al., 2010; Alberts et al., 2002). Structural carbohydrates make up the plant cell wall and include cellulose, hemicelluloses, and pectins. Cellulose serves as the foundational scaffold, reinforced by hemicelluloses, and this network is filled with pectins. Pectins also fill the space between adjacent plant cells, the middle lamella (Daher & Braybrook, 2015). Non-structural carbohydrates, such as inulin and starch—polymers of fructose and glucose, respectively—are stored within the plant cell vacuole. It was these intricate three-dimensional structures that early researchers like Rubner, Trowell, and Cummings, in their early work, described in fecal material as "plant cell membranes" and "skeletal remains of plants".

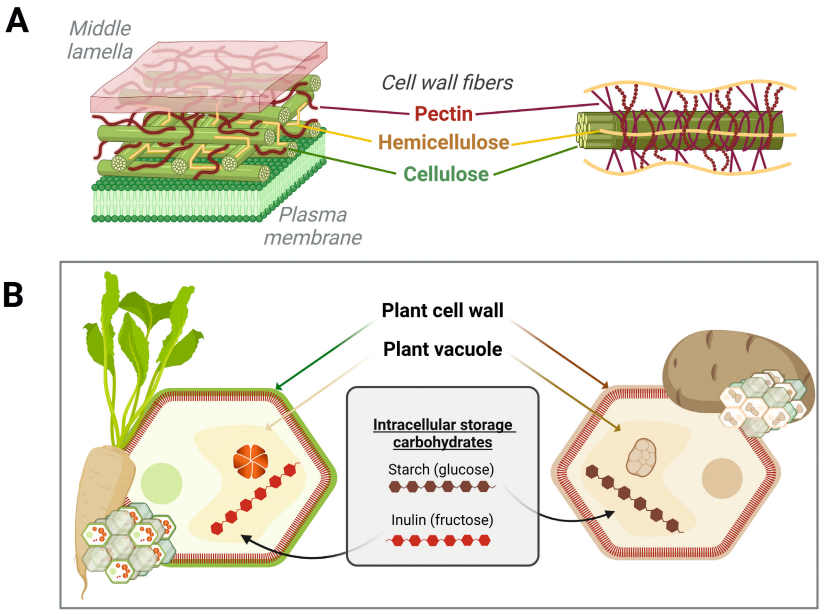

Figure 4. (A) Proposed model of the plant cell wall. The plant cell wall consists of the plasma membrane facing the inside of the plant cell. It is surrounded by a network of structural carbohydrates. Cellulose forms the basic scaffold of the plant cell wall and is reinforced by hemicelluloses. This network is filled with pectins, which "cement" both the hemi-/cellulose network and fill the spaces between adjacent plant cells (intercellular space), forming the middle lamella. **(B)** Within the plant cells, other non-structural carbohydrates are stored inside the plant vacuole. These are most commonly glucose polymers (starch) or fructose polymers (e.g., inulin). Created with Biorender.com

Recently, the nutrition community has once again embraced the three-dimensional concept of dietary fibers. A consensus paper of the International Carbohydrate Quality Consortium (ICQC) from 2020 states that "*[in] nutrition, a distinction needs to be made between intrinsic sources of dietary fibre and purified or chemically/physically modified forms of fibre, given that the three-dimensional (3D) matrix of the plant cell wall confers benefits above fibre isolates.*" (Augustin et al., 2020). To achieve this, a new terminology was introduced, which describes dietary fibers that are "[…] *intrinsically part of the cell wall material in edible plants such as fruits, vegetables, cereals, nuts, pulses, and even seaweed in some diets (from now on defined as* ***intrinsic fiber****)*". Differently put, 'intrinsic fibers' are dietary fibers found in the original plant cell matrix of whole plant foods and are not the same as isolated and purified single fibers extracted from the plant cell matrix. Food processing, whether domestic or industrial, can disrupt the plant cell matrix and reduce the amount of intrinsic fibers in the diet (Augustin et al., 2020). Throughout this PhD thesis, I will therefore use the term "plant foods" instead of "plant-based foods" when referring to the plant cell matrix in foods containing intrinsic

fibers, to avoid implying varying degrees of food processing that may not accurately convey my intended meaning.

The plant cell wall has an important impact on how food is digested, as it shields intracellular storage carbohydrates, proteins (e.g., in legumes), and lipids (e.g., in nuts) from human and bacterial enzymes. This was elegantly exemplified by Ellis and colleagues 20 years ago. They showed how lipids inside the plant cells of almonds can withstand digestion and that bacteria can open up the plant cell walls as found in excreted almond remnants in feces (Ellis et al., 2004). It is without doubt that the structural integrity of dietary fibers, and hence, the physical barrier imposed by intrinsic fibers, fundamentally affects how macronutrients provide or do not provide energy to the human body (Augustin et al., 2020). Hence, in 2024, Trowell's "*skeletal remains of plant cells*" are back on the table of nutrition research in their new terminological guise of "intrinsic fibers".

THE WORKING HYPOTHESIS: INTRINSIC FIBERS AND DISTAL FERMENTATION

Intrinsic fibers not only affect the extraction of energy from plant foods but also influence how gut microbiota access fiber as a substrate for energy, growth, and the production of SCFAs. Fiber fermentation, known as saccharolytic fermentation, is believed to primarily occur in the proximal colon (Korpela, 2018), which includes the ascending and transverse colon (Figure 5). Consequently, fermentation in the distal colon (descending and sigmoid-rectal colon) would shift towards the utilization of remaining proteins (proteolytic fermentation), resulting in an increase in intraluminal pH (Korpela, 2018). However, our understanding of bacterial fiber fermentation processes mostly stems from studies on single, isolated fibers. For example, inulin is known to be rapidly fermentable due to its simple linear structure as a fructose polymer (So et al., 2021). However, in minimally processed plant foods, inulin is stored inside plant cells and is therefore not readily available to gut microbiota.

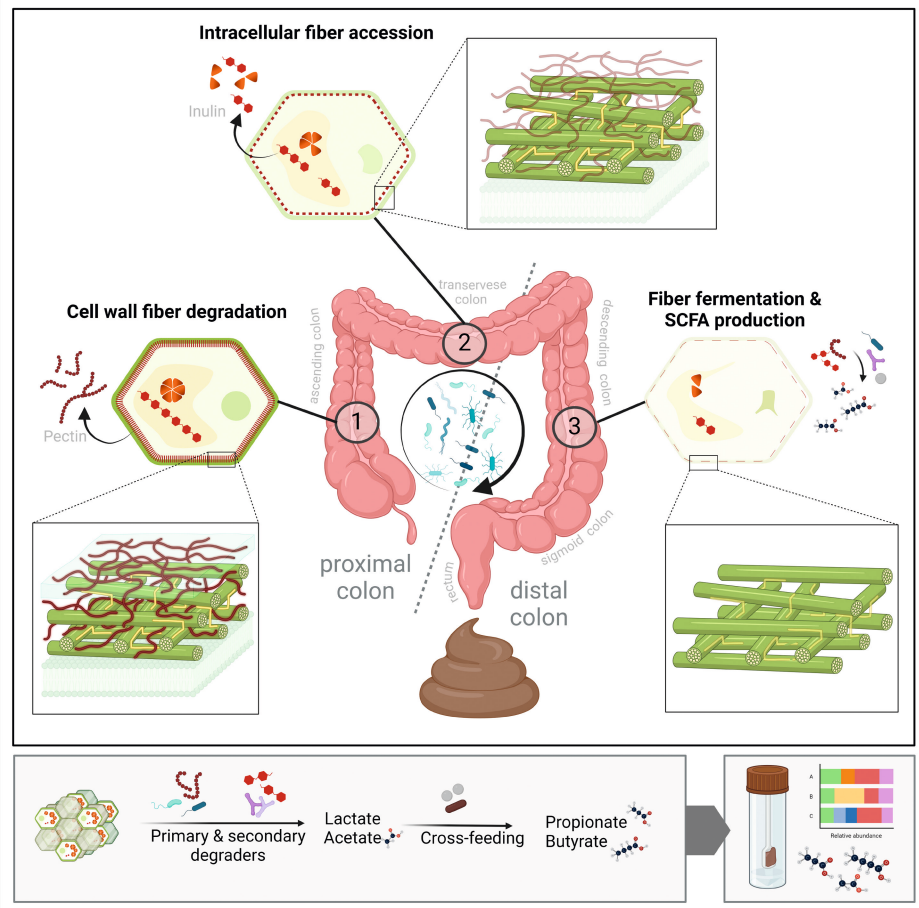

Figure 5. The potential impact of "intrinsic" dietary fibers on fiber fermentation by the human gut microbiota. The complex plant cell wall fibers create a physical barrier that slows down the degradation of the cell wall and the release of fibers, including intracellular fibers. Consequently, fiber release and fermentation occur gradually and extend from the proximal to the distal colon. As a result, one can expect considerable microbial activity in the distal colon, which leads to the distal production of SCFAs, notably those relying on cross-feeding, which are propionate and butyrate. In this thesis, I will study these processes by using fecal material to assess gut microbiota composition and metabolite production. Created with Biorender.com

Naturally, gut bacteria must first break down plant cell walls before accessing intracellularly stored inulin. Since the breakdown of cell walls containing intrinsic fibers has rarely been studied, the exact process remains unclear. Drawing from plant science, it is known that pathogens attacking plant tissues first degrade pectin, which serves as the "filler" of the hemi-/cellulose plant network (Alberts et al., 2002). Gut bacteria may use similar mechanisms. Pectin, being one of the most complex structural carbohydrates (Mohnen et al., 2008), requires gut bacteria to possess a variety of enzymes to break down this dietary fiber. This likely slows the release and fermentation of fiber from the plant

cell network. Consequently, dietary fiber fermentation may extend from the proximal to the distal colon, still producing substantial amounts of SCFAs in the descending and sigmoid colon. Moreover, higher distal microbial activity might favor the distal production of propionate and butyrate via cross-feeding (Figure 5). As more distal SCFA uptake has been linked to metabolic improvements, such distal fermentation appears beneficial (Canfora et al., 2017; van der Beek et al., 2016). Already Rubner noted that adding bran to bread changed the feces of his study subjects, resulting in softer stools with a stronger butyric acid smell, potentially indicating more distal microbial activity (Rubner, 1883).

This thesis will explore this potential shift in fermentation location within the colon resulting from the consumption of intrinsic fibers as a working hypothesis. I will use *in vitro* and *in vivo* methods, focusing on fecal microbiota to assess changes in microbial composition and metabolite production.

INTRINSIC DIETARY FIBERS AND HUMAN GUT AND METABOLIC HEALTH

This thesis will explore intrinsic dietary fibers in relation to two specific aspects of human health: human gut health, focusing on bowel function and gut microbiota, and human metabolic health, focusing on glucose homeostasis and insulin resistance.

One of the most well-known and immediately experienced effects of fibers on human health is its impact on bowel function. Throughout the 20th century, bran has been extensively studied as a modulator of transit time, fecal mass, water content, stool consistency, and frequency. The widely accepted mode of action of bran and other fibers at that time was their supposed capacity to hold water (Adiotomre et al., 1990; Cummings, 2001). According to this view, fibers would need to resist breakdown by the human gut microbiota in the colon because if their physical structure is broken down, they also lose their ability to hold water. Consequently, additional mechanisms beyond water-holding capacity must contribute to fibers' modulatory effect on bowel function, as evidenced by the well-established benefits of fiber intake on human bowel habit (Van Der Schoot et al., 2022). Dietary fibers play a fundamental role in shaping the intraluminal environment in which the gut microbiota resides by serving as substrates for energy and growth, and through the fermentation products produced (Daniel, 2022; Flint et al., 2017). As early as 1997, Heaton and Lewis demonstrated that accelerating or slowing gut transit time using wheat bran, senna, or loperamide impacted fecal butyrate levels, indicative of altered gut microbial function (Lewis & Heaton, 1997b).

This thesis will primarily focus on the effect of intrinsic fibers on bowel function in healthy adults and in individuals experiencing bowel function impairments such as hard stools and/or low stool frequency. To assess bowel function, self-reported outcomes, including the Bristol Stool Form Scale developed by Heaton and Lewis (Lewis & Heaton, 1997a) and other questionnaires aimed at evaluating gastrointestinal symptoms and their impact on daily life will be employed. Our aim is to advance the understanding of intrinsic dietary fibers to enhance bowel function through modulation of the gut microbiota.

An effect of dietary fibers that is less commonly known and not immediately experienced is its impact on metabolic health. Burkitt, 50 years ago, first proposed the link between fibers and metabolic health by suggesting that diabetes is one of the

fiber-deficiency diseases (Burkitt, 1971; Cummings & Engineer, 2017; O'Keefe, 2019), a notion supported by recent literature (Reynolds et al., 2019). Metabolic health involves numerous processes, with a central role played by the regulatory properties of the hormone insulin, which controls glucose levels and adipose tissue functioning (Saltiel & Kahn, 2001). Insulin resistance, where the body's sensitivity to insulin is impaired, is a critical aspect of metabolic health (Lebovitz, 2001). Within the context of metabolic health, this thesis will focus on poor glucose control, impaired insulin sensitivity, and aspects of adipose tissue regulation. Dietary fibers are reported to benefit glucose control and insulin signaling directly through modification of gastrointestinal transit time and indirectly via microbial fiber fermentation products. The latter include the uptake of SCFAs into the bloodstream and their role as modulators of gene expression through histone deacetylase (HDAC) inhibition, stimulators of hormone production (e.g., glucagon-like peptide-1 (GLP-1) and peptide YY), and activators of G protein-coupled receptors. Together, these actions can elicit a wide variety of physiological responses (Blaak et al., 2020; Canfora et al., 2015). While dietary fibers reportedly also impact immune function (Beukema et al., 2020; Deehan et al., 2024; Gill et al., 2020; Schley & Field, 2002) —a relevant player in metabolic health (Lackey & Olefsky, 2015)— this thesis will not cover this aspect as it is beyond our research scope.

This thesis will focus on the effect of intrinsic dietary fibers on metabolic health in individuals at risk for T2D, with or without obesity, particularly examining aspects of glucose control, insulin sensitivity, and adipose tissue function. To achieve this, we combine “classic” blood markers from fasting samples with “dynamic” outcomes from continuous glucose or hyperinsulinemic-euglycemic clamp measurements. Additionally, we will evaluate differences in clinical response as measured by these biomarkers and assess their correlation with gut microbiota differences. Our goal is to advance research on intrinsic dietary fibers to enhance metabolic health through modulation of the gut microbiota.

DRIED CHICORY ROOT AS A SOURCE OF INTRINSIC FIBERS

To explore the effect of the presence of the plant cell wall in intrinsic dietary fiber effects on human gut and metabolic health, we use dried chicory root particles in this thesis. Dried chicory root particles are produced from fresh chicory roots that have been minimally processed to safeguard the original plant cell matrix. From a plant science perspective, inulin is akin to starch in that both are storage carbohydrates stored within the plant vacuole and utilized for plant growth (Figure 2B). However, a fundamental difference for human consumption is that starch is readily digestible (except when present as resistant starch), whereas inulin is not. This characteristic dramatically increases the fiber content of fruits and vegetables containing inulin, making them valuable dietary fiber sources. Despite inulin's established role as a prebiotic, relatively few foods in our society are known to feature inulin as a primary storage carbohydrate, with many considered “forgotten foods”. Jerusalem artichoke (known as “aardpeer” in Dutch) originates from South America, as does the recently re-emerging yacon (known

as “appelwortel” in Dutch). Other root systems, such as dahlias and sunflowers, also contain inulin but are not consumed as they are primarily used as ornamental flowers. Chicory roots are the most common European vegetable rich in inulin, widely cultivated in our society. Consequently, dried chicory roots present a compelling plant food for studying the impact of intrinsic fibers on human gut and metabolic health.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to explore how dried chicory, a fiber-rich food product notably high in inulin, can benefit human metabolic and gut health and to revisit the added benefits of the plant cell matrix in enhancing the effects of dietary fibers.

Following the general introduction provided in **Chapter 1**, the thesis continues with revisiting our current understanding of dietary fibers as isolated structures and its distinction from fibers spatially organized within the plant cell matrix, distinguished by the term “intrinsic fibers” (**Chapter 2**). In this context, we summarize recent literature on the use of intrinsic fibers for human health modulation. Next, we explore dried chicory root as an intrinsic fiber product notable for its high inulin content, which distinguishes it from other fiber products **(Chapter 3**). Although dried chicory root is currently not well-known, we demonstrate its historically safe consumption by humans over hundreds of years.

To investigate the health benefits of dried chicory root, we first assess its gastrointestinal fate using a series of *in vitro* and *ex vivo* models (**Chapter 4**). We examine the integrity of the plant cell wall and its impact on the breakdown of dried chicory root by the gut microbiota, and how this is related to gut barrier function. Following these insights, we introduce the first *in vivo* assessment of dried chicory root on human gut and metabolic health in a three-week randomized, placebo-controlled parallel trial in subjects at risk for T2D (**Chapter 5**). Based on these findings, a longer follow-up trial was designed to further evaluate the effects of dried chicory intake in subjects with obesity and at risk of T2D. We document the 10-week changes in gut microbiota composition and function and investigate differences in individual responses (**Chapter 6**).

We then shed light on bowel function outcomes in relation to increased fiber intake. First, we present lessons learned from a randomized, placebo-controlled, cross-over trial using isolated inulin in subjects with functional constipation (**Chapter 7**). Following these findings, we outline the design of a randomized, placebo-controlled parallel trial to assess dried chicory root in a broader context of bowel function dissatisfaction (**Chapter 8**). Finally, I synthesize all our findings, reflect on the working hypothesis of distal fermentation, and evaluate our findings of dietary fibers' impact on bowel function and metabolic health in a wider scientific context. I continue by expressing my view on the role of food processing for the benefits of dietary fibers and conclude with an exploration of future prospects for dietary fiber research. (**Chapter 9**).

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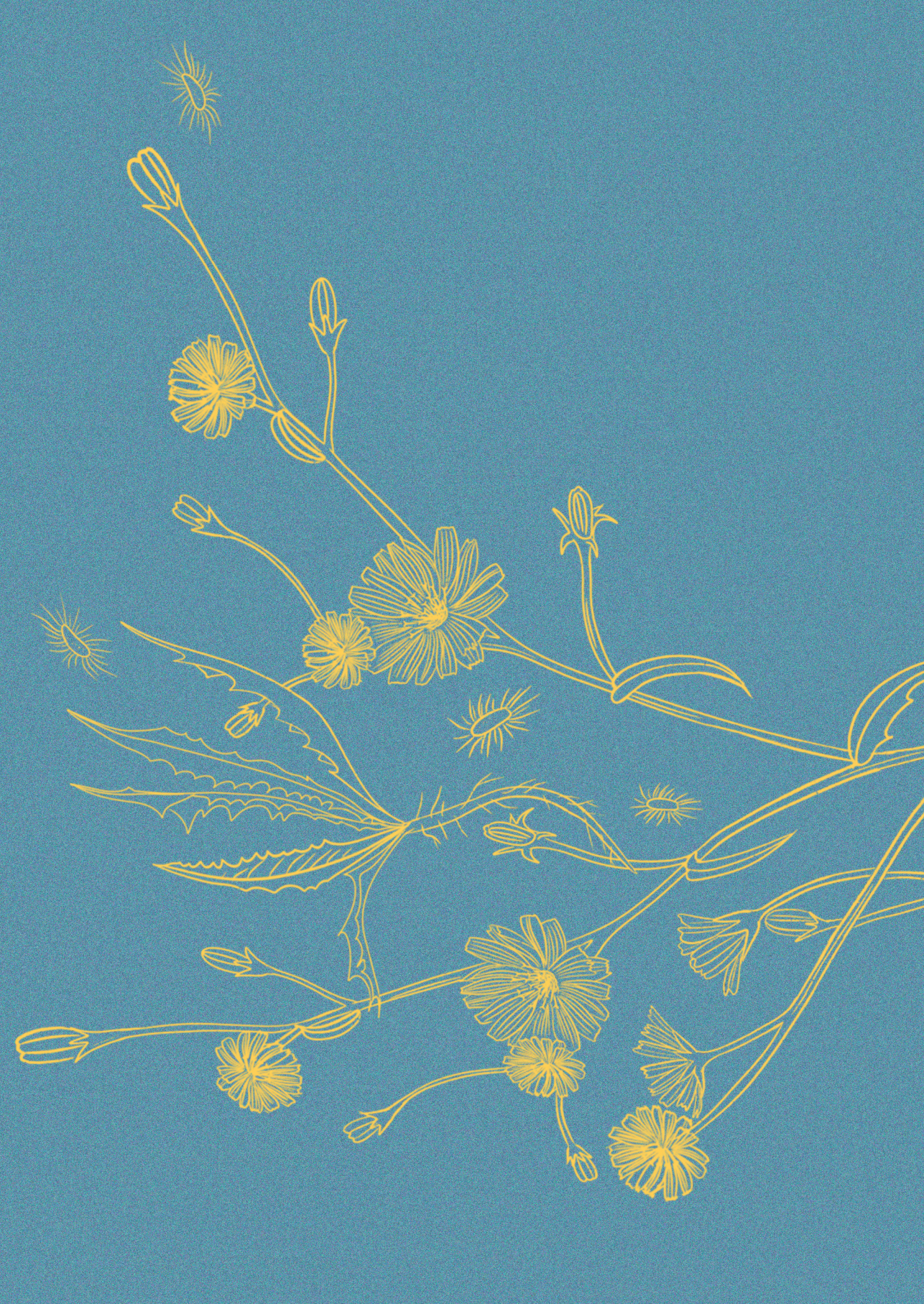

CHAPTER 2

Intrinsic dietary fibers and the gut microbiome: Rediscovering the benefits of the plant cell matrix for human health

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ABSTRACT

Dietary fibers contribute to structure and storage reserves of plant foods and fundamentally impact human health, partly by involving the intestinal microbiota, notably in the colon. Considerable attention has been given to unraveling the interaction between fiber type and gut microbiota utilization, focusing mainly on single, purified fibers. Studying these fibers in isolation might give us insights into specific fiber effects, but neglects how dietary fibers are consumed daily and impact our digestive tract: as intrinsic structures that include the cell matrix and content of plant tissues. Like our ancestors we consume fibers that are entangled in a complex network of plants cell walls that further encapsulate and shield intra-cellular fibers, such as fructans and other components from immediate breakdown. Hence, the physiological behavior and consequent microbial breakdown of these intrinsic fibers differs from that of single, purified fibers, potentially entailing unexplored health effects. In this mini-review we explain the difference between intrinsic and isolated fibers and discuss their differential impact on digestion. Subsequently, we elaborate on how food processing influences intrinsic fiber structure and summarize available human intervention studies that used intrinsic fibers to assess gut microbiota modulation and related health outcomes. Finally, we explore current research gaps and consequences of the intrinsic plant tissue structure for future research. We postulate that instead of further processing our already (extensively) processed foods to create new products, we should minimize this processing and exploit the intrinsic health benefits that are associated with the original cell matrix of plant tissues.

Keywords: intact plant cells, plant cell wall, minimal processing, colonic microbiota, gut health, short-chain fatty acids.

INTRODUCTION

Human health is substantially influenced by the food we eat. One food component that especially gained attention during recent years is the indigestible backbone of our plant foods: dietary fibers that cannot be directly utilized by our body. Dietary fibers appear to be an all-round talent reducing all-cause-mortality and protecting against different types of cancer, type 2 diabetes and cardiovascular diseases (Reynolds et al., 2019). The hypothesis that fibers have a crucial place in maintaining human health is not new. Already in the 1960's, Denis Burkitt developed the dietary fiber hypothesis placing dietary fibers at the origin of numerous diseases occurring in high-income countries with a Western lifestyle (Cummings & Engineer, 2017; O'Keefe, 2019). At that time, many beneficial effects of fibers were linked to gut microbiota-independent effects. These were based on physicochemical properties of fibers, like retaining water, which increases stool bulk and speeds up transit time, or bile-acid binding, which reduces cholesterol levels (Cummings & Engineer, 2017). Gut microbiota-dependent health benefits of fibers where hypothesized, but could not be determined as the present culture-independent high-throughput tools and mechanistic insights were lacking (Cummings & Engineer, 2017; De Vos et al., 2022; Zoetendal et al., 2008). This has changed during the last 25 years (O'Keefe, 2019). As dietary fibers are not broken down by human endogenous enzymes these are passed down to the lower gut where they are utilized by the gut microbiota, mainly consisting of bacteria residing in the colon (Flint, Scott, Duncan, et al., 2012). The gut bacteria thereby produce various metabolites, most importantly short-chain fatty acids (SCFA) that mediate some of the microbiota-dependent effects of fibers (Canfora et al., 2015; Gasaly et al., 2021; Venegas et al., 2019). These effects can range from locally interacting with epithelial and immune cells affecting barrier function and gut homeostasis (Gasaly et al., 2021; M. Li et al., 2018; Venegas et al., 2019) to peripherally impacting organs either by receptor-binding or by exerting direct effects (Blaak et al., 2020; Canfora et al., 2015). As a consequence, modulating gut microbiota activity and composition by fiber intake holds potential new therapeutic avenues. In this context, distinct fiber types have been studied to elucidate their specific microbiota-dependent and -independent effects on human health (Deehan et al., 2020). This has led to a continuous effort to further narrow down the underlying fiber-microbiota interactions *in vitro* and to understand the specific molecular make-up of fibers as for instance leading to the immunomodulatory potential of different fiber chain lengths and structural features (Beukema et al., 2020; Fransen et al., 2017). These efforts, however, follow a reductionist approach that considers fibers as loose entities (Eastwood & Kay, 1979; Hoffmann, 2003; Mozaffarian et al., 2018). This single-fiber-approach is in line with Western food processing and food design practices but neglects the original form in which fibers are present and consumed during our evolution: as part of whole foods and often only minimally processed.

Here we elaborate on the need to change our understanding of dietary fibers to unlock their full potential to modulate gut microbiota in relation to human health. We explain how dietary fibers exist in nature and discuss how this holistic view differs from the current approaches. Finally, we explore the existing intervention studies of intrinsic fibers in relation to human health and discuss potential consequences for future research avenues.

CURRENT VIEW OF DIETARY FIBERS: SINGLE, ISOLATED COMPONENTS AND THEIR IMPACT

Dietary fiber is an umbrella term for a group of polymers that are structurally and chemically very different. Numerous sugar molecules, such as glucose, xylose, mannose, galactose, arabinose and rhamnose are linked together by various glycosidic bonds following a specific or random pattern creating linear or branched structures that can be further decorated with phenolic acids or linked to other compounds (C. Li et al., 2021; Louis et al., 2021). For instance, inulin and starch consist of linear repetitions of a single molecule (fructose and glucose monomers, respectively). In contrast, rhamnogalacturonan – a type of pectin – is a highly branched molecule with various side-chains of different monomers (Zdunek et al., 2021). What makes them dietary fibers is the common trait of their bonds not being broken by human endogenous enzymes.

The enormous variation in fiber structures brings along physicochemical properties that are often classified into "soluble versus insoluble". This classification, despite being widely used, is only based on fiber content analysis of foods rather than functional behavior of the fibers in the human gut (Augustin et al., 2020; Capuano, 2017; McRorie & McKeown, 2017; So et al., 2021; Williams et al., 2019). Functional fiber properties are therefore better described in terms like bulking, viscosity and fermentability (So et al., 2021). Fermentation of the different fibers by the gut microbiota requires microbes to have at their disposal a set of enzymes that can break down the specific chemical linkages and types of molecules (Flint, Scott, Duncan, et al., 2012; Kaoutari et al., 2013). An often-observed feature of microbial communities is functional redundancy, meaning that taxonomically different members of the gut microbiota can perform similar functions, e.g. break down the same type of fiber (Flint, Scott, Duncan, et al., 2012; Louca et al., 2018; Reichardt et al., 2017). Microbes within the gut microbiota community can assist each other via cross-feeding, with primary degraders cleaving polymers into smaller compounds that can be further broken down by others and finally be converted into SCFA (Baxter et al., 2019; Flint et al., 2008; Flint, Scott, Duncan, et al., 2012; Reichardt et al., 2017). Since the vast majority of the gut microbiota consists of mainly anaerobic bacteria, these are the main contributors of the fiber degradation, occasionally supported by the action of methanogens that convert generated hydrogen into methane (Flint et al., 2008; Rajilić-Stojanović & de Vos, 2014). While some of these bacteria are adapted to metabolize a wide range of polymers, others appear to be more specialized (Flint, Scott, Duncan, et al., 2012; Reichardt et al., 2017). Moreover, specific bacteria have been coined to be so-called "keystone" species as they are unique players in the metabolic networks for the breakdown of compounds or the production of specific metabolites (Flint, Scott, Duncan, et al., 2012).

Multiple intervention studies with isolated, single fibers have been reviewed elsewhere (Armet et al., 2020; Flint, Scott, Louis, et al., 2012; Holscher, 2017; Jefferson & Adolphus, 2019; Le Bastard et al., 2019; Rastall et al., 2022; So et al., 2018). The emerging picture is that i) a range of different fibers are able to stimulate a more diverse range of gut bacteria (S. F. Chung et al., 2019), and ii) chemically and structurally complex fibers

can be used to specifically target bacteria relevant for human health (Cantu-Jungles & Hamaker, 2020; W. S. F. Chung et al., 2016; Hamaker & Tuncil, 2014). Using this approach, efforts have been made to define differences in the fine structure of fibers and relate these to the specific gut microbiota response and health outcomes (Cantu-Jungles et al., 2021; S. F. Chung et al., 2019; Deehan et al., 2020). For instance, in a recent human trial wheat bread was enriched with a variety of fibers like wheat dextrin, micronized wheat bran, oat flakes and bran, inulin, locust bean gum and pectin, which to some degree decreased cholesterol, insulin and HOMA-IR levels and these changes were linked to an increase in the gut microbiota able to break glycosidic bonds (Ranaivo et al., 2022). Similarly, another recent human trial in overweight individuals linked the gut microbiota's ability to ferment arabinoxylan but not crystalline cellulose to an observed increase in satiety (Deehan et al., 2022). Moreover, *in vitro* assessment of different fast-fermentable fiber types indicated a delayed fermentation rate for some fibers when presented in a mix instead of alone (Tuncil et al., 2017). A delayed fermentation rate is hypothesized but not yet shown to be a desired feature for the therapeutic application of fibers in the treatment of irritable bowel syndrome (So et al., 2021). All these studies have as a common principle that they rely on single fibers or fiber extracts that have been isolated from the plant tissue they originate from. Understanding the isolated effects of these single fiber types has its place in determining trophic chain interactions between microbes (Flint et al., 2008) and to unravel underlying mechanism for specific fiber application in e.g. the medical field (Sorbara & Pamer, 2022). However, looking at fibers as isolated components overlooks one aspect of dietary fibers: how we eat them.

INTRINSIC FIBERS: COMPLEXLY INTERTWINED THREE-DIMENSIONAL STRUCTURES

When we discuss dietary fibers, we mainly think of them as single, loose compounds. However, this contrasts with their “natural form” - the form already recognized by Burkitt to entail the crucial health benefits of fibers. The bulk of fibers we eat are not single, isolated fibers, but part of plant foods like vegetables, fruits, seeds, nuts, legumes and grains (An et al., 2022; Augustin et al., 2020; Grundy, Edwards, et al., 2016; Hansen & Sams, 2018; Qin et al., 2021). We consume the tissues of these plants, which are made up from a matrix of plant cells (Holland et al., 2020). The backbone of this matrix are dietary fibers that are complexly intertwined into a three-dimensional network creating plant cell walls (Holland et al., 2020). These plant cells can further encapsulate other fibers that are stored in the vacuoles, which are either fructans or starch serving the plant as reserve for growth (Eisenach et al., 2015; Yoshida, 2021). During recent years, awareness has risen that the existence of this three-dimensional plant cell matrix has important consequences for digestibility and health while very likely exerting different effects than the single, isolated fibers. For this purpose, a distinction was made between isolated fibers and fibers in their natural, three-dimensional form. The latter fibers were termed “intrinsic fibers” (Augustin et al., 2020) referring to these as being an intrinsic part of the plant cell wall.

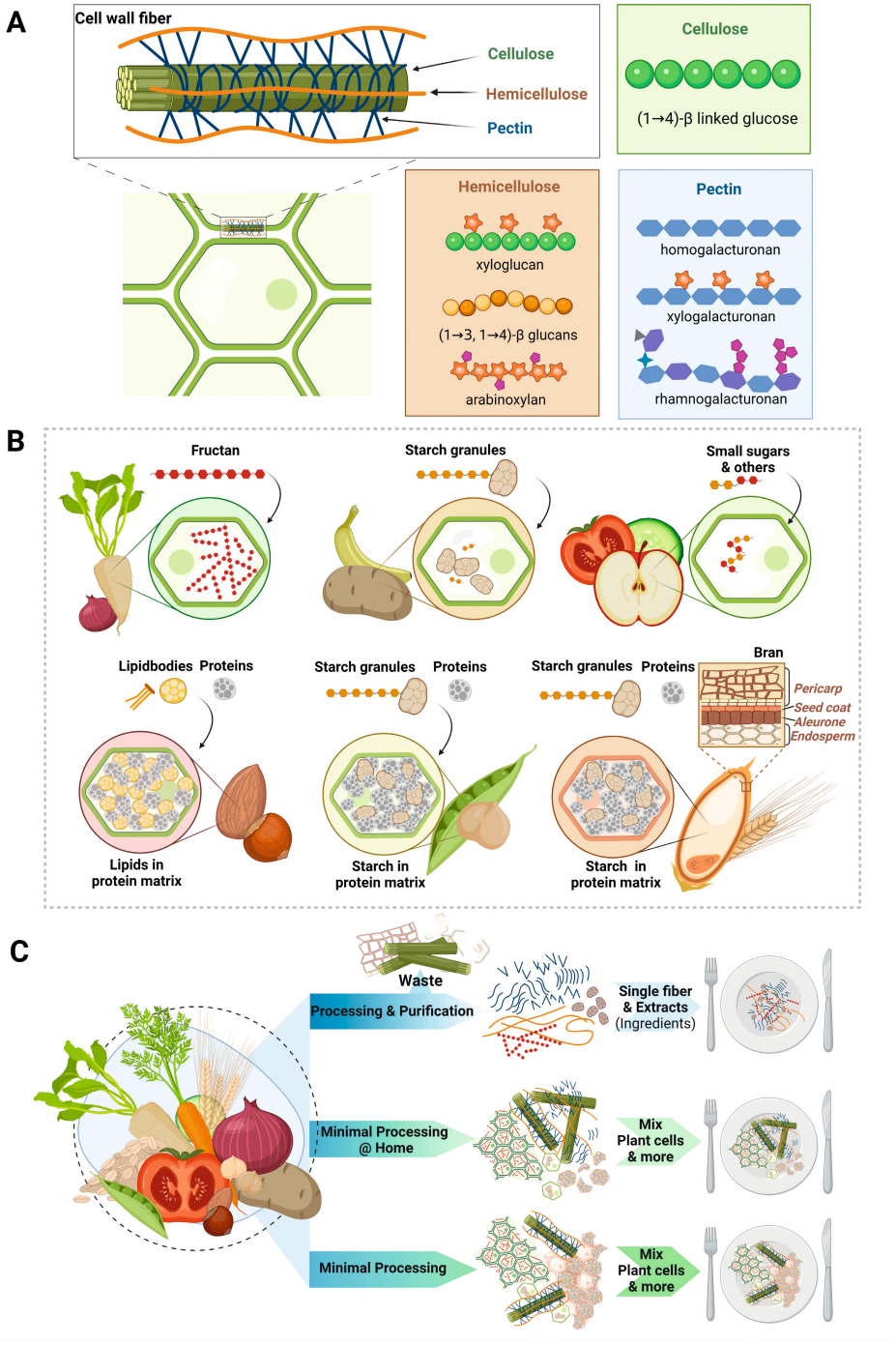

Figure 1. Intrinsic make-up of dietary fibers in plant foods (A) Dietary fibers are the backbone of plant foods that we consume, like grains, legumes, fruits, vegetables, nuts and seeds. The intrinsic

make-up of plant tissue is created by the plant cell-wall fibers cellulose, hemicellulose and pectin. Cellulose (green)forms fibrils, which are stabilized by hemicellulose (orange) and further strengthened by pectin (blue) forming a complexly intertwined three-dimensional structure. While cellulose is a structurally simple molecule made of glucose molecules that are β(1→4) linked, hemicellulose and pectin are two groups of structurally very diverse cell wall fibers. An abundant hemicellulose in dicot plants (like legumes) is xyloglucan, while monocot plants (grains) have higher amounts of mixed linkage β-glucans (e.g. oats) and arabinoxylans (e.g. wheat). Pectin can either consist of linear chains of monomers (homogalacturonan), that can be esterified and further decorated with other sugars (xylogalacturonan) or of several molecules with side-chains made of other compounds (rhamnogalacturonans). (B) The complex cell-wall fiber structure encapsulates various other nutrients in the plant vacuole like storage carbohydrates, which can be either fructan – a dietary fiber with a fructose backbone- or starch. Starch is stored in starch granules, which are found in vegetables and fruits like potatoes and (unripe) bananas. Other fruits and vegetables' plant cells contain vacuoles mainly filled with water and other nutrients (e.g. small sugars). Nuts contain lipid bodies that are embedded within the vacuole in a protein matrix, while in legumes starch granules are embedded in the protein matrix. Finally, in contrast to the before-mentioned dicot plants, grains, which are monocots, have different plant cell-wall but also have starch granules are embedded a protein matrix. This starch-protein matrix is surrounded by the so called aleurone layer, the seed coat and the pericarp that form together the so-called bran. (C) Dietary fibers can be processed in various ways that either disintegrate or maintain the three-dimensional plant tissue matrix. Common approaches in the industry are to extract and purify dietary fibers into single fibers creating (new) ingredients that are used to create new food products. Cooking and other domestic processing types maintain the tissue matrix to different extents but can generally provide intact plant cell wall structures. Finally, we propose that similar to domestic processing, plant foods should be minimally processed such that the plant cell-wall structure and overall plant tissue matrix are maintained. The latter opens avenues for new, convenient and minimally processed fiber products that exploit the health benefits of the intrinsic plant structure, while aligning with the need for more suitably produced, plant-based food solutions. Created with BioRender.com.

MAKE-UP OF INTRINSIC FIBERS

The make-up of plant cells follows a common principle, which consists of three main building blocks: cellulose, hemicellulose and pectin (Figure 1A) (Albersheim et al., 2010; Holland et al., 2020). Cellulose is considered as basic scaffold of the cell wall, being a linear polymer of β(1→4) linked glucose units that align into rigid microfibrils. These microfibrils are reinforced by hemicellulose (Figure 1A), which is a group of structurally very diverse polymers with either glucose-xylose (xyloglucans), a glucose (mixed-linkage glucans) or xylose (xylan) or mannan backbones (Capuano, 2017; Scheller & Ulvskov, 2010). To these backbones other sugar molecules and also phenolic acids can be linked, such as the hemicellulose type arabinoxylan (Collins et al., 2010; Scheller & Ulvskov, 2010). Finally, pectin serves like a filler giving the cellulose-hemicellulose scaffold stability, controlling permeability and together creating a matrix that can retain water (Figure 1A) (Albersheim et al., 2010; Xiong et al., 2022). Similarly to hemicellulose, also pectin is a group of structurally diverse polymers, which have a linear galacturonic acid backbone (homogalacturonans) or a galacturonic acid and rhamnose backbone (rhamnogalacturonans) that can be further decorated with side-chains forming complexly branched polymers (Figure 1A) (Capuano, 2017; Mohnen et al., 2008; Zdunek et al., 2021). It is essential to understand that these different polymers

are physically entangled and partly also chemically bound to each other, despite the fact that their precise organization is still not fully understood (Albersheim et al., 2010). These complex structures form together three-dimensional plant cells that are glued together by pectin, which fills the inter-cellular space between connecting plant cells (Albersheim et al., 2010). Besides the fiber polymers, plant cell walls also contain small amounts of proteins (arabinogalactan proteins) and minerals (Albersheim et al., 2010; Holland et al., 2020). Finally, certain specialized cell-types are reinforced with lignin, which is a dietary fiber consisting not of sugars but of different types of phenolic phytochemicals that are complexly linked (Albersheim et al., 2010; Holland et al., 2020). While all plants share these common aspects of the plant cell wall, their make-up differs depending on the type of plant (e.g. monocots versus dicots; see below), its maturation or ripeness and especially food processing (Holland et al., 2020; Louis et al., 2021; Xiong et al., 2022). Even within plants, differences in cell types exist between the tissue we consume (such as leaf, root, fruit, stem, seed) and within these tissues (Holland et al., 2020; Scheller & Ulvskov, 2010).

As hemicellulose and pectin are groups of very heterogenous polymers, the specific types of these fibers that are present in the plant cell walls distinguish different plant types (Holland et al., 2020; Xiong et al., 2022). Vegetables, fruits, nuts, seeds and legumes belong to the dicots, that have so-called type I cell walls, while grains are monocots having type II cell walls. Type I cell walls of dicots contain xyloglucan as the most abundant hemicellulose and the hemi-/cellulose network is further stabilized by pectin, which has been reported to make up a third of the cell wall weight (Holland et al., 2020; Louis et al., 2021). In contrast type II cell walls of monocots, have no or very low amounts of pectin and the abundant hemicelluloses are β-glucan and arabinoxylan (Holland et al., 2020; Xiong et al., 2022). Rice is one of the grains that contains some pectin, while oats and barley are particularly rich in β-glucan and wheat, maize and rye in arabinoxylan (Collins et al., 2010). Another distinguishing feature of grains is the coating that covers the grain kernel. This outer coating consists of several layers known as the aleurone layer, the seed coat and the pericarp, which together form what we know as "bran" (see Figure 1B)(Zitterman, 2003). Bran is therefore not a homogenous compound but contains different cell types that have a different ratio of hemicellulose types than the starchy endosperm (Collins et al., 2010).

The three-dimensional capsules that are created by the cell wall fibers contain the plant cell with its vacuole in which other nutrients like lipids, proteins and carbohydrates as well as phytochemicals are stored (Figure 1B) (Eisenach et al., 2015). Depending on the plant type, the encapsulated contents can profoundly differ. For instance, plants store reserve carbohydrates in their vacuoles. These can either be fructans (Yoshida, 2021), or starch, which is stored in the form of starch granules (Figure 1B). While fructans classify as dietary fibers as their glycosidic bonds cannot be broken down by human endogenous enzymes, most types of starch can be digested with some resistant to digestion due to their physical state (resistant starch). Many vegetables and fruits have plant cells whose vacuoles are filled with mainly water, which maintains

the internal pressure (turgor) and keeps an open cell structure that provides the food product its characteristic crispiness/crunchiness (Figure 1B). The vacuoles also contain other small molecules, such as sugars and phytochemicals (Eisenach et al., 2015). In legumes and nuts the vacuoles are filled with a protein matrix, embedding starch granules and lipid bodies, respectively (Figure 1B). Finally, the starch granules in grains are also embedded in a protein matrix within the cell walls (Figure 1B).

The three-dimensional make-up of plant cells, the physical and chemical entanglement and the encapsulation of other fibers and nutrients have important consequences for the gut microbes' ability to access the individual fibers. Food processing can substantially influence and destroy these intrinsic structures and is used to extract, isolate and purify single fibers. Moreover, in contrast to these isolated fibers, during upper gut digestion the cell wall fibers do not simply dissolve and become freely available as isolated components but remain to a considerable extent within their intertwined matrix. Hence, the make-up of the plant food cell matrix, food processing and digestion influence how intrinsic dietary fibers are altered in the gut and fermented by the gut microbiota.

FOOD PROCESSING IMPACTS THE INTRINSIC FIBER MAKE-UP

Whether a fiber can be classified as intrinsic fiber and how further digestive processes influence its digestive fate depends largely on its processing. As intrinsic fibers are an intrinsic part of the plant cell wall whole, raw foods, like unprocessed apples, bananas, cucumbers, tomatoes etc. contain by definition intrinsic fibers. However, any form of processing - industrial as well as domestic - can alter this three-dimensional structure (Capuano, 2017; Holland et al., 2020; Hu et al., 2022; C. Li et al., 2021; Tejada-Ortigoza et al., 2015; Xiong et al., 2022) and whether a fiber is still an intrinsic fiber needs careful checking of the applied methodology.

In general food processing has as aim to preserve foods, increase digestibility or extract compounds that can be used to create new foodstuffs. To do so, a plethora of techniques can be applied which include mechanical (physical), chemical, thermal and enzymatic processing as well as high pressure, ultrasound and microwave technologies (Maphosa & Jideani, 2015; Tejada-Ortigoza et al., 2015). While industrial processing often aims at extracting single fibers for specific food applications, domestic processing is usually gentle and does not fully disintegrate the intrinsic fiber structures (Figure 1C). The effects of different processing techniques on the physicochemical properties (Guillon & Champ, 2000) of fibers as well as cell wall integrity and overall cell structure has been reviewed by others (Capuano, 2017; Holland et al., 2020; C. Li et al., 2021; Xiong et al., 2022). Hence, here we give only a small overview of these techniques to provide a basic understanding of their consequences for intrinsic fibers.

Possibly the most gentle food processing techniques in terms of safeguarding the overall intrinsic tissue structure are drying, freezing and freeze-drying, which aim to conserve food products. While drying leads to shrinkage of the cell tissue, freezing and freeze-drying maintain the open cell structure, but the respective plastic changes of drying and water crystals formed during freezing can induce cracks in the cell wall

(Lewicki & Pawlak, 2003). However, the extent of the caused damage depends on the applied conditions. Consequently, despite cell wall cracks the overall tissue is largely maintained reducing the possible release of intracellular components. Similarly, hydrothermal processing can maintain the overall tissue structure, while inducing more profound damage to cell walls mainly by affecting pectin (Capuano, 2017). Boiling or steaming can result in swelling of cell walls and dissolution of pectin (Capuano, 2017; Holland et al., 2020). When pectin leaks out of the inter-cellular space it weakens the overall cohesion between cells. This can then lead to separation of plant cells, but also to cell wall rupture. Moreover, leaking of pectin increases the space between the fibers within cell walls, which is called cell wall porosity (Capuano, 2017; Holland et al., 2020; Xiong et al., 2022). Dry thermal treatments like baking, roasting, popping are more violent processing types, fundamentally damaging the cell wall, but can also maintain overall tissue structure (Holland et al., 2020).

Mechanical (physical) destruction, like milling/grinding, generally break cell walls releasing the encapsulated contents (Holland et al., 2020; Xiong et al., 2022; N. Zhang et al., 2018). The finer the particle size, the less likely it is that whole plant cells are still present and the more likely that parts of the three-dimensional organization of cell walls have undergone physical destruction. Fine milling of the starchy endosperm of grains for instance leads to complete disruption of plant cells, breaking the protein-starch matrix and releasing the enclosed starch granules (Figure 1B) (C. Li et al., 2021). Similarly, the complex structure of bran can be substantially destructed with fine milling (Qin et al., 2021). In this context we want to highlight that despite the suggestive name, whole grain products do not necessarily provide intrinsic fibers (Jefferson & Adolphus, 2019; Van Der Kamp, 2012). The reason is that for the production of whole grain products, first the components bran, germ and the starchy endosperm are separated and each of these components is milled (Van Der Kamp, 2012; Williams et al., 2019). After processing, these fractions are reconstituted together, which allows the product to be defined as whole grain (Van Der Kamp, 2012). Hence, when we read about whole grain products, we refer to the processed and reconstituted fractions of the whole grain instead of the intact grains (Bach Knudsen, 2015; Van Der Kamp, 2012; Williams et al., 2017). The intact cells in grains have, however, specific effects on starch accessibility and the gelatinization during heating and thereby impact whether starch escapes upper gut digestion becoming available to the colonic microbiota (Do et al., 2019; Holland et al., 2020).

In summary, mechanical processing, either domestic or industrial, has major detrimental effects on the intrinsic fiber structure and for instance, dried, pulverized fibers likely do not behave like dried fibers that have undergone minor mechanical destruction. Nevertheless, in these powdered preparations, cell-wall parts might still be present, but their particle size might be very small, increasing the accessibility for gut bacteria. Finally, extrusion, a processing technique where food is subjected to high pressure, high shear and high temperature, is one of the most violent processing techniques (Hu et al., 2022) as it can completely degrade even the rigid cellulose fibrils (Dang & Vasanthan, 2019).

In order to produce single, isolated fibers further extensive extraction techniques are needed that separate the specific fibers from the three-dimensional plant cell matrix (Maphosa & Jideani, 2015; Tejada-Ortigoza et al., 2015). Some of these fibers are water-extractable like certain arabinoxylans, which are hence termed "soluble", while others need the application of enzymatic, gravitational and/or chemical treatments in order to be extracted (Collins et al., 2010; Maphosa & Jideani, 2015). During these extensive extraction processes, a variety of waste streams are created that might still contain valuable residual fibers and intracellular components. However, the integrity of these structures depends on the harshness of the applied processing. Therefore, fiber products that are based on waste streams might or might not contribute intrinsic fibers. While in some cases the waste streams can be valorized (Campos et al., 2020; Sabater et al., 2021), in many cases the production of fibers have a considerable carbon foot print and do not contribute to a circular economy (Tejada-Ortigoza et al., 2015; Zhu et al., 2016).

It is crucial to realize that when we investigate the isolated behavior of single dietary fibers, we in fact study fibers that have undergone extensive processing in order to be released, separated and purified from plant cells (Tejada-Ortigoza et al., 2015). It is highly doubtable, that the effects and behaviors of these isolated fibers sufficiently reflect that of intrinsic fibers (Holland et al., 2020). To illustrate this, we will below describe the impact of intrinsic fibers on digestibility *in vitro* followed by insights from human interventions.

IMPLICATIONS OF INTRINSIC FIBERS ON DIGESTION

It is clear that food processing substantially impacts how fibers behave in the gut during digestion (Capuano, 2017). While the behavior of isolated, single fibers has extensively been studied, the digestive fate of plant tissues is far less understood. The limited research that assessed plant tissue and single plant cells has focused more on the upper gut mainly relying on *in vitro* models and animal *in vivo* models. There are some studies that assessed human ileostomy effluents (with carrots) (Tydeman et al., 2010) or ileal samples (with white beans) (Noah et al., 1998), but very few studies followed the digestion of intrinsic fibers until the colon (Ellis et al., 2004). Here, we will only highlight the main consequences of upper gut digestion for intrinsic fiber structures and refer the reader to the comprehensive reviews by others (Capuano, 2017; Do et al., 2018; Holland et al., 2020; C. Li et al., 2021; Xiong et al., 2022).

Our general understanding of digestion is the breakdown of food matrices into their building blocks and subsequent absorption. However, when consuming plant food tissues with an intact cell matrix, the constituents will not simply dissolve as they are intertwined within the cell wall and in this form not readily water-soluble under physiological conditions (Capuano, 2017; Williams et al., 2019). Similarly, encapsulated compounds cannot dissolve directly into the gut environment and will be shielded from immediate digestion, delaying their breakdown. In general, the orogastric processes lead to size reduction of plant tissue fractions, and dissolution of certain water-soluble

pectins (Holland et al., 2020). During chewing, plant tissues are degraded into smaller sizes, which depending on the physical state of the plant type (hard, soft), ripeness and preparation methods (like cooking) happens by cell rupture (hard foods) or separation along the cell walls (soft foods) (Capuano, 2017; Holland et al., 2020; Xiong et al., 2022). Hence, a mix of differently sized particles containing intact and broken cells and their contents arrives in the stomach. There, further particle breakdown can occur by the mechanical gastric forces. It is generally believed that particles smaller than a few millimeters pass unchanged into the small intestine and, hence, intact plant cells can arrive into the small intestine. Larger structures are retained for further size reduction (called gastric sieving)(Xiong et al., 2022). However, whether this also applies to soft plant tissue is not clear. Pectin can leak from the intercellular spaces (Capuano, 2017; Holland et al., 2020; Xiong et al., 2022) and thereby reduce overall cohesion within plant particles. Whether also water-extractable hemicelluloses, like arabinoxylan, can leak out is not known but rather unlikely (Capuano, 2017; Collins et al., 2010). That means that plant particles with intact cells arrive in the colon. Indeed this has been confirmed from analysis of ileostomy effluents and ileal samples (Noah et al., 1998; Tydeman et al., 2010). The mixture of broken and intact cells is available to the gastric and small intestinal digestive enzymes to be degraded further, but released fructans and certain resistant starches will resist digestion. Due to pectin leakage cell wall porosity might increase. However, based on available literature, the diffusion of enzymes into intact cells and the diffusion of nutrients out of the cell is believed to be limited by the cell wall pore size (Holland et al., 2020; Xiong et al., 2022). Interestingly, the presence of plant cell wall material has been shown to reduce enzymatic breakdown of starch due to adsorption of α-amylase to the plant cell material (Holland et al., 2020; C. Li et al., 2021; Xiong et al., 2022).

In cases of mild food processing, the fractions of intact plant tissue that we swallow likely arrive in the colon in a rather intact state, mainly affected by chewing and dissolution of pectin (figure 1C). Hence, the bacteria in the colon are confronted with a mix of plant tissue particles, intact plant cells and broken cell wall material and its contents. Dissolved fibers, like released fructans or starch (and possibly leaked pectin) can be directly used by the gut bacteria. However, since bacteria are too large to diffuse into intact plant cells, these must spatially interact with the plant tissue, to access intertwined polymers such as pectins and hemicelluloses. All these digestive aspects make it obvious that the breakdown and consequent physiological behavior of intrinsic fibers cannot or can only partly be explained by that of their isolated single counterparts (Holland et al., 2020; Qin et al., 2021).

GUT MICROBIAL INTRINSIC FIBER BREAKDOWN

The number of studies that have investigated the microbial breakdown of intrinsic fibers in the colon is impressively low especially regarding human *in vivo* studies. From *in vitro* studies we have a fair understanding of which type of bacteria and bacterial enzymes are generally involved in metabolizing specific isolated fiber types (Flint et

al., 2007; Rastall et al., 2022). The different enzymes employed by gut bacteria can be classified according to carbohydrate-active enzymes classes (CAZymes; see www.cazy.org (Drula et al., 2022)), and bacteria differ in the number and type of enzymes they have, as thoroughly reviewed elsewhere (Kaoutari et al., 2013). The most abundant microbial enzyme class are glycoside hydrolases, cleaving neighboring sugar and non-sugar moieties. Other less abundant enzymes are polysaccharide lyases specialized in the cleavage of uronic acid moieties as present in pectins, and carbohydrate esterases cleaving off esterified groups as present in pectins and hemicelluloses. These enzymes may be assisted by other so-called carbohydrate binding modules or auxiliary activities (Drula et al., 2022). The bacterial enzymes involved in storage carbohydrate breakdown are relatively well established, like the GH13 subclass for starch and the GH32 subclass for fructan utilization (Kaoutari et al., 2013), and the discovery of enzyme systems to disintegrate the cell-wall fibers pectin (Despres et al., 2016) and hemicellulose is ongoing (M. Zhang et al., 2014). However, our knowledge on how gut bacteria interact with the most robust compound of the plant cell-wall, the cellulose microfibrils, is still very limited (Flint et al., 2008; Flint, Scott, Duncan, et al., 2012). While amorphous cellulose is believed to be partly utilized by employing a complex enzyme system, crystalline cellulose has been reported to be not fermentable (Flint et al., 2008; Flint, Scott, Duncan, et al., 2012). Bacteria were found to adhere to cell-walls on almond particles (Ellis et al., 2004) and coarse wheat bran (Schel et al., 1980) recovered from human feces, which indicates that bacteria possibly use the crystalline cellulose backbone for particle adherence (De Paepe et al., 2017; Flint et al., 2008). A small set of studies tried to identify the bacterial communities colonizing these plant tissue fractions (De Paepe et al., 2017, 2019; Leitch et al., 2007; Walker et al., 2008). Bacteria recovered from plant particles in human feces differed in the detected phyla compared to the liquid fraction (De Paepe et al., 2017; Walker et al., 2008). Moreover, the type of recovered particles (bran versus resistant starch, differently processed brans) impacted which bacteria adhered to them (De Paepe et al., 2019; Leitch et al., 2007). Based on these observations the breakdown of plant cell walls is believed to be initiated by primary degraders able to interact with the insoluble plant cell material. Subsequently, material is released from cell-wall compounds which other bacteria use to cross-feed (De Paepe et al., 2017; Flint et al., 2008; Flint, Scott, Duncan, et al., 2012). Some studies have addressed these plant cell-bacteria biofilms and the action of bacterial enzymatic systems that would be needed to degrade cell walls (Y. Ben David et al., 2015; Flint et al., 2008; Ze et al., 2015). However, how exactly the spatial interaction of these cooperations would look like and how plant cells are exactly opened-up by gut bacteria is unclear. Future research distinguishing between particulate and liquid fecal phases and using imaging techniques combined with identifying bacterial communities will offer exciting insights into these aspects.

It is generally believed that fermentable fibers are readily metabolized in the proximal colon. However, if readily fermentable fibers are not in isolation but either part of plant cell walls or encapsulated by them, the fermentation rate is likely to be slowed down.

This has indeed been supported by the results of *in vitro* experiments. For instance, *in vitro* assessment of plant cell-encapsulated cereal starches revealed that microbial enzymatic activity was first directed towards cell wall fiber degradation (pectin, xylan and cellulose) followed later by the slow degradation of starch and involving different microbial communities (Warren et al., 2018). Accessible intracellular material from broken cells also impacts the fermentation kinetics of plant cell walls, as demonstrated *in vitro* with differently processed wheat bran. A reduced SCFA production was found in wheat bran from which remaining starch fractions had been enzymatically removed (De Paepe et al., 2019). In the same experiment, the effect of fine milling was demonstrated with micronized (very finely milled) bran leading to higher initial SCFA production *in vitro* compared to unmodified bran. This observation had been made previously and attributed to a higher bacteria-to-surface ratio with smaller particle sizes (Stewart & Slavin, 2009). However, at the end of the fermentations, SCFA levels were similar (De Paepe et al., 2019; Stewart & Slavin, 2009; Tuncil et al., 2018), but the ratios between SCFA differed, with larger bran particles producing more butyrate (Stewart & Slavin, 2009; Tuncil et al., 2018). This is particularly interesting since more extensive processing of bran, such as extrusion, is considered desirable as it makes the constituents more accessible for microbial fermentation by degrading the three-dimensional bran structure. However, this may reduce the potentially health-promoting butyrate production (Collins et al., 2010; Demuth et al., 2020; Roye et al., 2020). In summary, the intrinsic structural features of the plant cells likely slow down fiber fermentation, inducing a lag phase (Xiong et al., 2022), but do not necessarily reduce the absolute amount of SCFA produced. Consequently, there is a gradual release of SFCA, which means that SCFA production is not restricted to the proximal colon but spread throughout the whole colon, including its distal parts benefitting local, mucosal health. This likely translates into beneficial systemic, peripheral effects as distal SCFA infusion *in vivo* has shown to induce more pronounced effects on biomarkers than proximal (van der Beek et al., 2016). Also delayed fermentation has been proposed as a desired feature of fibers in the treatment of irritable bowel syndrome but lacks presently experimental verification (So et al., 2021). In summary, the delayed fermentation of intrinsic fibers presents a highly relevant feature that isolated, single fibers do not have.

INSIGHTS FROM EXISTING HUMAN STUDIES

Whether the observed features of intrinsic fibers *in vitro* also occur *in vivo* and how these effects are reflected in health outcomes is not yet clear. To advance our insight, we summarized human intervention studies that investigated the effect of intrinsic fibers on gut microbiota composition and activity and/or related metabolic and bowel function outcomes (Table 1; extended version in Supplementary Table S1). We included randomized-controlled trials (no acute testing, patient-control or single-arm designs) published during the last 20 years. These trials either assessed diets based on whole foods or used one specific food (including vegetables, fruits and nuts) either in its fresh, cooked or dried form. If whole foods were further processed during the study to be

incorporated into meals we did include those as well, but excluded pulverized versions of food products as the mechanical processing extensively destroys the plant matrix. Similarly, we excluded fruit and vegetable pastes, juices or extracts as these underwent extended processing. For each study we have presented the processing type based on the reported information as to understand the impact on intrinsic fiber structure. We did not include studies using waste-stream products as the level of information on the applied processing is generally insufficient to decide whether intrinsic structures are maintained or not. Based on provided information on fold-changes in relative abundance of gut microbial taxa we estimated the modulatory potential to be either small (<1.5-fold), moderate (1.5 - 2.5-fold) or major (>2.5-fold), which is summarized in an extended version of Table 1 (Supplementary Table S1).

Whole food interventions are particularly relevant in the context of intrinsic fibers as subjects do not consume one but a variety of differently processed intrinsic fibers. Most of these studies (Table 1, Diets) have in common that they emphasize whole grains, fruits and vegetables often combined with legumes and nuts (Berendsen et al., 2013), specific types of grains and berries (Uusitupa et al., 2013) as well as dairy and animal products, while others can be more restricted (macrobiotic diet) (Candela et al., 2016). These diets have been linked to numerous beneficial health outcomes, like improvements in markers of cognitive function (Ghosh et al., 2020), inflammation (Ghosh et al., 2020), lipid (Candela et al., 2016; Gürdeniz et al., 2022; Uusitupa et al., 2013) and glucose metabolism (Candela et al., 2016; Uusitupa et al., 2013), and in subjects adhering to these diets these changes were associated with increased levels of gut microbial taxa involved in fiber breakdown and short-chain fatty acid production (e.g. *Faecalibacterium* spp.; Supplementary Table S1).

Although strictly speaking wheat bran is not necessarily an intrinsic fiber as its intactness largely depends on the degree of processing, we also included old and recent wheat bran studies (Table 1, Bran). Bran has been investigated in the 1970's and 1980's for its modulatory effect on lipid metabolism and bowel function and current approaches potentially provide insights into underlying gut microbiota modulation. Especially the early studies assessed processing effects, like different milling degrees and concluded that only coarse bran reduced transit time and intraluminal pressure (Supplementary Table S1) (Kirwan et al., 1972). Also hydrothermal processing (cooking) impaired effectiveness of wheat bran and only raw but not cooked bran influenced bowel function (Supplementary Table S1) (Wyman et al., 1976). Surprisingly, with the available high-throughput technologies only very limited effects of bran on gut microbiota composition and activity were observed in a recent study with bran of different particle sizes (Supplementary Table S1) (Deroover et al., 2021). However, also in bread-based interventions and acute settings few effects are found (Boets et al., 2017; Lappi et al., 2013), likely linked to the applied processing (fine milling, and enzymatic treatment). Moreover, bran and enclosed starch might have a synergistic function in the whole kernel, as in isolation they have been found to distinctly and differently impact gut microbiota (Salonen et al., 2014; Walker et al., 2010).

Table 1. Human intrinsic fiber studies. We selected randomized-controlled trails that have either used diets or single whole foods to assess their modulatory potential on gut microbiota and/or related health effects. These trials were published during the past 20 years, except for two of the bran studies. Even though bran does not necessarily classify as intrinsic fibers (depending on its processing), we did include some old and recent bran studies due to the long and ongoing interest in this type of plant food. Details of the trials are provided in the Supplementary Material Table S1.

Intrinsic fiber	Processing	Study design	Gut microbiota composition	Microbiota activity	Metabolic markers	Bowel function	Reference
Whole diets							
Mediterranean diet	Whole foods; incorporated into meals @Home	RCT, 1 year, parallel (dietary advice & provided foods)	Yes ‡	-	Yes	-	(Berendsen et al., 2013; Ghosh et al., 2020)
Nordic diet	Whole foods; incorporated into meals @Home	RCT, 18 or 24 weeks, parallel (dietary advice & provided foods)	Not assessed	-	Yes	-	(Gürdeniz et al., 2022; Uusitupa et al., 2013)
Macrobiotic diet	Whole foods; incorporated into meals by cooks	RCT, 3 weeks, parallel (controlled diet)	Yes ‡	-	Yes	-	(Candela et al., 2016)
Bran							
Wheat bran (20 g/day)	Coarse vs fine	RCT, 4 weeks, parallel	-	-	-	Yes	(Kirwan et al., 1972)
Wheat bran (12-22 g/day)	Raw vs cooked	RCT, 2 weeks, cross-over	-	-	-	Yes (raw)	(Wyman et al., 1976)
Wheat bran (20 g/day)	Reduced in size	RCT, 4 weeks, parallel (normal/obese)	No change	No change	Yes (obese)	No change	(Deroover et al., 2021)
Grains							
Barley (75 g/day)	Whole kernels, boiled, in bread (no milling)	RCT, 4 weeks, cross-over	-	-	Yes	-	(Nilsson et al., 2016)
Barley vs brown rice vs mix of both (60 g/day)	Whole kernels, cooked	RCT, 28 days, cross-over	Yes, moderate Δfold	-	Yes (mix)	-	(Martínez et al., 2013)
Coix (160 g/day)	Whole kernels, cooked	RCT, 1 week, parallel	Yes, small Δfold	-	Yes	-	(Jinnouchi et al., 2021)

Intrinsic fiber	Processing	Study design	Gut microbiota composition	Microbiota activity	Metabolic markers	Bowel function	Reference
Nuts							
Walnut (42 g/day)	Whole	RCT, 3 weeks, cross-over	Yes, moderate changes	-	Yes	-	(Holscher, Guetterman, et al., 2018)
Almond (57 g/day)	Whole, roasted	RCT, 6 weeks, parallel	Yes, small & large Δfold	-	-	-	(Dhillon et al., 2019)
Almond (42 g/day)	Whole raw, whole roasted, chopped roasted, almond butter	RCT, 3 weeks, cross-over	Yes, moderate to large Δfold (most chopped)	-	-	-	(Holscher, Taylor, et al., 2018)
Almond or pistachio (43 or 85 g/day)	Whole	RCT, 2.5 weeks, cross-over	Yes *, stronger pistachio effect	-	-	-	(Ukhanova et al., 2014)
Legumes & Seeds							
Chickpea or raffinose (200 g vs 5 g/day)	Canned; incorporated into soups & desserts	RCT, 3 weeks, cross-over	Yes ‡	No change	-	-	(Fernando et al., 2010)
Linseed, sunflower & sesame seed, wheat grain, haricot & kidney bean, chickpea	Whole vs ground; incorporated into meals (no milling)	RCT, 1 week, cross-over (controlled diet)	-	Yes	-	Yes	(Hovey et al., 2003)
Vegetables							
Broccoli, cauliflower +/- green & red cabbage (~ 800 g/day)	Raw and incorporated in soup or microwaved	RCT, 2 weeks, cross-over (controlled diet)	Yes ‡	-	-	-	(F. Li et al., 2009)
Broccoli and Cauliflower (168 +/- 300 soup g/day)	Frozen & steamed or incorporated into soup	RCT, 2 weeks, cross-over	Yes ‡	-	-	-	(Kellingray et al., 2017)
Chicory root (30 g/day)	Dried, cut into cubes (3mm)	RCT, 3 weeks, parallel	Yes, large Δfold	Yes	Yes	Yes	(Puhlmann et al., 2022)

Intrinsic fiber	Processing	Study design	Gut microbiota composition	Microbiota activity	Metabolic markers	Bowel function	Reference
Fruits							
Avocado (1 piece/day)	Whole food	RCT, 12-weeks, parallel (hypocaloric diet)	Yes, moderate to large Δfold	-	Yes	-	(Henning et al., 2019)
Avocado (140-175 g/day)	Whole food, part of meal	RCT, 12 weeks, parallel (partly controlled diet)	Yes, small Δfold	Yes	Yes	-	(Thompson et al., 2021)
Mango (300 g/day)	Whole food	RCT, 4 weeks, parallel	-	Yes	Yes	Yes	(Venancio et al., 2018)
Kiwi (2 pieces/day)	Whole food	RCT, 3 days, cross-over	-	-	-	Yes	(Wilkinson-Smith et al., 2019)
Date (~50 g/day)	Dried	RCT, 3 weeks, cross-over	No change	Yes	-	Yes	(Eid et al., 2015)
Prune (80 or 120 g/day)	Dried	RCT, 4 weeks, parallel	Yes, small Δfold	No change	-	Yes	(Lever et al., 2019)
Raisin (120 g/day)	Dried	RCT, 3 weeks, cross-over	-	Yes	-	Yes	(Spiller et al., 2003)

RCT, randomized-controlled trial; Δfold, fold changes in relative abundances; ‡ no information on fold changes provided

Nuts are another increasingly studied source of intrinsic fibers as their plant cells are filled with lipid bodies (Figure 1B). Information on lipid-degrading gut bacteria is limited and, generally, the amount of fat reaching the colon is believed to be low as most fats we consume are of animal origin or extracted from plants (oil) (Oliphant & Allen-Vercoe, 2019). Lipid bodies in nuts, however, resist upper gut digestion (Ellis et al., 2004; Grundy et al., 2015; Grundy, Carrière, et al., 2016). Consequently, studying the impact of nuts on the gut microbiota is of interest and a considerable number of studies assessed the modulatory potential of especially walnuts and almonds in various processed forms, as has been reviewed comprehensively (Fitzgerald et al., 2021). Indeed, nuts generally appear to exert moderate effects on various taxa, yet these effects are not consistent within and between nut types (Supplementary Table S1). One study assessed the effect of different almonds preparations and as expected their most processed form, which was almond butter, had only a small to moderate modulatory effect on the affected taxa when consumed (Supplementary Table S1) (Holscher, Taylor, et al., 2018). In contrast, chopped and roasted almonds exerted the largest modulatory effect on the affected taxa (Holscher, Taylor, et al., 2018). It is tempting to hypothesize that increased particles size improves but roasting impairs the efficiency of the intrinsic matrix disintegration in the upper gut (Grundy et al., 2015; Grundy, Carrière, et al., 2016), which in turn impacts gut microbial breakdown. Unfortunately, these nut studies did generally not assess other parameters like fecal SCFA or bowel function (Table 1, Nuts).

For instance, intake of resistant starch increased the relative levels of *Ruminococcaeae* and decreased members of the *Lachnospiraceae* family, while wheat bran induced the opposite effect (Salonen et al., 2014; Walker et al., 2010). In contrast, two whole-kernel barely interventions with or without brown rice (Martínez et al., 2013; Nilsson et al., 2016) and a coix seed intervention (Jinnouchi et al., 2021) did modulate gut microbiota response by increasing health-associated fiber-degraders such as *Bifidobacterium, Roseburia* or *Faecalibacterium* spp. and further impacted metabolic outcomes, like decreasing inflammatory markers (Table 1, Grains; Supplementary Table S1). This indicates that the whole kernel of grains likely acts differently than the separated, milled and reconstituted kernel fractions in whole-grain products. Moreover, this confirms the notion described above that whole grain products do not necessarily provide intrinsic fibers (Jefferson & Adolphus, 2019; Van Der Kamp, 2012).

To the best of our knowledge only one human study tried to assess the difference between isolated and intrinsic fiber structure in adult volunteers (Fernando et al., 2010). In this study canned chickpeas were compared to raffinose (Table 1, Legumes & Seeds). While the intactness of the plant cell structure was likely already impaired in the canned chickpeas, we hypothesize that this was even further reduced by mechanical processing, as both fiber types were incorporated into soups and desserts. Both fibers slightly affected the gut microbiota composition compared to control, with the suggestion that chickpea induced a decrease of ammonium-producing strains (Fernando et al., 2010). Another study assessed a variety of whole seeds, legumes and wheat grains mixed together and compared them against their ground form incorporated into meals

(Hovey et al., 2003). Both types of meals improved bowel function but only the meals with unground seeds, legumes and grains increased fecal butyrate as well as total SCFA (Supplementary Table S1) (Hovey et al., 2003).

Vegetables have been rarely investigated in their intrinsic form and fruits are mainly assessed dried, but not fresh (Table 1, Vegetables). This might be due to the fact that more attention has been given to their phytochemicals, which require cell breakage to be released from the vacuole. Two studies assessing cooked cruciferous vegetables prepared in various dishes observed modulation of the gut microbiota composition, but did not indicate how affected taxa related to fecal SCFA levels and other outcomes (Supplementary Table S1) (Kellingray et al., 2017; F. Li et al., 2009). Recently, we reported on the intake of dried chicory root cubes (approximately 3 mm rib), as a palatable preparation of this root vegetable high in inulin-type fructans (Puhlmann et al., 2022). The product had major effects on gut microbiota composition and activity, inducing a butyrogenic trophic chain including *Bifidobacterium* and *Anaerostipes* spp. and improved bowel function (Supplementary Table S1). Of note, fecal SCFA levels were highly increased to an extent never observed with isolated inulin. While the variation of blood glucose levels was reduced by the dried chicory root, fasting plasma markers were only slightly impacted, which we found to relate to baseline gut microbiota composition (Puhlmann et al., 2022). Interestingly, a single-arm trial, using meals based on inulin-rich vegetables (e.g. onion, Jerusalem artichoke, leeks), did not observe changes in fecal SCFA levels despite a major increase in *Bifidobacterium* spp. (Hiel et al., 2019). This could relate to the low dosage (~15 g/day fresh vegetable) and damage to cell walls by cooking, releasing encapsulated inulin already in the proximal colon.

There are some studies that investigated various effects of fresh fruits like avocado, mango and kiwi and dried fruits like prunes, raisin and dates (Table 1, Fruits). Similarly to nuts, avocados are plant foods rich in fat, but despite their high fat content have been found to decrease circulating triglyceride levels and concomitantly increase fecal fat and bile acid output (Henning et al., 2019; Thompson et al., 2021). While fiber can exert these positive effects on lipid metabolism by microbiota-independent effects and the small intestinal microbiota is known to impact bile acid metabolism (Gasaly et al., 2021; Staley et al., 2017), the role of the colonic microbiota is not yet understood. As avocado intake was reported to increase levels of bacteria normally related to a diet high in fat from animal foods (e.g. *Bilophila* (L. A. David et al., 2014)) and associated with negative health outcomes, future studies will shed light onto the microbiota-mediated health benefits of these intrinsic fibers (Supplementary Table S1) (Henning et al., 2019; Thompson et al., 2021). Many other fruit studies have focused on the application of intrinsic fibers to stimulate bowel function, and for a comprehensive overview hereof we refer the reader to others (Katsirma et al., 2021). Unfortunately, the majority of these fruit studies did not address the gut microbiota and its products, such as butyrate and other SCFA (Chan et al., 2007; Chang et al., 2010; Wilkinson-Smith et al., 2019). One recent study compared mango to an equal fiber dose of psyllium (Supplementary Table S1) (Venancio et al., 2018). Psyllium likely relieves constipation by microbiota-independent

effects as only a minimal impact on gut microbiota composition and SCFA production has been reported (Jalanka et al., 2019). In contrast, the mango fruit improved bowel function and also increased fecal SCFA and decreased IL-6 levels (Supplementary Table S1) (Venancio et al., 2018). Also intake of dried raisins improved bowel function and increased fecal SCFA (Supplementary Table S1) (Spiller et al., 2003). These results suggests that (dried) fruits that are metabolizable by the gut microbiota in the colon have microbiota-dependent health impacts related to their intrinsic fibers.

Overall, human intervention studies assessing intrinsic fibers confirm that these fibers impact the gut microbiota in various ways despite the possible physical barrier and complexity of the plant cell matrix. The majority of the reported effects on gut microbiota composition and activity is small to moderate, with exception of the dried chicory root particles that had major effects (Table 1, Vegetables). Moreover, few studies assessed changes in gut microbiota composition and activity together with metabolic markers and bowel function, which makes the translation of the observed effects challenging. In future it is important to investigate the effect of processing including particle size of intrinsic fibers evaluated against isolated, single fibers.

CONCLUSIONS AND CONSIDERATIONS FOR FUTURE RESEARCH

Modulating the gut microbiota using dietary fibers is an exciting field likely resulting in new therapeutic avenues to maintain and improve human health. Research on fiber-microbiota interactions has followed for years a reductionist approach based on the concept that isolated fibers are needed to understand how dietary fibers impact human health. However, during digestion dietary fibers do not simply dissolve from the plant tissues making them available to the gut microbiota as single components. Instead, plant tissue fractions are maintained and their complexly intertwined cell walls and encapsulated fructans and starch polymers arrive in the colon. These intrinsic fibers likely slow down colonic bacterial fermentation as the gut bacteria cannot spatially access all cell wall fibers and encapsulated contents. Thereby fiber-derived SCFA production is likely spread throughout the entire length of the colon, notably the distal colon with described health benefits (Neis et al., 2019; van der Beek et al., 2016). However, how these processes evolve is barely understood. Research assessing intact plant tissue fractions and cells has focused mainly on the upper gut. Few studies have assessed the further breakdown of intrinsic fibers in the colon and are mainly based on *in vitro* or animal *in vivo* data. Hence, there is a clear lack of human *in vivo* data on the utilization of intrinsic fibers by the gut microbiota. Future research should focus on understanding (i) how intrinsic fiber structures differ from isolated single fibers in their fermentation kinetics, (ii) how the gut microbiota spatially colonizes intrinsic fiber particles and cooperates with other bacteria in the liquid and mucosal environment, and (iii) how intrinsic fibers from different plant sources and their processing affects microbial breakdown and related human health outcomes. Finally, with the shift from animal-based to more sustainable, minimally-processed plant-based diets we should put considerable effort in the understanding how plant cell-encapsulated proteins

and fats affect the gut microbiota in the distal colon in contrast to animal-derived equivalents.

Food processing can fundamentally affect the intrinsic fiber structure (Figure 1C). Elucidating how different domestic preparation techniques (e.g. raw versus cooked in water versus steamed vegetables) affect health status by modulating the gut microbiota is an exciting field that has rarely been explored (Pérez-Burillo et al., 2018; Tydeman et al., 2010). In this context it is also important to be reminded that food processing per se is not health-detrimental. Certain foods are barely digestible without any processing and for specific populations, e.g. those suffering from malnutrition or diseases, food processing is crucial. However, in the Western population that consumes an abundance of highly (over)processed foods, the increased digestibility has resulted in negative health outcomes related to obesity and welfare diseases (Monteiro, 2009). Unsurprisingly, focusing on assessing the isolated effects of fibers and relying on them to create new food designs stimulates food processing and the production of waste streams but not necessarily promotes the development of healthy foods. As fibers in their unextracted, minimally processed form of the intrinsic plant cell matrix provide health benefits by naturally encapsulating ingredients and slowing down dietary fiber fermentation, we need to rethink the way we use dietary fibers in healthy food design. Hence, we postulate that future food designs should rather reduce the extent of food processing and move towards exploiting the intrinsic plant cell matrix, which we find in any plant food (Figure 1A-C). By doing so, we might not only reduce the level of food processing, but also reduce waste and create new healthy products that are in line with more sustainable and plant-based oriented diets.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

MLP and WMdV conceptualization; MLP literature search, writing initial draft; WMdV supervision, critical revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The following table is an extended version of Table 1 in the main article. We summarized existing human intervention trial that assessed intrinsic fiber in their relation to the gut microbiota and related health outcomes. Most of these fibers are in the form of whole foods, either as single foods or part of a whole food based diet. For this purpose, we selected randomized-controlled trials (excluding single-arm designs and patient-control designs) that were published during the last 20 year. The only exception is bran which - depending on its processing – is not necessarily an intrinsic fiber and has been studied extensively in the 1970's and 1980's. Hence, we included also bran studies older than 20 years. We did not include waste stream-derive fibers as the available information is mostly insufficient to make conclusion regarding the intactness of the intrinsic fiber structure.

Supplementary Material Table S1. Dietary fiber interventions using whole foods. Intrinsic fibers have been rarely studied in human intervention trials. We selected human studies that have either used whole food diets or whole foods (fresh, cooked, dried) to assess their gut microbiota modulatory potential and related health effects. Even though bran does not necessarily classify as intrinsic fiber due to processing, we did include some old and recent bran studies here as bran has been widely tested in relation to human health and different processing conditions. If not indicated different the populations consist of healthy subjects.

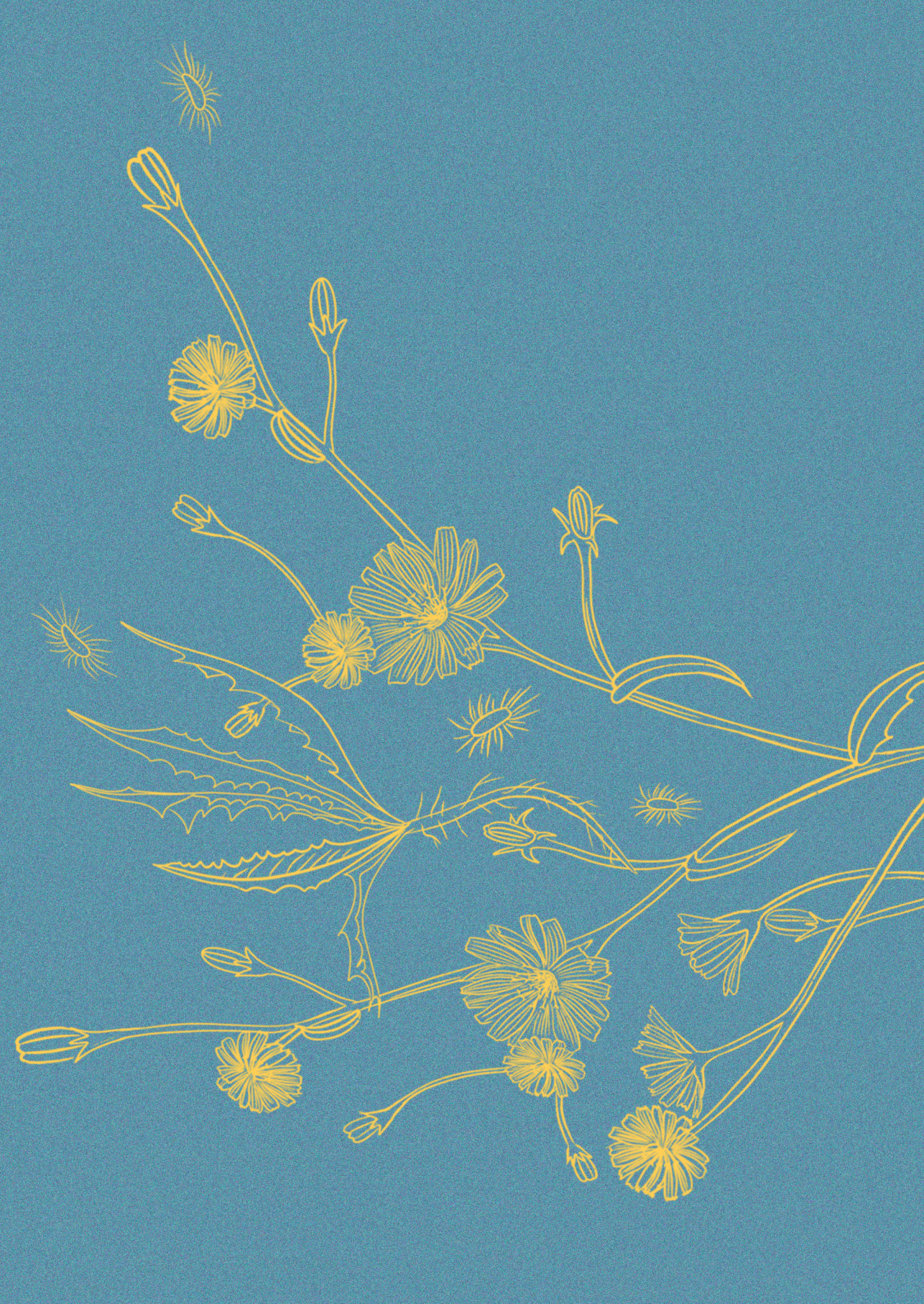
Intrinsic fiber	Processing	Study design	Gut microbiota modulation (Δfold)	Changes in microbiota activity	Changes in metabolic markers	Changes in bowel function	Reference
Whole diets							
Mediterranean diet (whole grains, fruits & vegetables, legumes and nuts)	Whole foods; incorporated into meals @Home	RCT, 1 year, parallel (dietary advice & provided foods)	No clear Δfold reported, adherence associated with ↑ *Faecalibacterium, Roseburia, Eubacterium* ↓ *Ruminococcus, Dorea Collinsella, Coprococcus*	-	Association with ↑ Cognitive function ↓ CRP, IL-17	-	(Berendsen et al., 2013; Ghosh et al., 2020)
Nordic diet (rye, barley, oats, berries, fruit, vegetable)	Whole foods; incorporated into meals @Home	RCT, 18 or 24 weeks, parallel (dietary advice & provided foods)	-	-	↓ cholesterol markers, association with lipid, glucose metabolism	-	(Gürdeniz et al., 2022; Uusitupa et al., 2013)
Macrobiotic diet (mainly vegetables & grains)	Whole foods; incorporated into meals by cooks	RCT,3 weeks, parallel (fully controlled)	No clear Δfold reported	-	↓ fasting & post-prandial glucose, triglyceride, cholesterol	-	(Candela et al., 2016)
Bran							
Wheat bran (20 g/day)	Coarse vs fine	RCT, 4 weeks, parallel	-	-	-	↑ colonic motility, ↓ transit time	(Kirwan et al., 1972)
Wheat bran (12 & 20 vs 13.2 & 22 g/day)	Raw vs cooked	RCT, 2 weeks, cross-over	-	-	-	↑ fecal weight, stool volume ↓ transit time (raw bran)	(Wyman et al., 1976)

Intrinsic fiber	Processing	Study design	Gut microbiota modulation (Δfold)	Changes in microbiota activity	Changes in metabolic markers	Changes in bowel function	Reference
Wheat bran (20 g/day)	Reduced in size	RCT, 4 weeks, parallel (normal weight and obese)	No change	No change	↑ fasting serum acetate, total SCFA (obese subjects)	No change	(Deroover et al., 2021)
Grains							
Barley (75 g/day)	Whole kernels, boiled, in bread (no milling)	RCT, 4 weeks, cross-over	-	-	↓ postprandial glucose, GLP-1, breath hydrogen	-	(Nilsson et al., 2016)
Barley vs brown rice vs mix of both (60 g/day)	Whole kernels, cooked	RCT, 28 days, cross-over	↑ α-diversity; Moderate Δfold: *Bacteroides*: 0.7-0.8 *Blautia*: 1.4-1.5 *Roseburia:*0.9-1.5 *Bifidobacterium:* 1.0-2.0	-	↓ IL-6 (mix)	-	(Martínez et al., 2013)
Coix (160 g/day)	Whole kernels, cooked	RCT, 1 week, parallel	↓ α-diversity; Small Δfold: *Faecalibacterium:* 1.4	-	↑ & ↓ in subset of lymphocytes	-	(Jinnouchi et al., 2021)
Nuts							
Walnuts (42 g/day)	Whole	RCT, 3 weeks, cross-over	Moderate Δfold: *Ruminococcus*: 0.8; *Dorea*: 0.8; *Roseburia*: 1.7	-	↓ fecal bile acids, cholesterol	-	(Holscher, Guetterman, et al., 2018)
Almonds (57 g/day)	Whole, roasted	RCT, 6 weeks, parallel	↑ α-diversity, Small & Large Δfold: *Mollicutes*: 1.5; *Alistipes:* 0.6; *Sutterella*: 3.7; *Bacteroides fragilis*: 1.2	-	-	-	(Dhillon et al., 2019)
Almonds (42 g/day)	Whole raw (**WR**), whole roasted (**RO**), chopped roasted (**C**), almond butter (**B**)	RCT, 3 weeks, cross-over	Moderate to large Δfold: *Roseburia*: 1.0-1.8 (**B<RO/WR<C**) *Lachnospira*: 1.1-1.6 (**B<WR<RO<C**) *Dialister*: 1.0-2.6 (**B<C<RO<WR**) *Oscillospira*:1.0-1.4 (**WR<RO<B<C**) **(order of Δfold indicated per processing type as abbreviated)**	-	-	-	(Holscher, Taylor, et al., 2018)

Intrinsic fiber	Processing	Study design	Gut microbiota modulation (Δfold)	Changes in microbiota activity	Changes in metabolic markers	Changes in bowel function	Reference
Almonds or pistachios (43 or 85 g/day)	Whole	RCT, 2.5 weeks, cross-over	No clear Δfold reported for stronger pistachio effect	-	-	-	(Ukhanova et al., 2014)
Legumes & Seeds							
Chickpeas or raffinose (200 vs 5 g/day)	Canned; incorporated into soups & desserts	RCT, 3 weeks, cross-over	No clear Δfold reported	No change	-	-	(Fernando et al., 2010)
Linseeds, sunflower & sesame seeds, wheat grains, haricot & kidney beans, chickpeas	Whole vs ground; incorporated into meals (no milling)	RCT, 1 week, cross-over, (fully controlled diet)	-	↑ fecal butyrate, total SCFA ↓ fecal pH	-	↑ stool weight (whole & ground)	(Hovey et al., 2003)
Vegetables							
Broccoli, cauliflower +/- green & red cabbage (up to 800 g/day)	Raw and incorporated in soup or microwaved	RCT, 2 weeks, cross-over (controlled diet)	No clear Δfold reported, association with ↑ *Eubacterium, Egerthella, Alistipes Phascolarctobacterium, Burkholderiales*	-	-	-	(F. Li et al., 2009)
Broccoli and Cauliflower (168 +/- 300 soup g/day)	Frozen & steamed or incorporated into soup	RCT, 2 weeks, cross-over	No clear Δfold reported for ↓ *Rikenellaceae, Ruminococcaceae, Mogibacteriaceae, Clostridium, Clostridiales*	-	-	-	(Kellingray et al., 2017)
Chicory root (30 g/day)	Dried, cut into cubes (~3mm)	RCT, 3 weeks, parallel	↑ β-diversity, large Δfold: *Bifidobacterium:* 4.1 & *Anaerostipes:* 3.2, trophic chain proof	↑ fecal acetate, propionate, butyrate	↓ HOMA-ir glucose variability, fasting glucose microbiota dependent	↑ stool frequency, consistency	(Puhlmann et al., 2022)
Fruits							
Avocado (1 piece/day)	Wholefood	RCT, 12-weeks, parallel (hypocaloric diet)	Moderate to large Δfold: *Bacteroides:* 1.4; *Dialister:* 1.4; *Sutterella*: 3.4; *Bilophila*: 3.5	-	↓ triglyceride levels, CRP, IL-1	-	(Henning et al., 2019)

Intrinsic fiber	Processing	Study design	Gut microbiota modulation (Δfold)	Changes in microbiota activity	Changes in metabolic markers	Changes in bowel function	Reference
Avocado (140-175 g/day)	Whole food, part of meal	RCT, 12 weeks, parallel (partly controlled diet)	Small Δfold: *Ruminococcus*: 0.7, *Faecalibacterium*: 1.3-fold, *Roseburia:* 0.7, *Lachnospira:* 1.4	↑ fecal acetate	↓ fecal bile acids, ↑ fecal fatty acids	-	(Thompson et al., 2021)
Mango (300 g/day)	Whole food	RCT, 4 weeks, parallel	-	↑ fecal valerate ↓ fecal endotoxins	↓ IL-6	↑ stool frequency, consistency	(Venancio et al., 2018)
Kiwi (2 pieces/day)	Whole food	RCT, 3 days, cross-over	-	-	-	↑ stool volume, consistency, frequency	(Wilkinson-Smith et al., 2019)
Dates (~50 g/day)	Dried	RCT, 3 weeks, cross-over	No change	↓ fecal ammonium	-	↑ stool frequency, consistency	(Eid et al., 2015)
Prunes (80 or 120 g/day)	Dried	RCT, 4 weeks, parallel	Small Δfold: *Bifidobacterium*: 1.0-1.1	No change	-	↑ stool weight, frequency	(Lever et al., 2019)
Raisin (120 g/day)	Dried	RCT, 3 weeks, cross-over	-	↑ fecal total SCFA, acetate, butyrate, propionate ↓ fecal bile acids	-	↑ stool consistency ↓ transit time	(Spiller et al., 2003)

-, not assessed; Δfold, fold-change in relative abundance; ↑, increase; ↓, decrease; +/-, with or without; CRP, C-reactive protein;, IL, interleukin; RCT, randomized-controlled trial, SCFA, short-chain fatty acids



CHAPTER 3

Back to the Roots: Revisiting the Use of the Fiber-Rich *Cichorium intybus* L. Taproots

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ABSTRACT

Fibers are increasingly recognized as an indispensable part of our diet and as vital for maintaining health. Notably, complex mixtures of fibers have been found to improve metabolic health. Following an analysis of the fiber content of plant-based products, we found the taproot of the chicory plant (*Cichorium intybus* L.*)* to be one of the vegetables with the highest fiber content, comprising nearly 90 % of its dry weight. Chicory roots consist of a mixture of inulin, pectin and (hemi-) cellulose and also contain complex phytochemicals, such as sesquiterpene lactones that have been characterized in detail. Nowadays, chicory roots are mainly applied as a source for the extraction of inulin, which is used as prebiotic fiber and food ingredient. Chicory roots, however, have long been consumed as a vegetable by humans. The whole root has been used for thousands of years for nutritional, medicinal and other purposes, and it is still used in traditional dishes in various parts of the world. Here, we summarize the composition of chicory roots to explain their historic success in the human diet. We revisit the intake of chicory roots by humans, and describe the different types of use along with their different way of preparation. Hereby we focus on the whole root in its complex, natural form, as well as in relation to its constituents, and discuss aspects regarding legal regulation and safety of chicory root extracts for human consumption. Finally, we provide an overview of the current and future applications of chicory roots, and their contribution to a fiber-rich diet.

Key words: chicory roots, inulin, dietary fiber, human nutrition, traditional medicine

INTRODUCTION

While the relation between nutrition and health has been studied since Hippocrates, scientific approaches focusing on what we eat and how our diets affect our body, only developed in the last century. Already during the early years of nutrition research, the focus shifted from the effects of whole foods towards their isolated ingredients, and from macronutrients towards micronutrients (Mozaffarian et al., 2018). Besides macro- and micronutrients, fibers are also a major component of food, and their contribution to human health and well-being has long been underestimated (O'Keefe, 2019). Here, we briefly discuss the role of fibers, their contribution to our diet, and their presence in our present-day foods. We further focus on the whole roots of the chicory plant that have one of the highest levels of fibers and are a versatile source of fibers and other bioactive compounds.

IMPACT OF FIBERS ON HUMAN HEALTH AND ROLE OF THE COLONIC MICROBIOTA

Fibers are macromolecules consisting mostly of carbohydrate polymers (except lignin) with 3 or more monomeric units, linked in such a way that endogenous human enzymes in the small intestine cannot break them down, rendering them not digested or absorbed at that site (Joint FAO/WHO Food Standards Programme, 2010; Stephen et al., 2017). Consequently, fibers end up in the large intestine predominantly in an undigested form, providing no direct energy, and hence have a low overall caloric load [~2 kcal/g or 8 kJ/g (FAO, 2003)]. Depending on their structure and related physicochemical properties fibers can exert different effects in the gastrointestinal (GI) tract (McRorie & McKeown, 2017). Soluble and water-retaining fibers (such as pectin) contribute to bulking of the digestive chyme and thereby slow down upper GI tract transit, which increases satiety and contributes to weight management (Wanders et al., 2011). In the lower GI tract, insoluble and nonfermentable fibers, such as cellulose or psyllium husks, mechanically stimulate mucus secretion or retain water in the stool, all leading to improved defecation regularity (McRorie & McKeown, 2017). Moreover, soluble fermentable fibers, such as inulin or resistant starch, in turn, are partially degraded by human colonic microbes, collectively termed the microbiota. The colonic microbiota forms one of the most metabolically active parts of our body, and therefore it has been termed a "forgotten organ" (O'Hara & Shanahan, 2006). In recent years, the colonic microbiota has been found to include over 1000 species, notably bacterial, harboring an enormous genetic potential which vastly exceeds that of our own body. Broadly, the colonic microbiota contributes to the maintenance of health and onset of disease (Qin et al., 2010; Rajilić-Stojanović & de Vos, 2014; Salonen et al., 2014; Sekirov et al., 2010), which is partly a result of the colonic conversion of fibers into SCFAs, such as acetate, propionate, and butyrate. Although some of these SCFAs are known to fuel enterocytes and impact GI processes, most are also taken up into the bloodstream leading to systemic effects on metabolic and immune health (Chambers et al., 2018; den Besten et al., 2013; Schroeder

& Bäckhed, 2016). Beyond the production of SCFAs in general, the location of production and related absorption into the bloodstream have been recently found to impact health. Experimental colonic infusion studies revealed that health markers were improved when SCFA concentrations were higher in the distal compared with the proximal colon (Canfora et al., 2017; C. M. van der Beek et al., 2016). Based on these observations, it has been suggested that there is a need for complex fibers that are converted distally in the colon into SCFAs, which may then directly enter the systematic circulation and exert peripheral effects (Hansen & Sams, 2018; Neis et al., 2019; Williams et al., 2017).

FIBER INTAKE DEFICIT IN THE WESTERN WORLD

With all these recent insights into the effect of fibers on human health, their reputation has changed from simply indigestible food ingredients to indispensable components of a healthy diet (Cummings & Engineer, 2017; Reynolds et al., 2019). Despite this, Western diets fall below the recommended fiber intake of ≤30–40 g/d (Mertens et al., 2019; O'Keefe, 2019; Stephen et al., 2017): the so-called "fiber gap" (Jones, 2014). One of the factors explaining this fiber gap is the limited availability of high-fiber foods in contrast to the plentiful and highly consumed refined products in the Western diet (Mertens et al., 2019). An easy solution to alleviate the fiber gap is the use of fiber supplements (McRorie, 2015). Such supplements generally comprise single types of fiber that have been isolated by disintegration of the original plant material and further purification via various processing steps. In the plant, however, fibers do not exist as isolated ingredients, but together with other fibers. The cell walls of plant material are made up of cellulose, hemicellulose, and pectin, which are intertwined in a complex network (Keegstra, 2010), encapsulating storage carbohydrates, such as glucose and fructose polymer fibers (see Figure 1) (Hansen & Sams, 2018). It is this complex network of fibers, rather than the isolated ingredients, that have been traditionally consumed in our daily diet, and has been associated with improved health outcomes (O'Keefe, 2019; Reynolds et al., 2019; Williams et al., 2017).

Observational and intervention studies of recent decades have addressed the health-promoting role of complex dietary fibers in the form of vegetables, fruits, and whole grains (Reynolds et al., 2019). High dietary fiber intake was found to be linked to decreased incidence of cardiovascular disease and related mortality, overall mortality, and type 2 diabetes (Reynolds et al., 2019; The InterAct Consortium, 2015). Similarly, clinical studies reported a reduction in metabolic risk factors related to high dietary fiber intake (Reynolds et al., 2019). Interestingly, a dose-dependent relation between fiber intake and health outcomes was observed, suggesting a higher fiber intake of >25 g per day entails more health benefits (Reynolds et al., 2019). These findings make the current Western fiber intake levels of particular concern and emphasize the need for nutritional solutions to overcome the fiber gap.

Product category	Fiber (g/100g product)	Water (g/100g product)	Fiber (%$_{dm}$)
Seeds&Nuts			
Linseeds	34.8	2.2	35.6
Chia seeds dried	34.4	5.8	36.5
Poppy seeds	24.0	5.2	25.3
Almonds with skin unsalted	10.2	4.6	10.7
Pecan nuts unroasted unsalted	9.6	3.5	9.9
Legumes			
Beans soya boiled	13.2	45.1	24.0
Beans white boiled	9.9	67.0	30.0
Beans kidney red boiled	9.8	66.0	28.8
Peas marrowfat boiled	8.9	64.0	24.7
Peas chick boiled	8.8	64.0	24.4
Grains			
Wheat bran	45.0	8.3	49.1
Wheat germ	16.0	6.1	17.0
Oat bran	15.4	6.6	16.5
Muesli with fruit	8.5	11.0	9.6
Oatmeal	7.3	12.0	8.3
Vegetables			
Chicory root raw	17.5	80.0	87.6
Jerusalem artichoke raw	17.4	78.0	79.3
Tomato sun-dried	12.3	14.6	14.4
Beans broad tinned	5.3	84.1	33.3
Celeriac boiled	4.9	88.0	40.8
Fruits			
Prunes dried	16.1	36.9	25.5
Apricots dried	14.4	25.0	19.2
Goji berries dried	13.0	7.5	14.1
Olives ripe in brine tinned/glass	12.5	68.5	39.7
Figs dried	9.8	30.1	14.0
Bread and Crackers			
Crispbread	11.9	5.8	12.6
Bread linseed	9.6	31.5	14.0
Bread brown/wholemeal with sunflowerseeds	8.7	34.0	13.2
Bread rye	8.2	46.2	15.2
Bread brown/wholemeal with pumpkinseeds	7.8	37.3	12.4
Starchy roots			
Tannia prepared without fat	7.2	69.8	23.8
Taro boiled	3.0	72.9	11.1
Potato sweet boiled	2.3	74.7	9.1
Potatoes boiled with skin	1.8	81.0	9.5
Potatoes without skin boiled	1.6	78.0	7.3
Others			
Seaweed nori dried	36.3	6.5	38.8
Cocoa powder	34.0	15.4	40.2
Almond/imitation paste	8.6	14.2	10.0
Cocoa powder sweetened	6.0	6.2	6.4
Yeast extract Marmite	3.1	39.2	5.1

Figure 1. Fiber content of foods. An overview is presented of the fiber content of foods based on 100 g edible product and on a dry matter basis by correcting for water content. Data is retrieved from the Dutch Food Composition Database NEVO-online (RIVM, 2019) and for inulin-containing vegetables from the FoodData Central of the U.S. Department of Agriculture (USDA) (U.S. Department of Agriculture - Agricultural Research Service, 2019) as referred to by van Loo et al. 1995 (Van Loo et al., 1995). %$_{dm}$, percentage on a dry matter basis

CICHORIUM INTYBUS L. TAPROOTS – A FIBER-RICH ROOT VEGETABLE

Although all plants and their parts contain fibers, corresponding fiber levels vary considerably. We reviewed the food composition data of eight food groups and selected the top five fiber-rich food products from each group, based on their fiber content per 100 g edible product (Figure 2). Certain foods clearly stand out with respect to their fiber content per weight. Seeds, nuts, and wheat bran are all high in fiber, but also contain very small amounts of water, making it difficult to compare them to water-rich foods like fruits and vegetables. Hence, it would be more appropriate to express the fiber content on a dry matter basis (Figure 2). When this is done, vegetables with the highest fiber content on a dry matter basis include chicory taproots, and, to a lesser extent, Jerusalem artichokes. Both root vegetables are rich in fiber, especially due to their high inulin content. Inulin is a storage carbohydrate, which consists mainly of fructose molecules linked via a β(2 ← 1) linkage that cannot be broken down by human intestinal enzymes, and is therefore classified as a dietary fiber (Roberfroid, 2005). Chicory inulin has been instrumental in the discovery and subsequent definition of prebiotics, which are substrates that are selectively utilized by GI microorganisms and confer a health benefit (Gibson et al., 2017). The health benefits of chicory inulin-type fructans on the composition and activity of the gut microbiota have been addressed in a great number of studies, and have recently been reviewed (Le Bastard et al., 2019). These include the well-known stimulatory effect of inulin on potentially beneficial bifidobacteria, increased production of SCFA, and improved stool frequency and consistency (Le Bastard et al., 2019; So et al., 2018). Moreover, systemic effects of inulin consumption on satiety and insulin sensitivity in relation to obesity have also been documented (Rao et al., 2019).

Chicory taproots are, however, much more than just inulin. Besides inulin, chicory roots also contain the cell-wall fibers pectin, cellulose, and hemicellulose that have been studied and acknowledged for their health-promoting effects, such as the reduction of cholesterol or glycemic response by pectins (EFSA, 2010; Femenia et al., 1998; Ramasamy et al., 2013). Within cell walls, inulin is trapped as a storage carbohydrate (Figure 1). This mix of dietary fibers in the chicory root results in a total fiber content of 15–20% wet weight and ≤90% on a dry weight basis (Baert & Van Bockstaele, 1992; Douglas & Poll, 1986; Van Loo et al., 1995). Consequently, chicory roots outscore other commonly consumed vegetables, fruits, seeds, or nuts for fiber content.

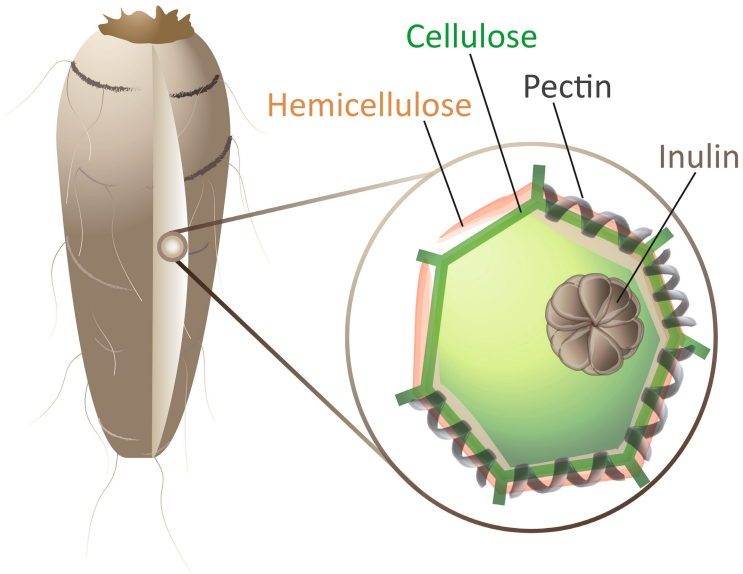

Figure 2. Schematic representation of fiber types in the chicory root. Visualized is the network of the cell wall fibers (cellulose, hemicellulose and pectin) that encapsulates the storage carbohydrate inulin, which is also a dietary fiber.

Chicory taproots are by no means new to human consumption. They have a long history of use in the Western world, and were consumed in Ancient Egypt, Ancient Greece, and the Roman Empire (Hammer et al., 2013). Chicory roots are the taproots of the plant *Cichorium intybus* L., which belongs to the Asteraceae (or Compositae) family. The plant is easily recognizable due to its blue flowers and can be found as a wild or cultivated plant in numerous regions around the world, with various local names (Hegi & Wagenitz, 1987; Kiers, 2000). The cultivated plant is either grown for its leaves (leaf chicory) or its white fleshy taproots (root chicory) (Kiers, 2000). Leaf chicory can have, depending on the variety of *C. intybus* L., either green leaves (sugarloaf), red leaves (radicchio), or white chicons (Belgian endives also known as witlof or witloof) (Hammer et al., 2013; Kiers, 2000). The latter is produced by forcing chicory roots indoors in the dark, which results in blanched tasteful shoots (Hammer et al., 2013). Root chicory is cultivated for the production of root vegetables and inulin (Hammer et al., 2013).

Beside fibers, chicory taproots also contain various micronutrients such as potassium and calcium, as well as many phytochemicals (Leclercq, 1992). The major phytochemicals are sesquiterpene lactones [≤0.81% per dry weight (Rees & Harborne, 1985)], a group of well-known bitter compounds with medicinal activities (Chadwick et al., 2013), for which several structures have been elucidated (Ripoll et al., 2007; T. A. Van Beek et al., 1990). Other phytochemicals include polyphenols, such as chlorogenic acid, coumarins, like cichoriin, and other organic acids (Leclercq, 1992), which are known to have various health-protective effects (Poumale et al., 2013; Tajik et al., 2017). All these fiber and phytochemical components of chicory roots have been exploited by humanity

for medicinal, culinary, and other uses. Some of these uses are still well-known, such as the consumption of chicory root coffee, whereas others are less well-known or have been forgotten.

In this review, we re-examine the use of *C. intybus* L. taproots, hereafter referred to as chicory roots, for human intake. We reconstruct the historical uses of chicory from Ancient Egypt to the beginning of the 21st century. Thereafter, we review safety issues and assessment by legal authorities. We end by detailing the current uses of the components of chicory roots and an outlook for the future applications of the whole chicory root.

HISTORICAL USE OF CHICORY ROOTS

C. intybus L. is believed to be one of the oldest cultivated vegetables in human history (Bostock & Riley, 1855; Hort, 1916). One of the first descriptions of chicory root cultivation dates from around the 3rd century before Christ (BC) when the Greek Theophrastus, a student of Aristotle and one of the first botanists, described chicory and its growth (Hort, 1916). Beside botanists, physicians, cooks, and even poets have also written about the chicory plant, which indicates that the plant enjoyed great popularity.

Chicory roots have been historically used for 3 purposes: 1) as a food product for culinary vegetable dishes and later for the production of ingredients, 2) as a medicinal plant prepared either as a whole or as a base for extraction, and 3) for miscellaneous use, such as cosmetic applications and spiritual intentions (for an overview see Figure 3).

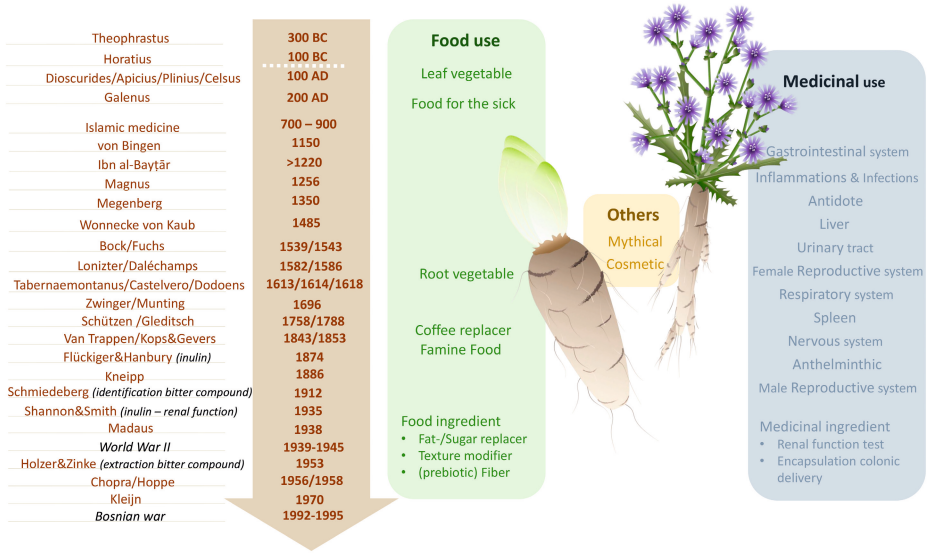

Figure 3. Historical overview of chicory use. An overview is presented of the use of roots from both the wild and cultivated *C. intybus* L. since ancient times in culinary, medicinal or other applications.

HISTORICAL USE OF CHICORY ROOTS AND THEIR INGREDIENTS AS FOOD

The first application of chicory roots for any type of intake seems to relate to their culinary use. Whether they were first used for the production of leaves or as a root vegetable is unclear. Most of the very early references do not specify the plant part that was used. Only Theophrastus specifically mentioned that the leaves were used as food (Hort, 1916). This is also what Horatius, a Roman poet living two centuries later, most likely refers to when he cherishes chicory in his ode to Apollo (Horatius, 1753). The first record of the culinary use of chicory dates back to the 1st century anno domini (AD), in the ancient Roman cookbook Apicius–De re coquinera (Vehling, 2012). During harvesting time, the vegetables were preserved with oil and onions, and during winter consumed with honey and vinegar. Celsus, a Greek writer living in the 1st century AD, also mentioned in his De Medicina a roasted version of chicory (Celsus et al., 1906). This preparation was consumed alone or together with other foods. The bitter taste of the vegetable was also well-known in both the wild and cultivated plant, as described by the Greek physician Galenus in the 2nd century AD (Galenus & Kühn, 1826).

More detailed information on the culinary use of chicory started to appear in the Renaissance and includes a well-cited 1614 cookbook of the Italian, Giacomo Castelvero (Castelvetro & Riley, 1989). He appraised two seasonal vegetable dishes made from chicory roots. In spring, the leaves together with the young roots were harvested and served raw with oil, vinegar, and salt. In autumn, the roots were boiled, cut, seasoned, and eaten with raisins to neutralize the bitter taste. The tradition of eating the cooked root in the autumn months still exists in Italy, where these are considered a regional specialty (Bianco, 2009; Hammer et al., 2013). Contemporaries of Castelvero, such as the German, Tabernaemontanus, described the culinary use of chicory in 1613 and mentioned, similar to Castelvero's recipes, that both the chicory roots and the chicory leaves were consumed in salads (Theodorus & Bauhin, 1613). Moreover, chicory was prepared in mushes, cooked and served as a vegetable dish alone, or used as a condiment in meat and chicken dishes. He concluded his elaboration on the culinary practices by stating that the leaves and the root can be prepared for food use in any way desired (Theodorus & Bauhin, 1613). After the Renaissance, chicory roots continued to be used culinarily in salads and soups in Germany until the beginning of the 19th century (Gleditsch, 1788; Schützens, 1758). Chicory roots even found their way onto confectioners' shelves, where they were sold as confected and candied sweets (Schützens, 1758). In the south west of the Netherlands (Province of Zeeland) chicory roots were also consumed frequently until the end of the 19th century, prepared with vinegar, syrup, or sugar (Kleijn & Brouwer, 1970).

In the second half of the 18th century, the use of chicory roots as a food product started to change, as did the general image of chicory roots. They were still used in vegetable dishes, although the dishes were prepared more out of need in periods of food shortage, and chicory roots thereby became known as a famine food. Chicory roots were one of the few foods available during wartime, for instance during World War II (Vorstenbosch et al., 2017), and the more recent Bosnian War (Redžić & Ferrier, 2014).

Aside from the cooked versions, chicory roots also started to be dried and ground to powder, which was used as flour replacement for breadmaking (Kops & Gevers Deynoot, 1853; van der Trappen, 1843). The chicory root powder eventually also found its way into a new food application: chicory drinks, which were made by roasting the root powder and infusing it with hot water, either with or without real coffee (Gleditsch, 1788; Law, 1850). It is likely that such a drink was known for a longer period of time, but it gained popularity at the end of the 18th century when Frederick the Great prohibited coffee import into Germany (Ebertn, 1870; König, 2000). In the ensuing years, chicory roots were increasingly cultivated for the production of chicory coffee in Europe (Law, 1850). Eventually, the food use of the chicory root changed completely at the end of the 19th and beginning of the 20th century when inulin was discovered (Flückiger & Hanbury, 1874), together with its versatile physicochemical properties as a food ingredient. Consequently, chicory roots started to be used mainly as a source for inulin production, and their use as a root vegetable became less common, apart from certain regions, notably in Italy (Hammer et al., 2013).

MEDICINAL HISTORICAL USE OF CHICORY ROOTS AND THEIR EXTRACTS

Similar to its use as food products, chicory roots also have a long-standing use in medicinal preparations. Beginning in the 1st century AD, the Greek physician, Dioscurides, and the Roman physician, Plinius, mentioned chicory as a medicinal plant (Berendes, 1902; Bostock & Riley, 1855). The sites of application and the types of preparation described in their work laid the foundation of today's core medicinal uses of chicory roots, which predominantly concern the GI tract, the liver, and their anti-inflammatory properties. The same medicinal uses and preparations of chicory were described in the following centuries in various Islamic medicine books, e.g., by the influential Persian physician, Rhazes, in the 9th century AD, and were later summarized by the Andalusian pharmacologist, Ibn al-Baytār (Baithar & von Sontheimer, 1842). Based on the documentation, in the Middle Ages, various European religious authorities expanded the portfolio of chicory's medicinal applications, as described by von Bingen (Throop, 1998), Magnus (E. H. F. Meyer, 1867), and Megenberg (Pfeiffer, 1861). However, it was only in the Renaissance that detailed descriptions of ingredient ratios, preparation methods (e.g., cooking time), and dosing began to be documented as precise recipes in medicinal books. Chicory is mentioned in almost every pharmacopeia written in the 16th and 17th centuries by influential botanists and physicians of the time (Figure 3) (Bock, 1630; Daléchamps & Des Moulin, 1653; Fuchs, 1543; Lonitzer, 1582; Wonnecke von Kaub & Schreiber, 1924). In their recipes, all plant parts were used: roots, leaves, stems, flowers, seeds, and the milky juice. The most impressively detailed documentations of chicory as a medicinal plant were those of the German, Tabernaemontanus, and his Dutch contemporary, Dodoneaus, followed by the Swiss, Zwinger (Zwinger, 1696), and the Dutch, Munting (Munting, 1696).

As a medicinal plant, chicory was historically used mainly in 3 ways: 1) as a preparation of “food for the sick," for consumption by ill or weakened individuals; 2) as

a medicinal preparation for internal use to treat specific organs or diseases; and 3) as a medicinal preparation for external use. "Food for the sick" mainly included a cooked version of the vegetable that was consumed with vinegar to help a malfunctioning GI tract ("weak stomach," bowel movements) (Berendes, 1902; Bostock & Riley, 1855). Later in the Middle Ages, eating the crushed leaves and drinking the juice from the whole plant was also advised for liver and spleen ailments (Baithar & von Sontheimer, 1842; Pfeiffer, 1861). Many more specific preparations existed for the internal treatment of various organs as summarized in Figure 3 (Berendes, 1902; Bock, 1630; Bostock & Riley, 1855; Dodoens & Clusius, 1618; Fuchs, 1543; Lonitzer, 1582; Munting, 1696; Pfeiffer, 1861; Theodorus & Bauhin, 1613; Throop, 1998; Wonnecke von Kaub & Schreiber, 1924; Zwinger, 1696). These medicinal preparations were mostly based on decoctions made from the roots and leaves in water, vinegar, and/or wine, the extracts of which were consumed alone or with wine (Berendes, 1902; Bostock & Riley, 1855; Lonitzer, 1582; Theodorus & Bauhin, 1613; Throop, 1998; Wonnecke von Kaub & Schreiber, 1924). In the Middle Ages, the first preserved medicinal preparations were described. Dried, powdered chicory was mixed with fermented honey and salt to produce a drink (Throop, 1998). Later, in the Renaissance, the juice and leaves were cooked to syrup, the roots were conserved, and flowers confected (Bock, 1630; Fuchs, 1543; Lonitzer, 1582; Theodorus & Bauhin, 1613). Another application included the consumption of the roots with dried cherries but does not specify whether they were cooked or eaten raw (Daléchamps & Des Moulin, 1653). Finally, distillates were also made from the roots, flowers, or a mix of roots and leaves (Bock, 1630). There was even a sugar aromatized with chicory flowers that was used as a universal remedy (Zwinger, 1696).

External applications of the leaves, roots, and juice of the plant were used to alleviate pain (Bock, 1630; Fuchs, 1543; Lonitzer, 1582), to treat infections of the eye and skin (Berendes, 1902; Bock, 1630; Bostock & Riley, 1855; Fuchs, 1543; Pfeiffer, 1861; Wonnecke von Kaub & Schreiber, 1924), and as an antidote against animal bites (Bock, 1630; Pfeiffer, 1861; Theodorus & Bauhin, 1613; Wonnecke von Kaub & Schreiber, 1924). For this purpose, bandages made with the plant material or its extracts, and ointments made from the juice and oily substances were prepared.

Following these extensive documentations of chicory as a medicinal plant around the 17th century, the development of new medicinal recipes ceased. Similar to the developments of its food use, chicory's use as a medicinal plant began to change in the 18th century. On the one hand, chicory entered pharmacies and pharmacognosy handbooks as a herbal drug (Fischer & Hartwich, 1900; Hoppe, 1958; Madaus, 1938), while on the other hand, chicory-derived products became increasingly recognized as folk medicine, which they currently still are in Europe (Gleditsch, 1788; Kneipp, 1921; Schützens, 1758), as well as Asia and Africa (Chopra et al., 1956; Van Wyk et al., 2009). Finally, at the beginning of the 20th century, the medicinal focus shifted completely from the whole root to the isolated ingredients, due to the discovery of inulin as an agent for the testing of renal function (Shannon & Smith, 1935), and the identification (Schmiedeberg, 1912) and extraction (Holzer & Zinke, 1953) of the first chicory root phytochemicals.

OTHER HISTORICAL APPLICATIONS OF CHICORY ROOTS

Beside its food and medicinal use, chicory was also used for various other purposes. The milky juice that is secreted from the root was applied externally for cosmetic reasons, e.g., to prevent hair loss of the eyebrows (Bock, 1630) or to provide skin-firming properties for the female décolleté (Dodoens & Clusius, 1618).

As the plant was part of folk medicine for centuries, mythical properties were also attributed to it, such as attracting people (Bostock & Riley, 1855) or repelling them (Throop, 1998). Over the years, beliefs surrounding the power of the chicory plant entered many sagas and traditions in various countries (Gleditsch, 1788; Hegi & Wagenitz, 1987; Schützens, 1758; Vintler, 1874; von Perger, 1864) . All in all, chicory roots have been part of everyday human life for many centuries, and have been used to feed, heal, and rejuvenate the human body.

SAFETY AND LEGAL ASPECTS OF CHICORY ROOT USE

As indicated above, chicory roots have evidently had a long history of safe use, and are still being consumed in raw and processed forms as part of normal diets in various parts of the world. With the shift away from the use of whole roots toward the extracted, concentrated components of chicory, safety concerns have also arisen over the last century.

ALLERGIES TO THE CHICORY PLANT

A rare allergy to the chicory plant has been documented in ~20 cases over the last 100 y. Most of these cases occurred in adults who were in contact with chicory due to their occupation (Cadot et al., 1996; Friis et al., 1975; Helbling et al., 1997; Herman & Baeck, 2017; Jimenez-Diaz & Cuenca, 1935; Morita et al., 2007; Nemery & Demedts, 1989; Pirson et al., 2009; Willi et al., 2009), and only a single case involved a child reacting to inulin (Streeks et al., 2017). Allergic reactions to the chicory plant have miscellaneous clinical explanations, being either immediate [IgE-mediated (type 1)] or delayed [T-cell mediated (type 4)], or sometimes both (Denisow-Pietrzyk et al., 2019; Herman & Baeck, 2017; Paulsen, 2017). Depending on the individual, allergic symptoms can be systemic and/or local, ranging from rhinoconjunctivitis, to asthma and anaphylactic reactions, to contact dermatitis. As individual as the allergic reactions are, the chicory preparations and routes of exposure are also unique and varied. There are only two cases where fresh chicory roots topically induced an allergic reaction (Friis et al., 1975; Nemery & Demedts, 1989). The majority of reactions occurred in response to leaves (raw and cooked) after skin contact or inhalation (Cadot et al., 1996; Friis et al., 1975; Helbling et al., 1997; Herman & Baeck, 2017; Morita et al., 2007; Nemery & Demedts, 1989). Sometimes, reactions were also caused by the inhalation of dried chicory roots and inulin (Jimenez-Diaz & Cuenca, 1935; Nemery & Demedts, 1989; Pirson et al., 2009), consumption of inulin-containing products (Franck et al., 2005; Gay-Crosier et al.,

2000), and once by intravenous inulin administration during a standard renal function test (Chandra & Barron, 2002).

It is not yet clear exactly how allergic reactions to chicory are triggered. Proteins from chicory or newly formed inulin-protein compounds (arising during production) (Franck et al., 2005; Herman & Baeck, 2017), as well as sesquiterpene lactones could be potential allergens (Denisow-Pietrzyk et al., 2019; Paulsen, 2017). Sensitization might arise from repeated exposures (Herman & Baeck, 2017; Paulsen, 2017) or from cross-sensitization with birch pollen (Cadot et al., 2003; Pirson et al., 2009) or lettuce (Friis et al., 1975; Morita et al., 2007). Due to all this ambiguity, the general advice is that people with allergies or occupational exposure to Asteraceae family members, people with birch-pollen allergies (Cadot et al., 2003), and people with atopic dermatitis should be cautious when coming into contact or consuming chicory- and inulin-containing foods (Paulsen, 2017).

TOXICOLOGICAL ASSESSMENTS OF CHICORY ROOT EXTRACTS

Since the intake of concentrated root extracts may pose a health risk, several safety and toxicological evaluations have been performed in recent years on chicory root inulin, its phytochemical extracts, and chicory root coffee. The toxicological safety of inulin was tested in several *in vitro* and animal models and summarized 20 years ago to pose no toxicity in the amounts administered (Carabin & Flamm, 1999). Human data from clinical studies indicated that inulin has been safely administered intravenously as a renal function agent for nearly 100 years (Shannon & Smith, 1935), even in pregnant women (Lopes van Balen et al., 2019). As a prebiotic fiber, inulin has been tested up to an intake of >50 g per day (Briet et al., 1995) and concluded to be safe (Carabin & Flamm, 1999). The only concerns comprised GI symptoms (such as flatulence and diarrhea), that appeared to be dose- and individual-dependent, but generally arose at doses of 20–30 g per day (Carabin & Flamm, 1999).

The safety of phytochemicals from chicory roots has mainly been evaluated based on concentrated ethanolic extracts of chicory roots using *in vitro* and animal models. Sesquiterpene lactones extracted from chicory roots did not reveal any mutagenic effect in an Ames test, nor any toxicological adverse effects in a rat model, establishing a no-observed-adverse-effect-level (NOAEL) of 1000 mg/(kg*d) (Schmidt et al., 2007). One human study included the safety of a sesquiterpene-rich chicory extract in a one-month phase I trial on osteoarthritis (Olsen et al., 2010). Only one of the participants receiving the highest dose (1800 mg/d) reported adverse effects, headaches, and diarrhea. The other 24 subjects in the intervention group showed no adverse change (Olsen et al., 2010). Consequently, it was concluded that there are no safety concerns in the clinical use of chicory root extracts (Olsen et al., 2010). Additionally, the external application of sesquiterpene lactones for human use has recently been studied in skincare formulations and found to be safe (Patrícia M B G Maia Campos et al., 2019).

Two trials evaluated the safety of a combination of inulin and phytochemicals in chicory coffee. It should be noted that the composition of chicory coffee is different

from raw chicory roots since inulin and phytochemicals are partly broken down during the roasting process forming new chemical compounds (Ripoll et al., 2010; Schumacher et al., 2011). The effect of chlorogenic acids on thrombosis prevention was tested in 27 subjects who consumed 20 g of chicory coffee in 300 mL for one week. They did not observe any negative side effects during this short time period and found some, but variable, effects on the measured thrombosis markers (Schumacher et al., 2011). Similarly, the effect of inulin on GI tolerance was tested in chicory coffee with a higher inulin content produced by a new method (Ripoll et al., 2010). Short-term (six days) consumption of ≤500 mL chicory coffee containing ≤7.8 g inulin, as well as long-term (four weeks) consumption of 500 mL chicory coffee containing 5 g inulin, did not lead to any adverse GI symptoms in any of the 35 subjects.

LEGAL STATUS OF CHICORY ROOT EXTRACTS

From a legal perspective, chicory inulin obtained from *C. intybus* L. has been assigned the status of generally recognized as safe (GRAS) by the FDA in their list of Substances Added to Food (U.S. Food & Drug Administration, 2003). GRAS is a status given to food components that have been proven to be safe due to a long history of consumption in a meaningful number of people. The FDA also lists a chicory root extract [Chemical Abstracts Service Registry Number (CAS Number) 68650-43-1] with GRAS status as a coloring and flavoring agent (U.S. Food & Drug Administration, 2018). The European Food Safety Authority (EFSA) has approved a health claim for unfractionated chicory root inulin obtained from *C. intybus* L. (called "native chicory inulin") at a dosage of 12 g per day in relation to stool regularity (EFSA, 2015). The European Medicines Agency (EMA) provided an assessment of the traditional medicinal use of chicory roots in Europe (EMA, 2013), and concluded that the traditional use of chicory roots for the treatment of GI complaints and to stimulate appetite is supported by scientific evidence and does not pose any safety risk. Nevertheless, based on the scientific data included in this assessment, it was not recommended to enter chicory root as a medicinal product, due to insufficient scientific information (EMA, 2013). Nevertheless, chicory root is still regarded as folk medicine in several European countries, such as Germany, France, and the Czech Republic, and is listed by their respective governmental authorities as a medicinal substance (Bundesgesundheitsamt, 1987; Ministère des affaires sociales et de la solidarité, 1990; Ministerstva zdravotnictvi Ceske republiky se spolupracovniky, 1993).

CONTEMPORARY USES OF CHICORY ROOTS

Presently, chicory inulin-based ingredients and root coffees are the most recognized applications of chicory roots. However, new methods of using chicory roots seem to be evolving, fueled by the combined interest in the nutritive and medicinal value of chicory roots, as well as increasing demand for autochthonous, traditional, and natural foods (Albuquerque et al., 2018; Almli et al., 2011).

FOOD USE IN THE 21ST CENTURY

As mentioned earlier, chicory roots are currently mainly used for the production of inulin, which is a highly versatile food ingredient and is generally extracted by a hot water process (D. Meyer & Blaauwhoed, 2009). After extraction, inulin is purified in a rather intense process, and can then be further refined based on its chain lengths, requiring additional solvents like ethanol, methanol, or acetone (Mensink et al., 2015b; Zhu et al., 2016). Inulin of different chain lengths has different physicochemical properties and, hence, can be added to food products for various purposes (Mensink et al., 2015b), which can be generally categorized into sugar replacement (short-chain inulin), fat replacement (long-chain inulin), or texture modification (long-chain inulin) (Leyva-Porras et al., 2015). Besides changing the sensory properties of a product, inulin can also be added to a food product to increase its prebiotic dietary fiber content, or consumed as a food supplement (Roberfroid, 2005). Due to its importance as a food ingredient, optimization and sustainability of the, until now, energy- and time-consuming extraction process of inulin from chicory roots is an ongoing research topic (Zhu et al., 2016). In addition, the cell wall fibers in chicory pulp, which emerge as waste during inulin production are currently being examined for their potential as a food ingredient (Pi et al., 2019) or fiber supplement (Wijlens et al., 2013). Chicory pulp was reported to increase satiety *in vivo* (Wijlens et al., 2013) and is rapidly fermented *in vitro* (Ramasamy et al., 2014).

Besides using chicory roots in the isolated form of inulin, they are also still used for the production of chicory coffee, which is valued today as a caffeine-free alternative to coffee (Löhmar et al., 2003). It is also often used to add a characteristic flavor to coffee or alcoholic beverages (Leclercq, 1992), such as beer or vodka. For its use in beverages, chicory is dried and roasted (Löhmar et al., 2003). During the roasting process, depending on the conditions used, different flavor compounds can be formed, the characterization of which is a developing research area (Wei et al., 2016).

Today, the use of whole fresh roots is limited to areas where these have been traditionally consumed. Despite this, efforts have been made to preserve genetic resources and revive the culinary use of *C. intybus* L. roots, for instance in Italy where contemporary consumption in Sicily, Liguria, and Lombardy has been noted (Gaetano et al., 2012; Laghetti et al., 2018). Of interest, an annual festivity for chicory roots (Sagra delle Radici) is organized in autumn in Socino, in the Lombardy region (Bianco, 2009). Furthermore, various recipe ideas are promoted on websites of organizations and hobby cooks. More than 25,000 tons of chicory are still produced in Italy annually for consumption of the roots (Bianco, 2009). It is likely that chicory roots are still traditionally consumed in other countries, but it is not broadly known or described due to their existence as a regional specialty.

MEDICINAL USE IN THE 21ST CENTURY

Chicory inulin, similar to its food use, also forms the main application of chicory roots for medicinal purposes. Chicory inulin is still used for the measurement of renal

function (glomerular filtration rate), and for the colonic delivery of drugs. Chicory's inulin suitability as an agent for renal function tests originates in its low molecular weight, being neither absorbed nor metabolized by the kidney but readily excreted (Carabin & Flamm, 1999; Mensink et al., 2015a). As a colonic delivery medium, inulin can be chemically modified to carry or encapsulate drug compounds. Drugs that would be absorbed on their own, early in the GI tract, are now transported to the colon, where inulin is broken down by gut bacteria and the compound is released (Leyva-Porras et al., 2015; Mensink et al., 2015a).

Besides chicory inulin, the relevance of the phytochemical compounds in chicory roots has also been increasingly recognized in recent years. There is a large body of research on the medicinal effects ascribed to the phytochemicals of chicory roots, which have been recently reviewed (Das et al., 2016; Saeed et al., 2017; Seetaloo et al., 2018; Street et al., 2013). The majority of those studies are based on *in vitro* or animal models (Das et al., 2016). A single human intervention study has been reported that assessed the anti-inflammatory potential of chicory sesquiterpene lactones in patients with osteoarthritis (Olsen et al., 2010). All 3 tested dosages (600, 1200, and 1800 mg/d) led to an improvement of pain, stiffness, and general functionality assessment, with the highest improvement in the highest dose group, albeit without reaching statistical significance (Olsen et al., 2010). The medicinal field is still evolving, and present interest is focused on reproducing effects in humans and elucidating the underlying mechanisms.

OTHER USES OF CHICORY ROOTS IN THE 21ST CENTURY

Besides human food use, chicory roots are also still used for cosmetic purposes. Although the juice of chicory roots was advised in the 17th century to be a skin-firming agent, chicory root extract has recently been rediscovered as a protective and regenerative component in skincare formulations (P M B G Maia Campos et al., 2017).

In recent years, the health benefits of inulin have also been discovered and proven in animals, and consequently chicory inulin is nowadays added into feed for domestic and companion animals (Verdonk et al., 2005). For an even longer time, chicory pulp has been added to animal feed formulations as a cheap, fiber-rich ingredient (Fone, 1998). Recently, the use of whole chicory roots has also been evaluated for nutritive and medicinal benefits for animals (Nwafor et al., 2017; Uerlings et al., 2019). For instance, in trials with companion animals it has been shown that dried whole chicory roots, as a source of prebiotic fibers, provided health benefits and contributed to longevity (Cupp et al., 2008; Grieshop et al., 2004). Consequently, whole chicory roots are now also tested in studies with domestic animals for their potential as nutritive and health-promoting feed components (A Lepczyński et al., 2016, 2017; Adam Lepczyński et al., 2015; Robak et al., 2019).

FUTURE USE OF CHICORY ROOTS FOR HUMAN INTAKE

Although isolated chicory root compounds are still widely investigated for their functionality, studies have started emerging that focus on the whole vegetable instead of the isolated ingredient, with the aim of increasing the intake of multiple fibers and exploiting the bioactive potential of the phytochemicals present in the chicory root. One report focused on the prebiotic effect of inulin in combination with probiotic feta cheese on lipid metabolism (Mohammad Moradi et al., 2013). However, instead of consuming inulin, participants drank a watery extract made from a decoction of raw chicory roots. The consumption of this combination led to a significant reduction in total and LDL cholesterol, as well as a reduction in serum triglyceride concentrations, which the probiotic cheese alone did not achieve (Mohammad Moradi et al., 2013). Another report studied the effect of inulin from roasted chicory roots on glucose and lipid metabolism and intestinal health (Nishimura et al., 2015). For this purpose, chicory coffee, instead of conventional inulin supplementation, was used. A significant improvement of glycated hemoglobin (HbA1c) and stool frequency was found, however, no improvements in fasting glucose or insulin concentrations and lipid metabolism were observed. Chicory coffee was also used in relation to its phenolic acid content (chlorogenic acids), which was thought to beneficially affect thrombosis prevention, as was previously demonstrated *in vitro* and *in vivo* in mice using synthesized compounds (Schumacher et al., 2011). In the human intervention, it was found that the *in vivo* effects were far lower than expected and blood values remained unchanged. Despite this, chicory coffee had a positive effect on whole blood and plasma viscosity, as well as RBC deformability (Schumacher et al., 2011). Furthermore, a very recent preliminary report mentioned the use of dried and ground chicory roots to replace fat in burgers. Interestingly, a replacement of 75% of the fat could be achieved, which improved water retention during cooking. This also affected sensory properties, although within an acceptable range (Zeny et al., 2019). No study has yet examined the effect of whole chicory roots on human health. One very recent study investigated the effect of inulin-containing vegetables instead of isolated inulin on food intake and GI health (Hiel et al., 2019), but did not include chicory roots. It was observed that a 2-wk intake of, on average, 15 g/d inulin via whole foods increased fecal bifidobacteria and satiety ratings, similar to the effects of isolated inulin, without leading to negative effects on GI tolerability (Hiel et al., 2019). Consequently, it was concluded that the inclusion of inulin-rich whole foods in the diet might be a health-benefitting food-behavior strategy.

CONCLUSION

Chicory roots have a long history of traditional use for human consumption. Although they have enjoyed broad popularity for >2000 y, chicory roots have become increasingly connoted as a famine food over the last 3 centuries, and have today been nearly

forgotten as a food source apart from their use in some regional specialties, notably in Italy. With the recent recognition of the importance of dietary fiber for the improvement and maintenance of human health, the fiber-rich chicory roots offer an attractive option to close the gap between fiber recommendations and consumption. Moreover, chicory roots meet consumer interest in being minimally processed, autochthonous, and natural foods. The mix of cell wall fibers and the phytochemical content, in addition to the inulin content of chicory roots, offer interesting nutritional options for improving human health. Revisiting chicory roots in the 21st century as a rich and versatile source of complex dietary fibers opens up opportunities to combat a range of chronic metabolic diseases originating in the current fiber-poor Western diet.

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The authors report no conflicts of interest. The authors' responsibilities were as follows: MLP searched and collected the literature and wrote the draft manuscript; WMdV conceived the idea and corrected the manuscript; all authors read and approved the final manuscript.

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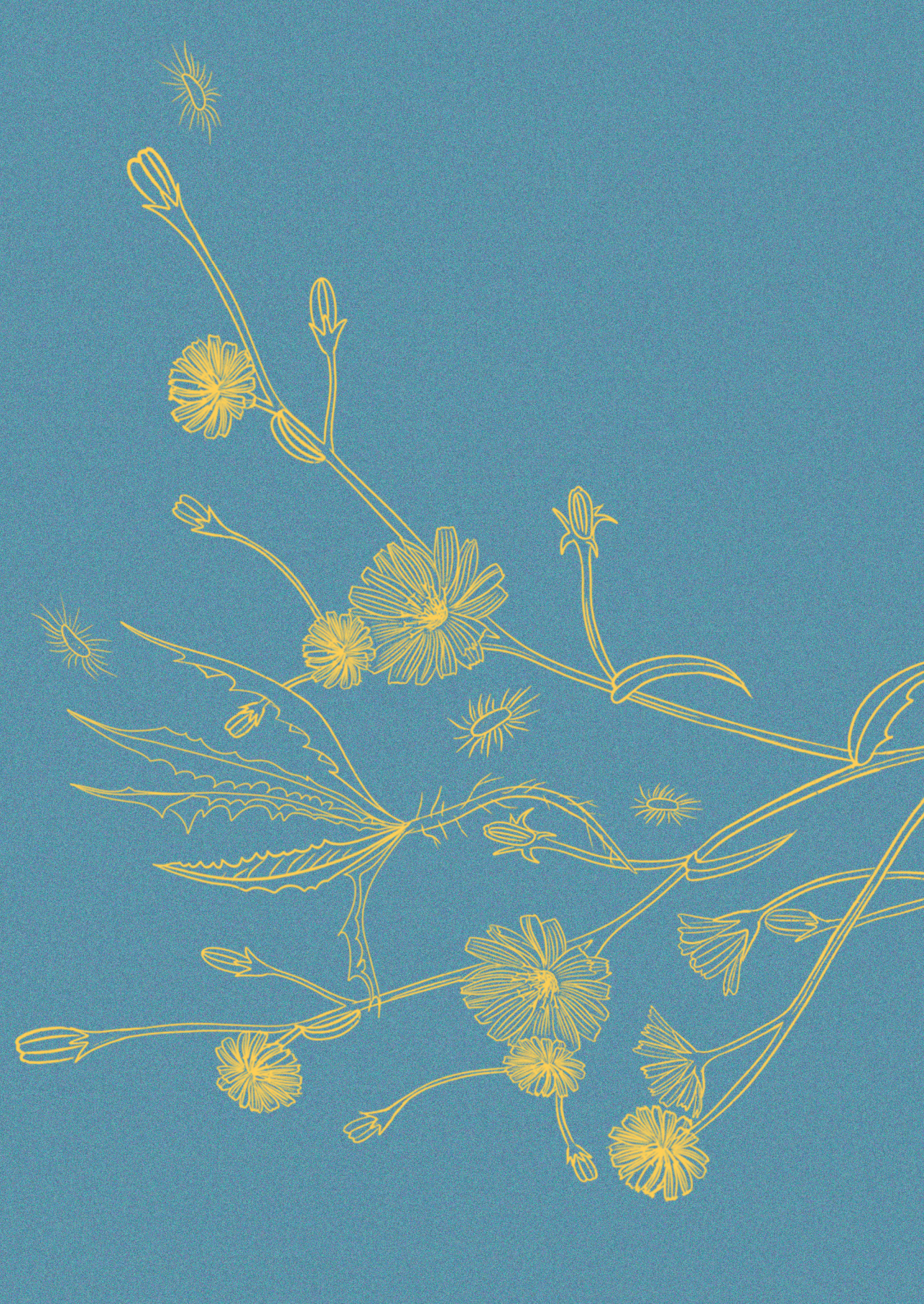

CHAPTER 4

Analysis of the fermentation kinetics and gut microbiota modulatory effect of dried chicory root reveals the impact of the plant-cell matrix rationalizing its conversion in the distal colon

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ABSTRACT

Aim: The cell matrix of plant foods has received little attention in prebiotic fiber research. We aimed to understand the impact of the plant cell matrix in dried chicory root on its breakdown in the human gut to explain its reported beneficial effects on gut and metabolic health.

Methods: We applied in vitro digestion and fermentation models together with an ex vivo gut barrier integrity model. Plant cell matrix intactness in the upper gastrointestinal tract was investigated by scanning electron microscopy. Colonic breakdown of inulin, and chicory root cubes and powder was assessed by gut microbiota analysis using 16S rRNA gene amplicon sequencing and determining the kinetics of changes in pH, gas, and short-chain fatty acid production. Finally, effects on gut barrier integrity were explored by exposing colonic biopsies to fermentation supernatants in an Ussing chamber model.

Results: The plant cell matrix of dried chicory root cubes remained intact throughout upper gastrointestinal transit. Dried chicory root fermentation resulted in higher final relative abundances of pectin-degrading *Monoglobus* and butyrate-producing *Roseburia* spp. compared to inulin and a seven-fold increase in *Bifidobacterium* spp. in donors where these species were present. Dried chicory root cubes yielded similar total SCFA but higher final butyrate levels than chicory root powder or isolated inulin with less gas produced. No uniform but donor-specific effects of fermentation supernatants on maintenance of gut barrier integrity were detected.

Conclusion: The intact plant cell matrix of dried chicory root affected its colonic breakdown kinetics and microbiota, underpinning its beneficial effect *in vivo*.

Keywords: plant cell wall, chicory root, intrinsic fiber, gut health, gut microbiota, colonic fermentation, butyrate production.

INTRODUCTION

Dietary fibers are omnipresent in human food products and play a fundamental role in maintaining human health. The health benefit of fibers was originally attributed to their physicochemical properties improving satiety by water-binding and regulating lipid homeostasis by binding cholesterol and bile acids (Gill et al., 2020). However, research of the past two decades revealed that a large part of the beneficial effect of fibers is mediated by the human gastrointestinal tract microbiota. As dietary fibers are by definition compounds that cannot be broken down by human endogenous enzymes, they reach the lower gut undigested (Gibson et al., 2017; Joint FAO/WHO Food Standards Programme, 2021). There they are used as substrates by the human gut microbiota, a collective of fungi, protozoa, archaea, and mostly bacteria that have the enzymatic machinery to break down dietary fibers and thereby produce a range of microbial metabolites (Flint et al., 2012). The interaction between the gut microbiota, its metabolites and the human body mediates the beneficial effect of fibers on human health beyond their physical interaction with the upper gastrointestinal tract. A major group of fiber-derived bacterial metabolites are short-chain fatty acids (SCFAs), including acetate, propionate and butyrate that signal to GPR receptors and may have local or systemic effects (Blaak et al., 2020). Notably butyrate has been recognized to be essential for maintaining human gut health as it serves as fuel for colonocytes, strengthening the gut lining and improving gut barrier function (Hamer et al., 2008). That has led to rising efforts to understand how butyrate production in the human colon can be stimulated using dietary fibers.

Approaches to study fiber-microbiota relationships have progressively become reductionist with a focus on single fibers' molecular breakdown (Cantu-Jungles et al., 2021). One particularly studied fiber is inulin, known for its prebiotic effect, which means it is selectively used by human gut bacteria – notably bifidobacteria - thereby conferring health benefits (Gibson et al., 2017) contributing to a health claim for stool frequency maintenance (EFSA, 2015). Another well-studied group of fibers includes pectins that have been recognized to regulate postprandial glucose response, blood cholesterol levels and satiety (EFSA, 2010), while some of their fragments have immune-modulatory benefits (RG-I; (Lutter et al., 2021)). For the purpose of establishing such structure-function relationships and health outcomes fibers are often studied in isolation, which means they have been extracted and purified from the original plant food matrix. It is hypothesized that these isolated fibers are mainly fermented in the proximal colon with subsequently low levels of SCFAs in the distal colon, which favors fermentation of residual proteins (Korpela, 2018; So et al., 2021). While these reductionist approaches allow us to understand how specific fibers elicit specific microbial responses, they ignore how fibers are commonly consumed: intrinsically present in plants foods with each their unique plant cell matrix (Augustin et al., 2020; Puhlmann & de Vos, 2022). Fibers like pectin and inulin in plant foods make up an intrinsic, complex plant cell network in which pectin is intertwined with hemicellulose and cellulose forming

the structure of the plant cells encapsulating other non-structural fibers, like inulin (Augustin et al., 2020; Puhlmann & de Vos, 2022). This complex network does not dissolve into its isolated compounds in the human gastrointestinal tract but rather functions as a vehicle transporting these fiber structures to the lower gut (Capuano, 2017; Williams et al., 2017). For these reasons, fibers originally present in the plant matrix have been termed intrinsic fibers to distinguish them from isolated fibers extracted from the plant cells (Augustin et al., 2020).

Chicory root is a root vegetable with a plant cell matrix encapsulating particularly high amounts of inulin inside the plant cell vacuoles. While chicory roots are nowadays mainly used for the production of isolated inulin, they have long been used as medicinal and culinary root vegetable (Puhlmann & de Vos, 2020). In its dried form, chicory root consists of up to 85% fiber of which 70% inulin, which makes it an excellent source of intrinsic dietary fiber (Puhlmann & de Vos, 2020). We hypothesize that the presence of the plant cell wall can potentially function as a physical barrier, shielding inulin from immediate contact with the gut microbiota thereby impacting intrinsic fiber's breakdown kinetics and location in the human gut. Food processing steps like particle size reduction (Day et al., 2012; De Paepe et al., 2019; Stewart & Slavin, 2009; Tuncil et al., 2018; Yao et al., 2023) or thermal treatment (Day et al., 2012; Rovalino-Córdova et al., 2020) have the potential to affect gut bacteria accessibility and related breakdown kinetics due to the induced damage of the plant cell matrix. Until now, *in vitro* assessments of plant food breakdown kinetics have focused on pectin-, starch- and lipid-containing whole foods (Low et al., 2015; Solvang et al., 2023; Widaningrum et al., 2020; Yao et al., 2023) but never inulin-rich vegetables like chicory root. Previously, we have shown in a placebo-controlled human trial that the intake of dried chicory root particles dramatically modulated gut microbiota composition by stimulating a trophic chain involving members of *Bifidobacterium* spp. and *Anaerostipes* spp. towards butyrate production and improved both gut and metabolic health (Puhlmann et al., 2022). We attributed these changes to a slow release of fibers from dried chicory root particles. This would prolong fermentation of the gradually rehydrated particles rationalizing a rather distal location of their breakdown. In humans, a slow and gradual fiber fermentation has been hypothesized to benefit gut health by distributing fiber fermentation from the proximal throughout the distal colon (So et al., 2021). Moreover, SCFAs delivered to the distal colon have been shown to confer more pronounced systemic health benefits compared to a proximal delivery (Canfora et al., 2017; Neis et al., 2019).

Our aim was to assess whether the plant cell matrix of dried chicory root remains intact in the upper gastrointestinal tract and how its presence affects lower gut microbial composition and fermentation kinetics as well as the potential effect of dried chicory root fermentation supernatants on gut barrier integrity. For this purpose, we executed a series of experiments using *in vitro* and *ex vivo* models for the upper and lower gastrointestinal tract that were primed with dried chicory root particles with two different degrees of cell wall intactness in the form of cubes and milled into powder (Figure 1).

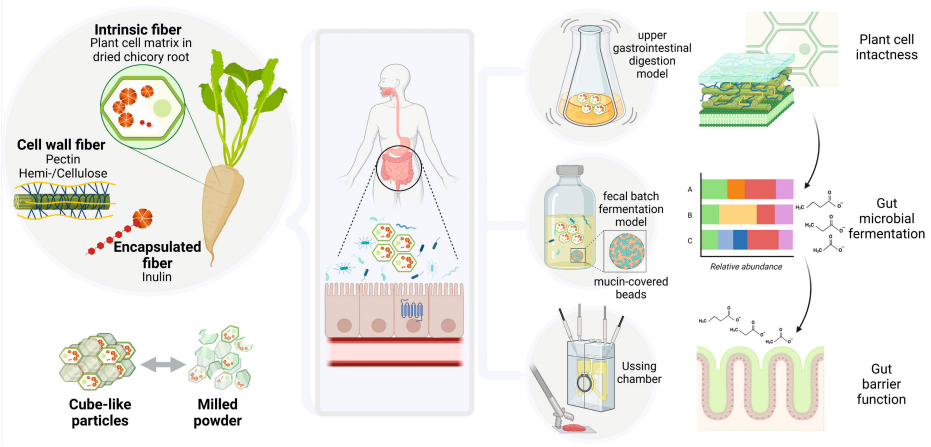


Figure 1. Study approach. Schematic depiction of the study approach to understand how the plant cell matrix in dried chicory root cubes and powder affects its upper digestion and lower gut microbial fermentation kinetics and subsequent interaction with the gut barrier. An upper *in vitro* digestion model was used to assess the intactness of the plant cell matrix upon entering the lower intestine (colon), where using an *in vitro* fecal batch fermentation model we studied gut microbial breakdown. Finally, we investigated how the fermentation metabolites potentially interacted with the human colonic epithelium using an Ussing chamber. Created with BioRender.com.

METHODS

DRIED CHICORY ROOT

Dried chicory root was provided by WholeFiber BV (Emmeloord, The Netherlands). The product is made from chicory roots that have been washed, cut and dried, producing cube-like pieces of approximately 3 mm rib. The final product has a dry weight of 93 %w/w of which 70 %w/w is native inulin, 10 %w/w pectin and 5 %w/w hemicellulose and cellulose and 4 %w/w mono- and disaccharides, 5 %w/w proteins, and remaining minerals, polyphenols and vitamins. To assess whether dried chicory root in its structure was similar to fresh, unprocessed chicory root, also fresh chicory root was provided by WholeFiber BV. To study the effect of particle size, dried chicory root cubes were ground to a mean particle size of <0.5 mm.

UPPER GASTROINTESTINAL DIGESTION MODEL

To assess potential physical changes during upper gastrointestinal digestion, we mimicked digestive processes during the oral, gastric and small intestinal phase using an adapted version of the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014). Experiments were executed in triplicate and samples were taken at the end of the oral phase (2 min) and after 30, 60 and 120 min of both the gastric and small intestinal digestion by separating the liquid from the solid digesta using a sieve. Liquid digesta were heat-treated and snap frozen in liquid nitrogen and solids were fixated in 70 %

ethanol for immediate image processing. We visualized the plant cell matrix of fresh and dried undigested and digested chicory root cubes using scanning electron microscopy (SEM) and light microscopy to assess structural changes. Potential leakage of pectin from the plant cell matrix was estimated by measuring uronic acid concentration in the gastric and small intestinal phase liquid digesta using an automated colorimetric *m*-hydroxydiphenyl assay (Thibault, 1979) and expressing measured levels as percentage of an expected total uronic acid content (5 %w/w) in chicory root as determined according to Ramasamy et al. (Ramasamy et al., 2013). Potential differences between inulin from dried chicory root powder versus cubes leaking into digestive fluids was estimated by measuring mono-/disaccharides and fructan-oligomers and polymers of different chain lengths (degree of polymerization, DP) using High Performance Anion Exchange Chromatography (HPAEC) according to established methods (Logtenberg et al., 2020). Details on the adapted INFOGEST method, the pectin and inulin measurements can be found in the online Supplementary Material.

LOWER GASTROINTESTINAL FERMENTATION MODEL

To investigate the effect of the presence and intactness of the plant cell wall matrix on the lower gut microbial fermentation of the dried chicory root, a fecal *in vitro* batch fermentation experiment was performed at ProDigest (Gent, Belgium) using dried chicory root cubes, powder and isolated chicory inulin as comparison. For this purpose, feces of a healthy human donor with low bifidobacteria count (assessed by qPCR to be $<10^8$ 16S rRNA gene copies/g corresponding to a relative abundance of <0.01 %) was chosen. This was done to exemplify how such a gut microbiota would respond to an intrinsic fiber product since high baseline bifidobacteria levels reportedly affect related fiber-responses (De Preter et al., 2008; Healey et al., 2018; Kolida et al., 2007; Korpela et al., 2014). Experiments were executed in triplicate and samples were taken at baseline (t = 0 h), after 6, 24 and 48 h. Details on the execution of the experiment including mucin-covered microsomes (Van Den Abbeele et al., 2012) and the subsequent analysis of gut microbiota composition in fermentation pellets, pH and gut microbial metabolites SCFAs, lactate, ammonium and branched-chain fatty acids (BCFAs) in fermentation supernatants, and gas production are given in the online Supplementary Material.

HUMAN GUT BARRIER FUNCTION MODEL – USSING CHAMBER

To explore possible effects of fermentation products on gut barrier function we performed an *ex vivo* Ussing chamber experiment using human colonic biopsies according to previously described methods (Tabat et al., 2020). Biopsies from four healthy donors (mean age 42 years) were obtained by endoscopy without prior bowel cleansing and exposed to fermentation supernatants from *in vitro* fermentation of dried chicory root cubes. The same donor's feces were used to obtain fermentation supernatants for the respective colonic biopsy to study the individual interaction between the donor's gut epithelium and their gut microbiota induced fiber fermentation. Changes in transepithelial resistance (TER; measure of overall gut integrity(Thomson

et al., 2019)) and paracellular permeability (assessed by Fluorescein isothiocyanate–dextran concentration; assessing passage between cells) in control biopsies over time were compared at 60 and 90 min to biopsies previously exposed (20 min) to the fermentation supernatant and biopsies stressed with sodium deoxycholate (SDC) as well as biopsies both exposed to the fermentation supernatant and stressed with SDC. Details on the human donors, the fecal *in vitro* batch fermentation model as well as analysis of the gut microbiota composition in fermentation pellets, pH and gut microbial metabolites in fermentation supernatants, and gas production are given in the online Supplementary Material.

STATISTICAL ANALYSIS

Data were analyzed using R version 4.2.3 (R Core Team, 2023). Normality was checked by inspecting QQ-plots. Descriptive statistics were calculated using the rstatix package (Kassambara, 2023) and data was expressed as mean with standard error of the mean (SE). While the use of statistical hypothesis testing for small sample sizes as commonly used in *in vitro* triplicate experiments is debatable, statistical inference was done for the purpose of understandability. Difference in metabolites (SCFA, BCFA, lactate and ammonium) and other outcomes (pH, gas production, α-diversity) between products at each timepoint were tested using robust ANOVA with corresponding post-hoc and Benjamini-Hochberg correction from the WRS2 package (Mair & Wilcox, 2019) and implemented in the ggstatsplot package (Patil, 2021). Robust ANOVA was chosen as it can handle violations against normality and homoscedasticity and as sample sizes were too small (triplicates) for reliable non-parametric testing implementing Chi-square distributions. Differences in gut integrity and permeability at each timepoint between conditions were tested using ggstatsplot within-subject ANOVA accounting for the biopsies' paired nature. Graphs were made using ggplot2 (Wickham, 2016) or Microsoft Office 365 Excel. Gut microbiota outcomes were analyzed as described previously (Puhlmann et al., 2022) using the mare (Korpela, 2016) and vegan package (Oksanen et al., 2022). Multivariate community analysis was done on genus level by using Principal Coordinate Analysis (PCoA) and constructing a Principal response curve based on Bray-Curtis dissimilarity (β-diversity) as well as calculating gut bacterial richness (α-diversity) based on the number of detected taxa. For univariate analysis at the genus level taxa counts were converted into relative abundances (%) and differential abundance testing with false-discovery rate (fdr) correction was performed as implemented in mare (Korpela, 2016).

RESULTS

We employed a series of complementary *in vitro* and *ex vivo* gastrointestinal models primed with dried chicory root cube-like particles or milled into powder as to determine

the intactness of the plant cell walls, its impact on gut microbial fermentation kinetics and composition, and finally the effect on gut barrier integrity in human colonic biopsies.

INTACTNESS OF THE PLANT CELL WALL IN DRIED CHICORY ROOT AS ASSESSED IN AN UPPER GASTROINTESTINAL IN VITRO DIGESTION MODEL

First, we set out to assess whether the plant cell matrix was still intact after drying the chicory root particles. Using scanning electron microscopy (SEM), we indeed observed that the overall plant cell structure was intact in both freshly cut chicory root pieces (Figure 2A-C) and rehydrated chicory root cubes (Figure 2D). Plant cells were open (Figure 2B) with small level of damage resulting most likely from cutting the plant particles for image preparation. Within the plant cells, inulin was clearly visible as a crystalline structure due to precipitation in ethanol (Figure 2C). Next, we hydrated the chicory root products as to mimic its consumption and conducted an upper *in vitro* digestion with an oral, gastric and small intestinal phase using the well-established INFOGEST procedure (Brodkorb et al., 2019; Minekus et al., 2014). After oral and gastric digestion with a pH lowered from 3 to 2 (to mimic the fasted state/end of gastric digestion), the overall plant structure remained largely intact with densely packed plant cells filled with inulin (Figure 2E). Similarly, at the end of the small intestinal phase, no obvious damage to plant cells in the form of holes or cracks in the cell wall was observed (Figure 2F). The same was confirmed by light microscopy (Supplementary Figure 1). However, throughout the gastric and small intestinal phase, the macrostructure (Supplementary Figure 2) weakened overall, with plant cells appearing less round and robust (Figure 2E-F). To estimate how much pectin was potentially leaking from the plant cell structure of the dried chicory cubes compared to the powder, we measured uronic acid content as proxy for pectin in the liquid part of gastric and small intestinal digesta. Estimated based on the total uronic acid (UA) content in dried chicory (Supplementary Figure 3), we found that on average (mean ± SD) 16.71 ± 2.91 % of pectin (UA: 8.36 ± 1.46 mg/g product) leaked from dried chicory root powder, which was slightly higher than for chicory root cubes with 10.20 ± 0.70 % of pectin (UA: 5.10 ± 0.35 mg/g product). Throughout the small intestinal phase, the leaked pectin increased for powder to 27.56 ± 0.73 % (UA: 13.782 ± 0.37 mg/g product), which was nearly twice as high as for chicory root cubes with 14.48 ± 1.25 % (UA: 7.24 ± 0.63 mg/g product). Besides pectin, we also investigated differences in inulin of various chain lengths (DP) detected in the digesta of dried chicory root powder and cubes by HPAEC chromatograms (Supplementary Figure 4). We observed a consistently higher total area under the curve of the HPAEC chromatograms for dried chicory root powder compared to the cubes, representing all detected mono-/disaccharides and fructan-oligomers and polymers. No changes in chain length distribution throughout the digestion phases were observed but dried chicory root powder had highest amounts of fructose mono- and oligomers (DP2 – 5) and longer-chain inulin (> DP16) compared to dried chicory cubes.

DIFFERENCES IN GUT MICROBIOTA RESPONSE TO DIFFERENT PARTICLE SIZES OF DRIED CHICORY ROOT COMPARED TO INULIN.

Following the assessment of changes in the upper gastrointestinal tract, dried chicory root cubes and powder were subjected to breakdown by the gut microbiota and compared to inulin using a fecal batch fermentation experiment. At baseline, all samples had a similar microbiota composition as determined by 16S rRNA gene amplicon sequence analysis (online Supplementary Figure 5). In line with the selection of a donor with low bifidobacteria count no *Bifidobacterium* spp. were detected at baseline (Figure 3). Following inoculation, we observed a rapid increase in *Escherichia-Shigella* spp. in all conditions (Control, dried chicory root cubes and powder and inulin), which was highest at 6 h, making up approximately half the microbial community, and decreasing to about 30% at 48 h (online Supplementary Table 1). These changes were not statistically significantly different between conditions (online Supplementary Table 2) and likely represent a model-induced outgrow of this facultative aerobic bacteria taxon.

When addressing changes in the overall microbiota composition, we observed a rapid modulation in all conditions within 6 h, which peaked at 24 h before leveling of at 48 h (between-sample β-diversity analysis based on Bray-Curtis dissimilarity, online Supplementary Figure 5 and within-sample α-diversity analysis based on gut bacteria richness, online Supplementary Figure 6). This time-dependent changes were reflected in the changes in relative abundance levels of individual gut microbiota members over time (Figure 3). At genus level numerous taxa in all conditions decreased rapidly within 6 h and leveled of at 24 h of fermentation resulting in the largest observed fold-changes in this time period (online Supplementary Table 1). Especially, *Streptococcus* spp. peaked at 6 h with significantly higher levels for dried chicory root cubes (14.9 %) compared to inulin (11.8%). In addition, *Parasutterella* spp. increased to their highest levels at 6 h in dried chicory root cubes (3.3%) and powder (3.0%; online Supplementary Table 1) and had still statistically significantly higher levels at 24 h compared to inulin (online Supplementary Table 2). A number of genera first decreased between 0 to 6 h but then re-increased between 6 to 24 and 48 h. Notably bacteria from the *Eubacterium hallii* group (now also known as *Anaerobutyricum* spp.(Shetty et al., 2018)) decreased in all conditions and were not detected at 6 or 24 h, but then re-increased above baseline levels at 48 h in inulin (4.5-fold) and dried chicory root powder (1.8-fold) and cubes (2.2-fold). *Monoglobus* spp. decreased also in all conditions, but re-increased from 6 to 48 h only for dried chicory root powder and cubes, despite reaching (slightly) lower levels than at baseline. The same was true for bacteria from the *Eubacterium eligens* group and *Roseburia* spp. (online Supplementary Table 1), which were after 48 h significantly higher in powder and cubes due to their virtual absence in inulin and control fermentations (online Supplementary Table 2). Taking all increases in all taxa together, we observed the highest number of significantly increasing genera at 48 h for dried chicory root cubes with 15 genera increasing in relative abundance compared to inulin with 12 genera increasing and powder and control with 11 genera (online Supplementary Table 3).

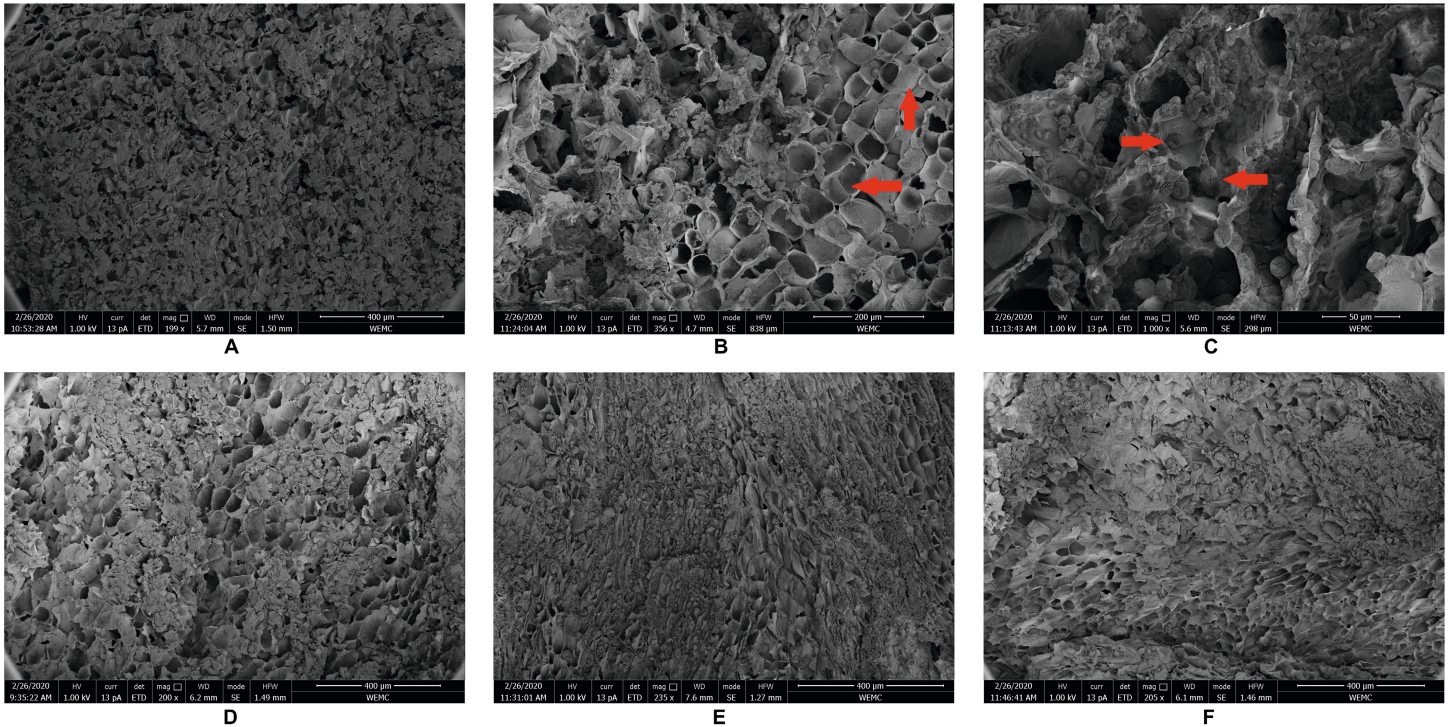


Figure 2. Scanning electron microscopy micrographs of fresh and digested dried chicory root. (A-C) Plant cell matrix of fresh chicory root (A) with intact plant cells (B) and intracellular inulin (C) as indicated by red arrows; D: intact plant cell matrix of dried chicory root cubes after rehydration prior to upper gastrointestinal digestion; E: plant cell matrix after gastric digestion of dried chicory root cubes; F: plant cell matrix after gastric and small intestinal digestion of dried chicory roots. While the macrostructure appeared to weaken over time (online Supplementary Figure 2), the overall structure remained visually unchanged throughout the gastric and small intestinal phase with no observed holes or cracks in the plant cell walls. Note the different magnifications in panels B and C.

The used system with mucin-covered beads also allowed for the analysis of the mucus-adhering microbiota, of which notably *Roseburia* spp. had significantly higher levels in dried chicory root powder and cubes after 48 h of fermentation (online Supplementary Figure 7 and online Supplementary Table 2). The use of mucin-covered beads also explains the increase in relative abundance of mucin-degrading *Akkermansia* spp. in control incubations lacking additional fermentable substrates.

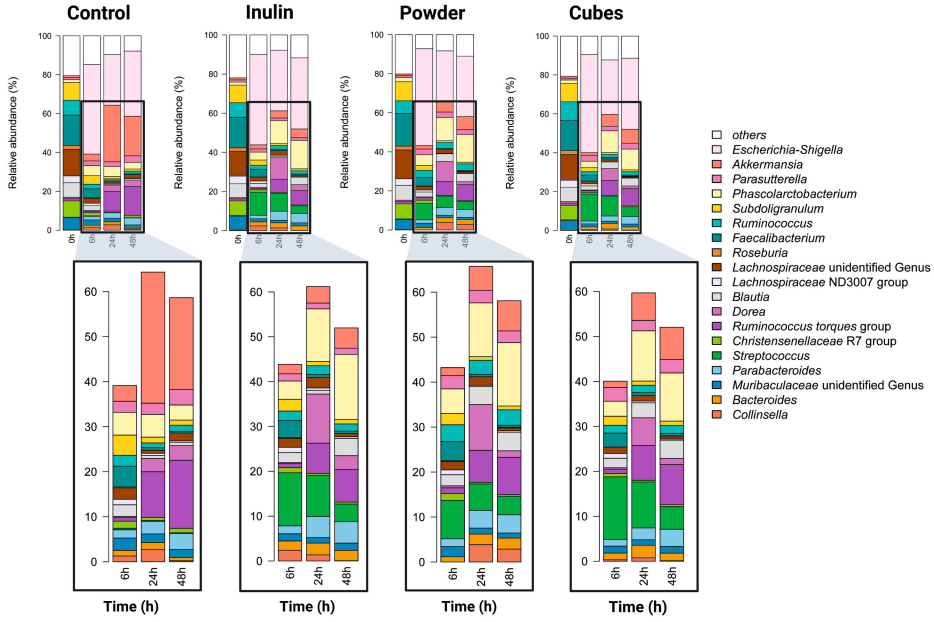


Figure 3. Differences in microbiota response to inulin and different dried chicory root particle sizes. Microbiota composition observed during fecal batch *in vitro* fermentations using a donor low in *Bifidobacterium* spp.. Mean relative abundance (%) of common genera (at least 1% relative abundance and 50% prevalence in all samples) detected in *in vitro* fermentations over time (t = 0, 6, 24 and 48 h in the control (Control; only inoculum) and for inulin (Inulin) versus dried chicory root powder (Powder) or cubes (Cubes). The lower panel zooms in on the common taxa omitting the contribution of *Escherichia-Shigella* spp., which dominated the community between 0 and 6 h of fermentation.

DIFFERENCES IN PH, GAS PRODUCTION AND FERMENTATION METABOLITES OF DIFFERENT PARTICLE SIZES OF DRIED CHICORY ROOT COMPARED TO INULIN.

Concomitantly with the time-dependent development in the microbiota composition, we also observed changes in pH, gas production and metabolites such as SCFA's and other organic acids produced (Figure 4). The pH decreased in all conditions from 0 to 6 and 24 h, but then slightly re-increased at 48 h. Fermentation of inulin induced the largest pH decrease, especially at 6 and 24 h (Figure 4). Consequently, inulin pH levels at 24 and 48 h were statistically significantly lower than those of the control, the

dried chicory root powder and cubes (online Supplementary Figure 8 and Table 4). Gas production increased rapidly and peaked at 24 h with inulin resulting in the highest gas production between 0 and 48 h, which was statistically significantly higher than control and dried chicory root products (Figure 4; online Supplementary table 4). Along with pH and gas, we measured the SCFAs acetate, propionate and butyrate. Butyrate started to increase between 6 to 24 h, but the most butyrate was produced between 24 and 48 h for inulin and the dried chicory root products. The highest butyrate levels were recorded at 48 h for dried chicory root cubes with (mean ± SE) 5.22 ± 0.21 mM, which differed statistically significantly from control (3.67 ± 0.18, p = 0.031) and inulin (4.21 ± 0.11, p = 0.049). Additionally, the increase in butyrate between 24 to 48 h was the highest for dried chicory root cubes (+3.13 ± 0.16 mM; online Supplementary Table 4). Compared to inulin, also the butyrate increase between 6 to 24 h was statistically significantly higher for dried chicory root cubes (p = 0.009) and powder (p = 0.009; online Supplementary Table 4). In contrast to the dynamics observed for butyrate, propionate increased substantially already between 0 to 6 h. At 6 h, propionate levels were statistically significantly highest for dried chicory root cubes but then at 24 h for inulin. At 48 h propionate levels remained higher for inulin despite not differing anymore from dried chicory root products (Figure 4; online Supplementary Table 4). Similarly to propionate, also acetate increased rapidly from 6 h onwards reaching highest levels at 48 h, but not differing between inulin and dried chicory root products (online Supplementary Table 4). As acetate is the most abundant SCFA, its changes dominated also the observed increases in total SCFAs, which were similar for inulin and dried chicory root products (Figure 4). Lactate also increased rapidly between 0 and 6 h with the highest levels at 6 h for dried chicory root cubes that differed statistically significantly from control, inulin and dried chicory root powder. Between 6 h to 24 h lactate was partially consumed in all conditions with the highest decrease in dried chicory root cubes (-1.39 ± 0.18 mM), which was statistically significantly different from inulin (- 0.34 ± 0.18), p = 0.003) but not dried chicory root powder (- 0.72 ± 0.02). At 48 h lactate had largely disappeared in all conditions (Figure 4). The production of BCFAs started from 6 h onwards and was the highest in the control, followed by dried chicory root powder, cubes and inulin (Figure 4). Yet, their levels only differed statistically significant for dried chicory root powder and not between cubes and inulin at any timepoint (online Supplementary Table 4). Finally, ammonium increased mainly between 0 to 24 h and was highest for control and least for inulin concomitant with the largest decrease in pH in inulin while dried chicory root cubes and powder did not differ. In summary, dried chicory root cube fermentation yielded similar total SCFAs but higher final butyrate levels compared to chicory root powder or isolated inulin with less gas produced.

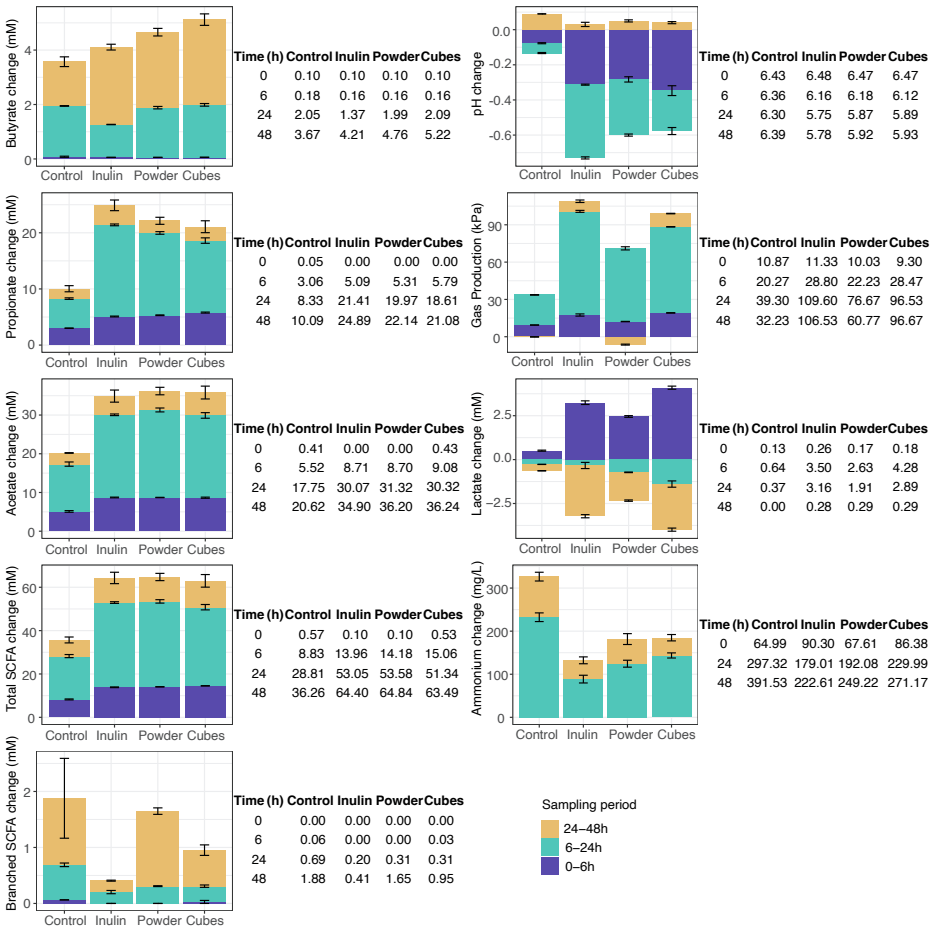


Figure 4. Differences in fermentation metabolites, pH, and gas production between inulin and dried chicory root particle sizes. Fermentation metabolites, pH and gas production measured during *in vitro* fermentation of control (control; only inoculum), inulin (Inulin) and dried chicory root powder (Powder) and cubes (Cubes) at baseline (t = 0 h), 6 h, 24 h and 48 h and the respective consecutive changes between each sampling period. Details on statistical testing are provided in online Supplementary Table 4.

EFFECT OF FERMENTATION SUPERNATANT PRODUCED FROM IN VITRO FERMENTATION OF DRIED CHICORY ROOT ON GUT PERMEABILITY

We continued to assess the effect of fermentation supernatant produced during the fermentation of dried chicory root on gut permeability in an *ex vivo* model using human colonic biopsies from four donors. Dried chicory root cubes were chosen as they were found to yield the highest butyrate levels *in vitro*.

Gut microbiota changes underlying the fermentation supernatants

To produce the fermentation supernatant, dried chicory root cubes were again fermented in a fecal *in vitro* batch fermentation model and compared to a negative control (control; only inoculum), however at higher inoculum concentration. Again, we observed an increase in *Escherichia-Shigella* spp., which dominated especially the control fermentation (Figure 5A). We observed a similar time-dependent modulation of the overall microbiota composition with changes relative abundance levels of individual taxa mostly peaking at 6 h and plateauing at 24 h (online Supplementary Table 5; online Supplementary Figure 9 and 10). In contrast to the single-donor *in vitro* fermentation, donors for this experiment were not chosen based on a low bifidobacteria count, and all four donors had considerable levels of *Bifidobacterium* spp. present in their feces (mean relative abundance across all conditions of 4.3 ± 1.5%; Figure 5A and Supplementary Figure 11). Following dried chicory root cube fermentation, we observed the highest changes at genus level in *Bifidobacterium* spp. (6 h: $p = 0.018$, $q = 0.216$; 24 h: $p = 0.081$, $q = 0.452$; 48 h: $p = 0.029$, $q = 0.152$). *Bifidobacterium* spp. levels increased rapidly up to a third of the whole microbiota community and were statistically significantly higher (up to 10-fold) from control levels at 6 and 48 h (online Supplementary Table 6 and for individual microbiota profiles Supplementary Figure 11). Constructing a principal response curve (Figure 5B), which assessed the combined response over time of gut bacterial genera to the product using redundancy analysis on the first principal component, confirmed that relative levels in *Bifidobacterium* spp. clearly differentiated the microbial community of dried chicory root cubes from the control. Besides the changes in *Bifidobacterium* spp. levels, changes in *Coprococcus* spp. levels differed, too, but reaching the highest levels in the control. We also observed several taxa decreasing in relative abundance in both conditions, but notably butyrate-producing genera decreased only in the control to statistically significantly lower levels (online Supplementary Table 5). *Butyricicoccus* spp. levels fluctuated but were statistically significantly higher at 24 h in the dried chicory root cube fermentations and virtually absent in the control at 48 h (online Supplementary Table 6). Again we observed that *Roseburia* spp. re-increased at 48 h for dried chicory root cubes but not control, while bacteria from the *Eubacterium hallii* group *(*renamed to *Anaerobutyricum* spp. (Shetty et al., 2018)) increased only for cubes at 6 and 24 h but then decreased at 48 h reaching lower levels than the control (online Supplementary Table 5 and 6). The dynamics of these gut microbiota changes were in line with those observed for the single donor low in bifidobacteria and simultaneously demonstrate the difference in gut microbiota modulation when *Bifidobacterium* spp. are present.

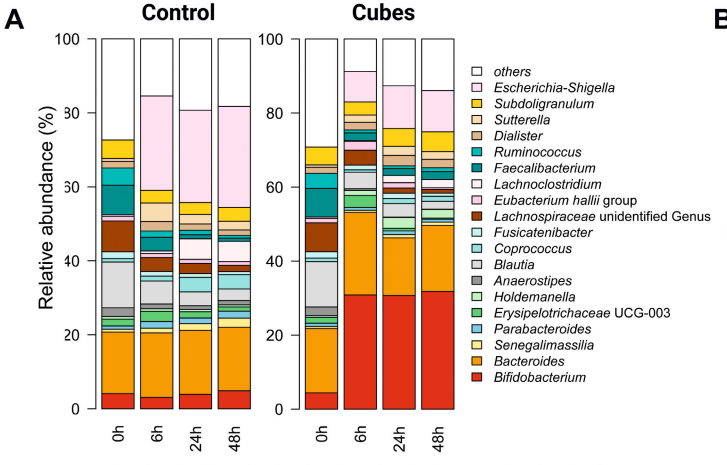
Changes in pH, gas production and fermentation metabolites underlying the fermentation supernatants

We determined pH, gas and production of SCFAs to understand the changes underlying the metabolites present in the fermentation supernatants (online Supplementary Table 7). The pH dropped again rapidly within 6 h, while gas production plateaued already

between 6 h and 24 h. Dried chicory root cubes yielded on average 12.56 ± 1.50 mM butyrate, 12.12 ± 1.97 mM propionate and 38.60 ± 2.03 mM acetate, which were up to three times higher than for the control fermentation (online Supplementary Table 7). Butyrate production was largest between 6 to 24 h with 8.04 ± 1.71 mM. We observed lactate to be produced at 6 h (5.83 ± 1.70 mM) during fermentation of dried chicory root cubes but this metabolite was completely consumed at 24 h. Formate followed the same dynamics as lactate, while the BCFA iso-butyrate levels peaked in dried chicory root cubes at 24 h before decreasing again at 48 h (online Supplementary Table 7). The fermentation outcomes based on four different donors were similar to the previous experiment with a single donor but followed a faster kinetic related to the higher concentration of fecal inoculum used.

Changes in gut barrier integrity of colonic biopsies following the stimulation with fermentation supernatants

Fermentation supernatants at 48 h were used to test their effect on gut barrier integrity of human colonic biopsies in an *ex vivo* Ussing chamber model. Human colonic biopsies were stressed with sodium deoxycholate (SDC), a secondary bile acid known to increase paracellular gut permeability and thereby affecting gut barrier integrity, simulating low-grade inflammation (Münch et al., 2007; Raimondi et al., 2008; Stenman et al., 2013; Zeng et al., 2022). Based on the measured SCFA concentrations in the fermentation supernatant at 48 h, biopsies stimulated with the fermentation supernatant (dilution to 2% v/v) were on average exposed to 0.20 to 0.34 mM butyrate (mean ± SE: 0.2 ± 0.03 mM), 0.17 to 0.34 mM propionate (0.24 ± 0.04mM) and 0.74 to 0.86 mM acetate (0.77 ± 0.04 mM). In all biopsies, transepithelial resistance (TER), as a measure of overall gut integrity, decreased over time with the least decrease after 90 min in the control biopsies (ΔTER = -2.09 ± 0.64) followed by the biopsies that were previously exposed to the fermentation supernatant (unstressed: ΔTER = -3.32 ± 0.50; stressed: ΔTER = -4.11 ± 0.55). Notably, in these exposed biopsies the decrease in TER was smaller and less rapid (from 60 to 90 min) compared to the stressed biopsies without previous exposure to the fermentation supernatant (t = 90 min ΔTER = -5.58 ± 1.79; online Supplementary Table 8).


A
Control
Cubes
Relative abundance (%)
0h
6h
24h
48h
others
Escherichia-Shigella
Subdoligranulum
Sutterella
Dialister
Ruminococcus
Faecalibacterium
Lachnoclostridium
Eubacterium hallii group
Lachnospiraceae unidentified Genus
Fusicatenibacter
Coprococcus
Blautia
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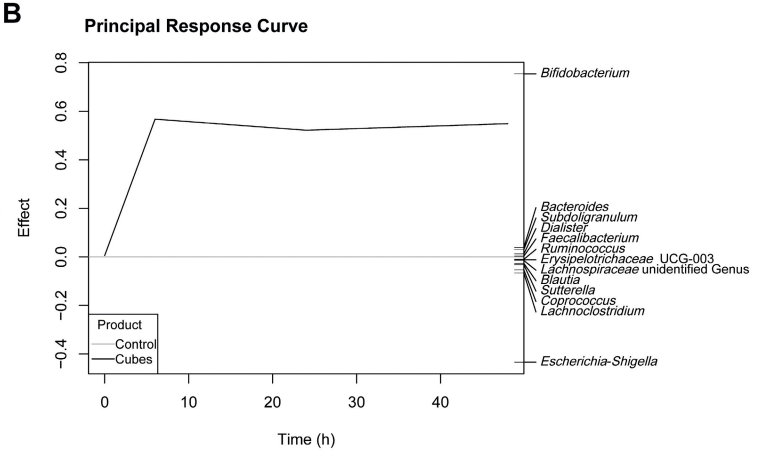
Bacteroides
Bifidobacterium


B
Principal Response Curve
Effect
Time (h)
Product
Control
Cubes
Bifidobacterium
Bacteroides
Subdoligranulum
Dialister
Faecalibacterium
Ruminococcus
Erysipelotrichaceae UCG-003
Lachnospiraceae unidentified Genus
Blautia
Sutterella
Coprococcus
Lachnoclostridium
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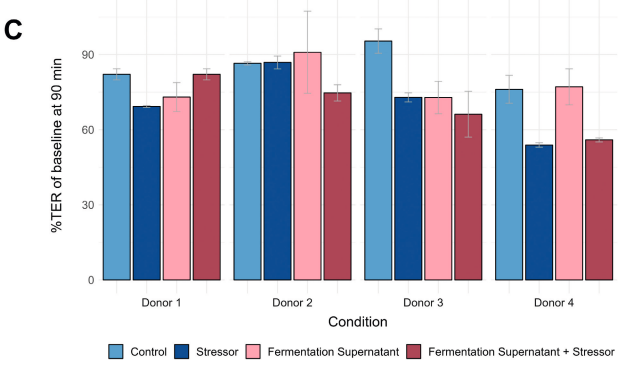
C
%TER of baseline at 90 min
Donor 1
Donor 2
Donor 3
Donor 4
Condition
Control
Stressor
Fermentation Supernatant
Fermentation Supernatant + Stressor

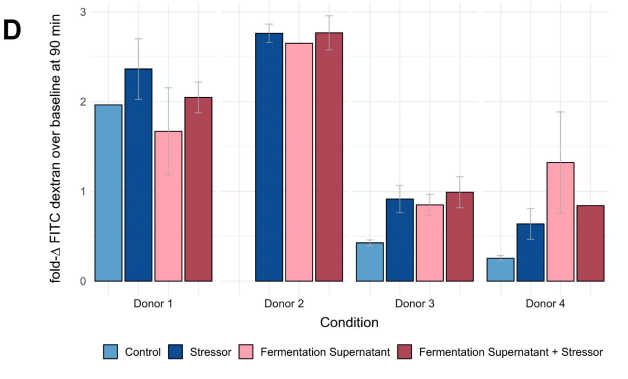
D
fold-Δ FITC dextran over baseline at 90 min
Donor 1
Donor 2
Donor 3
Donor 4
Condition
Control
Stressor
Fermentation Supernatant
Fermentation Supernatant + Stressor

Figure 5. Gut barrier function model outcomes. Gut microbiota composition of *in vitro* fermentations (A-B) to produce fermentation supernatants and their effect on colonic biopsies during the Ussing chamber experiment (C-D). (A) Common genera (mean relative abundance of at least 1% and mean prevalence of 50% in all samples) detected in the control (Control; only inoculum) and dried chicory root cube (Cubes) *in vitro* fermentation at baseline (T = 0), 6 h, 24 h and 48 h. (B) Principal response curve assessing the combined response of all genera over time (relative abundance (%)) and thereby revealing genera differentiating between dried chicory root cube and control fermentation. Genera driving the difference are depicted on the right scale with taxa equally present in both dried chicory root cubes and control having small weights and taxa deviating most having larger weights. (C) Change in transepithelial resistance after 90 min as percentage of baseline (%TER) in control or stimulated colonic biopsies using either the stressor SDC, the fermentation supernatant or their combination (fermentation supernatant + stressor). (D) Change in FITC-dextran concentration between 0 and 90 min expressed as factor of the baseline concentration (fold-Δ). Information on all timepoints of all four donors can be found in online Supplementary Figure 12. TER: Transepithelial resistance; %TER: TER at 90 min expressed as percentage of each biopsies respective baseline; FITC: Fluorescein isothiocyanate–dextran; fold-Δ FITC-dextran: relative change in FITC-dextran concentration between 0 to 90 min expressed as factor of each biopsies respective baseline (respective level expressed as factor of its baseline)

As the baseline TER differed considerable between donors, we calculated the relative decrease in TER at 90 min (expressed as percentage of baseline, %TER; Figure 5C). When biopsies were stressed with SDC no uniform difference in %TER decrease was found between exposed und unexposed biopsies (stressor: 70.72 ± 6.77 % versus fermentation supernatant + stressor: 69.72 ± 5.63 %). However, we observed considerable heterogeneity between donors in the response of unstressed biopsies exposed to the fermentation supernatant. Of these biopsies, for donor 2 and 4 the %TER decrease was similar or smaller when exposed to the dried chicory root fermentation supernatant compared to no exposure (control condition; Figure 5C). Notably, these two donors showed the highest increase in relative abundance of bifidobacteria.

Concomitantly with the decrease in overall gut integrity, we also observed an increase in gut permeability (online Supplementary Figure 12 and Table 8) and heterogenous responses (Figure 5D). Paracellular permeability assessed by serosal FITC-dextran concentration increased in all biopsies over time with the least increase again in the control biopsies (online Supplementary Figure 12B). FITC-dextran concentration and absolute change at 90 min over baseline was highest for biopsies previously exposed to the fermentation supernatant (stressed and unstressed; online Supplementary Table 8). However, their relative change in FITC-dextran concentration from 0 to 90 min (expressed as factor over baseline) was similar to the biopsies stimulated only with the stressor SDC (Figure 5D; online Supplementary Table 8).

None of these changes in TER and FITC-dextran concentrations were found to be statistically significantly different between stimulations (online Supplementary Table 8). In summary, in the Ussing chamber model, exposure to the fermentation supernatants in the presence of the stressor SDC had no uniform effect on gut barrier integrity of human colonic biopsies, as assessed by TER and paracellular permeability. However, TER decreased less rapid over time when exposed to the fermentation supernatant. Considering the observed heterogeneity between donors, we also addressed the individual responses and in two donors we found smaller relative TER decreases when

biopsies were exposed to their fermentation supernatant, indicating donor-specific effects on maintaining overall gut integrity (online Supplementary Figure 12).

DISCUSSION

Here we investigated how the plant cell matrix in dried chicory root impacts its breakdown in the human gut by assessing its intactness in the upper gastrointestinal tract and determining its microbial breakdown kinetics and effect on gut barrier integrity by a series of *in vitro* and *ex vivo* models. We observed that the plant cell matrix of cubes as well as powder of dried chicory root remained intact during upper gastrointestinal transit in the INFOGEST model. Dried chicory root rapidly modulated the microbial community, yielding the highest butyrate levels for cubes and co-occurring with higher levels of *Roseburia* spp. and the pectin-degrader *Monoglobus* spp. at 48 h of fermentation. For donors with *Bifidobacterium* spp. present at baseline we observed a seven-fold increase following dried chicory root cube fermentation compared to control. Using the fermentation supernatant of the dried chicory root cubes to stimulate human colonic biopsies in an Ussing chamber model did not uniformly prevent stressor-induced impairment. Instead, donor-specific differences in unstressed biopsies emerged, notably from two donors with highest final bifidobacteria levels.

The cell matrix of plant foods is a complex structure made of cellulose strengthened by hemicelluloses and pectin intrinsically intertwined into plant cell walls that encapsulate other non-structural carbohydrates, macro- and micronutrients. Dried chicory root is a food product that is particularly high in fiber due to its intra-cellular inulin content being part of the intrinsic plant cell matrix (Puhlmann & de Vos, 2020, 2022). While inulin is a fiber known to be easily fermentable by the human gut microbiota, the presence of the plant cell wall in dried chicory root forms a physical barrier that gut bacteria need to open as to access intracellular inulin and other cellular components. Consequently, the breakdown of dried chicory root differs from that of isolated inulin. Dietary fibers are by definition not digested in the upper gastrointestinal tract but they can still be affected by the prolonged incubation in digestive juices and gastric and small intestinal pH changes leading to, for instance, the dissolution of pectin (O.A. Patova et al., 2023; Olga A. Patova et al., 2022). We observed that an estimated 15% of the total pectin leaked from the dried chicory root matrix during the *in vitro* gastric and small intestinal digestion. This was nearly twice as high for chicory root powder, likely due do the larger damage of the plant cell matrix induced by milling, breaking more plant cells open. Pectin that leaks out from plant foods is believed to be mainly soluble pectin from the intercellular space, which glues the plant cells together enforcing the overall plant cell matrix (Capuano, 2017; Capuano & Pellegrini, 2019). Indeed, concomitantly with the leakage of pectin over time, we observed an overall weakening of the macrostructure. Nonetheless, no obvious damage to the plant cell matrix in the form of cracks or holes in plant cell walls was visible, which indicates that dried chicory root cubes are likely

to arrive in the lower gastrointestinal tract as intact particles. A weakened plant cell matrix may favor the release of intracellular inulin, and we observed higher amounts of fructose-monomers and fructo-oligosaccharides together with longer-chain fructan-polymers for dried chicory root powder, which we hypothesize to represent and relate to the higher plant cell damage. Thus, it is likely that dried chicory root cubes function as delivery system of inulin and pectin that remain primarily encapsulated inside the intact plant cells to reach the distal parts of the colon.

This plant matrix intactness challenges the breakdown by the gut microbiota as the opening of the plant cell wall requires the degradation of the chemically more complex pectins and hemicelluloses. Chemical complexity selects for the action of specialist bacteria that have the functional machinery to access and metabolize diverse sugar constituents (Cantu-Jungles et al., 2021) and the presence of different dietary fibers slows their gut microbial breakdown (Lu et al., 2020, 2021; Tuncil et al., 2017). Therefore, we hypothesized that the kinetics of microbiota-mediated fiber breakdown may differ in dried chicory roots compared to inulin.

After an initial rapid modulation in overall gut microbiota composition and decrease in gut bacterial richness within six hours, distinct differences between fiber products started to emerge between 6 to 24 h when most of the gas and total SCFAs were produced. Inulin resulted in the largest pH decrease and highest gas production, with propionate production surpassing that from dried chicory root cubes and powder at 24 h. While acetate and total SCFA production did not differ, both dried chicory root products produced significantly more butyrate than inulin. This was paralleled by significantly larger lactate production (up to 6 h) and consumption (from 6 h onwards) for dried chicory root cubes compared to hardly any consumption between 6 to 24 h for inulin, and little changes for dried chicory root powder. Comparing dried chicory root particle sizes, gut bacterial richness decreased less rapidly for powder than cubes. This may be due to the presence of more readily available substrate as the plant cell matrix in powder is more damaged. Consequently, more fibers are exposed (intracellular inulin alongside with pectin and hemi-/cellulose cell wall fibers) and more surface area is created for bacterial adherence compared to the cubes, where bacteria have to diffuse between the plant cells to break them down from the outside.

Between 24 to 48 h the overall gut microbiota community composition hardly changed, but we observed remarkable distinctions in butyrate production and relative abundance of individual taxa. Gut bacteria richness increased again at 48 h for dried chicory root cubes and powder but not inulin. This coincided with higher levels of the pectin-degraders *Monoglobus* spp. and bacteria from the *Eubacterium eligens* group as well as the butyrate-producing *Roseburia* spp. in both the fermentation liquid and mucin-covered beads . The re-increase in gut bacteria richness and appearance of specialist bacteria, s suggests that the more intact plant cell matrix of dried chicory root results in prolonged fermentation preventing substrate depletion at a later timepoint. Interestingly, lactate consumption and butyrate production were also highest between 24 to 48 h, resulting in butyrate being the only SCFA to notably increase during this

time period and to reach the levels for dried chicory root cubes. Increased butyrate production levels after prolonged fermentation has been previously reported for large wheat bran particles (Stewart & Slavin, 2009; Tuncil et al., 2018). It appears that the three-dimensional organization plays a role herein as exemplified for alginate-entrapped starch (Rose et al., 2009) and wheat plant cell walls (Lu et al., 2020) leading to higher butyrate production than their extracted single fiber alone. Especially larger particle sizes (>2 mm) often result in higher butyrate and lower acetate proportions (Yao et al., 2023), which is attributed to reaching a fermentation plateau and allowing more time for lactate/acetate-to-butyrate conversion through cross-feeding (Yao et al., 2023). Lactate conversion into propionate or butyrate is limited *in vitro* at a more acidic pH (5.5 vs 6.5) (Ping Wang et al., 2020), but it is debatable whether the slightly lower pH in inulin (5.75) compared to dried chicory root (5.89) fermentations, was physiologically relevant to be the main driver of higher lactate consumption and butyrate production. However, as we observed for dried chicory root cubes the largest lactate production and consumption to coincide with the largest butyrate production, lactate-to-butyrate conversion could play an essential role in the breakdown of the dried chicory root cubes.

Finally, we observed a lower increase in BCFAs and ammonium for inulin compared to the dried chicory root products and control. This may attributed to the significantly larger pH decrease for inulin, as the formation of these protein fermentation compounds is known to be less favored at lower pH (Korpela, 2018). In the *in vivo* situation the production of BCFAs is linked to the availability of fiber substrate in the proximal versus distal colon and inulin, due to its simple structure, is believed to be fermented more proximally (Korpela, 2018; So et al., 2021). Consequently, BCFA formation may still occur *in vivo* in the distal colon due to substrate depletion from inulin but not from dried chicory root, as a consequence of its slower breakdown.

Butyrate produced by gut bacterial fiber fermentation can be used by colonocytes as energy source and thereby potentially strengthen the colonic epithelium. Studying this interaction relies on the use of cultivated cell-line models (possibly combined with mimicking mucus secretion) or the removal of *in vivo* colonic tissues (yet losing the protective mucus layer). Previous studies using colonic biopsies revealed acute distinct effects of fiber on permeability in biopsies of healthy and/or diseased donors (Ganda Mall, Casado-Bedmar, et al., 2018; Ganda Mall, Löfvendahl, et al., 2018), but also considerable heterogeneity in the individual responses (Ganda Mall, Löfvendahl, et al., 2018). Here, we applied a similar acute model to study the interaction between the individuals' gut microbial fiber breakdown products and their human colonic epithelium. To model this, we investigated whether previous exposure to dried chicory root fermentation supernatants could acutely counteract or diminish stressor-induced impairments in the gut barrier function similar to a low-grade inflammatory state. We did not observe an acute, uniform difference between human biopsies stressed with SDC alone and those that were previously exposed to the fermentation supernatant, despite a less rapid decrease in TER over time in the latter. Based on the measured SCFA concentration in fermentation supernatants and dilution in the Ussing chamber

we estimated butyrate concentration to be 0.25 mM. Previous studies using sodium butyrate at a concentration of 5 mM and 25 mM (factor 20 to 100 higher, representing physiological concentrations) did also not demonstrate a protective acute effect against stressor-induced impairments using the mast cell degranulator Compound 48/80 (C48/80) in healthy donors' biopsies (Tabat et al., 2020). It is possible that the acute exposure in this model was too short to positively modulate gut barrier integrity. Butyrate has a considerable history of well-reported improvements on human colonic function *in vivo* (Hamer et al., 2009) and two-week *in vivo* exposure to butyrate using direct delivery by enemas has shown to reduce oxidative stress in the colonic mucosa (Hamer et al., 2009).

In line with an expected heterogeneity in the individual responses, we observed donor-specific differences, which became apparent in unstressed biopsies. Notably, for two donors (donor 2 and 4) exposure to the dried chicory root fermentation supernatant did not compromise, but maintained overall gut integrity measured by TER compared to the changes observed in control biopsies (%TER change). These donors also had the highest final relative levels of *Bifidobacterium* spp. at 48 h of fermentation. Bifidobacteria can positively impact gut epithelium proliferation via direct interaction of their tight adherence pili with colonic cells in mice (O'Connell Motherway et al., 2019). Moreover, bifidobacteria are known to strengthen the gut mucosa (despite being absent in this acute model) via the proposed interaction of neurotransmitter GABA (γ-aminobutyric acid) and the SCFA acetate with goblet cells (Gutierrez et al., 2023). It is hence possible that a beneficial effect of dried chicory root fermentation on the gut barrier integrity in such an acute model may become more apparent by using donors who previously consumed the dried chicory root product Furthermore, studying a larger number of donors would enhance our understanding of the overall significance of the present observation.

Understanding time-dependent digestive changes of food products relies on *in vitro* systems, with static (batch) incubations that are commonly used due to cost-effectiveness. However, these systems only model, not represent, human digestive processes (Isenring et al., 2023). Here, we used the standardized upper gastrointestinal *in vitro* Infogest model (Brodkorb et al., 2019; Minekus et al., 2014) tailored (as recommended) to our high dietary fiber product indigestible to human enzymes. The observed minimal structural changes supported the simplified incubations under anoxic, sterile conditions before *in vitro* fermentations. Additionally, we used simple fecal batch *in vitro* fermentations, common for individual and pooled microbiota response assessments. However, these batch fermentations are prone to known model-induced shifts in bacterial taxa (Shkoporov et al., 2023) limiting *in vivo* generalizability. Particularly, we noticed an increase in the common fast-growing, aerotolerant sugar-fermenter *Escherichia-Shigella* spp. in the control fermentations. It remains to be assessed how these higher relative levels in the control would reflect in absolute numbers as we did not measure bacterial load. Various other studies have observed this Enterobacteriaceae overgrowth (Ács et al., 2022; Gao et al., 2021; Gnanasekaran

et al., 2021; Long et al., 2015; Pirkola et al., 2023; Poppe et al., 2023), which reflects the model's limitations including the nature of the inoculum (low bacterial cell numbers favoring *Escherichia-Shigella* spp. (Poppe et al., 2023) and potential residual or re-entering oxygen. It is unlikely that the 4% mono-/disaccharides naturally present in the dried chicory root products substantially contributed to fermentation, although they might be absorbed *in vivo* in the small intestine. Moreover, we used two *in vitro* fermentation systems that differed in their set-up. The Ussing chamber fermentation were used to produce fermentation supernatants and contained a higher amount of fecal inoculum (1:5 versus 1:13) than the single donor experiment. This exposed more bacterial cells to the same amount of fiber substrate reaching the fermentation plateau faster, as observed from a higher amount and rate of butyrate production. Yet, in spite of these limitations and different model configurations remarkably similar responses were obtained across donors resulting in increased levels of microbial butyrate producers and butyrate levels, in line with previous *in vivo* results (Puhlmann et al., 2022). This demonstrates the donors' individuality in gut microbiota composition and at the same time the potential of dried chicory root to increase butyrate production across gut bacterial communities from different donors.

In all donors, lactate increased at 6 h and was subsequently consumed throughout the fermentation coinciding with butyrate production. For the low bifidobacteria donor, lactate originated possibly by the action of *Streptococcus* spp. (Louis et al., 2022). while for the donors in the Ussing chamber fermentations *Bifidobacterium* spp. increased by seven-fold and coincided with lactic acid production. Previously we showed that dried chicory root had a strong bifidogenic effect increasing *Bifidobacterium* spp. by four-fold *in vivo* (Puhlmann et al., 2022). For all donors we also observed an increase in butyrate and propionate production with significant changes in the relative levels of *Roseburia* spp. (single donor) or *Butyricicoccus* spp., both known butyrate producers (Louis et al., 2022; Louis & Flint, 2017). However, neither *Roseburia* spp. nor *Butyricicoccus* spp. reportedly use lactate for butyrate production (Louis et al., 2022). We hypothesize that the ongoing activity of known lactate-utilizing bacteria from the *Eubacterium hallii* group (renamed to *Anaerobutyricum* spp.) (Engels et al., 2016; Shetty et al., 2018, 2020, 2022) contributed to butyrate formation from lactate despite their relative levels not continuously increasing. The same has been observed in synthetic communities where the gene expression of the lactate-to-butyrate pathway was highly increased rather than their cell numbers (Shetty et al., 2022). Our previous *in vivo* results demonstrated that the increase in *Bifidobacterium* spp. was concomitant with a three-fold increase in the well-known butyrate-producing *Anaerostipes* spp. having the same lactate-to-butyrate pathway as *Anaerobutyricum* spp. (Louis et al., 2022; Shetty et al., 2020). Then, we demonstrated using a synthetic community that representative members with the canonical functionality of these genera formed a trophic chain yielding butyrate from dried chicory root (Puhlmann et al., 2022). It is hence possible that similar trophic chains involving lactate/acetate producers and butyrate producers were formed here, too, but representing donor and model-specific cross-feeding networks.

In this study we demonstrate how the presence and intactness of the plant cell matrix in dried chicory root affects the bacterial breakdown of its dietary fiber and differs from isolated inulin. Dried chicory root, particularly in the form of cubes, resulted in lower gas and more butyrate production compared to isolated inulin, although cumulating into similar final total levels of SCFAs . Lower gas production has been postulated to be a beneficial outcome *in vivo* especially for fiber-related therapies in irritable bowel syndrome (So et al., 2021). The observed butyrate production throughout the later stage of fermentation together with the detection of a re-increase in pectin-degraders may translate *in vivo* into a prolonged fermentation during which chicory root fibers are transported into the distal colon where butyrate production could benefit gut health. This would also explain the high levels of butyrate and other SCFAs in the fecal samples of our previous dried chicory root randomized-controlled *in vivo* trial. We were not able to demonstrate a uniformly strengthening effect of butyrate containing fermentation supernatant in the Ussing chamber using biopsies stressed with SDC, but observed donor-specific effects for overall gut integrity. Yet, a high production of butyrate from the dried chicory root cubes could still benefit gut barrier integrity *in vivo*. In conclusion, dried chicory root is an intrinsic fiber product containing high amounts of inulin that remain encapsulated within its plant cell matrix in the upper intestinal tract – this affects its breakdown kinetics by the human gut microbiota rationalizing a more distal fermentation and production of butyrate benefitting human health *in vivo*.

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AUTHORS' CONTRIBUTIONS

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Puhlmann M-L, van de Rakt E, Kerezoudi E, Rangel I, Brummer RJ, Smidt H, Kaper FS, de Vos WM

Performed data acquisition, as well as provided administrative, technical, and material support: Puhlmann M-L, van de Rakt E, Hutnik E, Kerezoudi E, Rangel I, Brummer RJ, Smidt H

Wrote original draft, reviewed and edited: Puhlmann M-L, de Vos Willem.

AVAILABILITY OF DATA AND MATERIALS

Data is available in the online Supplementary material and 16S rRNA gene amplicon sequences are submitted to the European Nucleotide Archive (ENA) under accession number PRJEB74436 and PRJEB74437.

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CONFLICTS OF INTEREST

F.S. Kaper is founder and CEO of WholeFiber BV, and W.M. de Vos is scientific advisor to WholeFiber BV.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The Research Ethics Committee of Örebro University (dnr 2021-05142) approved the study using human colonic biopsies. The study was executed conform with the Declaration of Helsinki and written informed consent was obtained from all subjects prior to the study.

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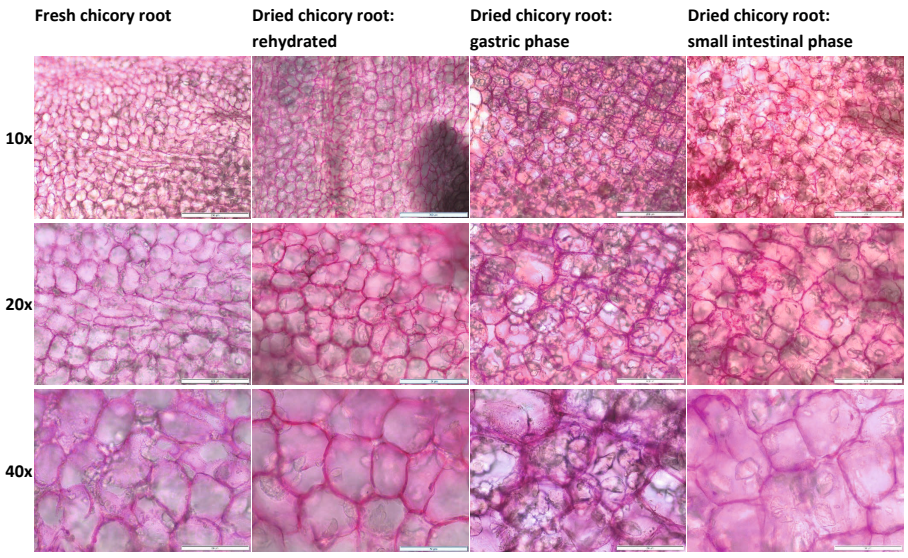
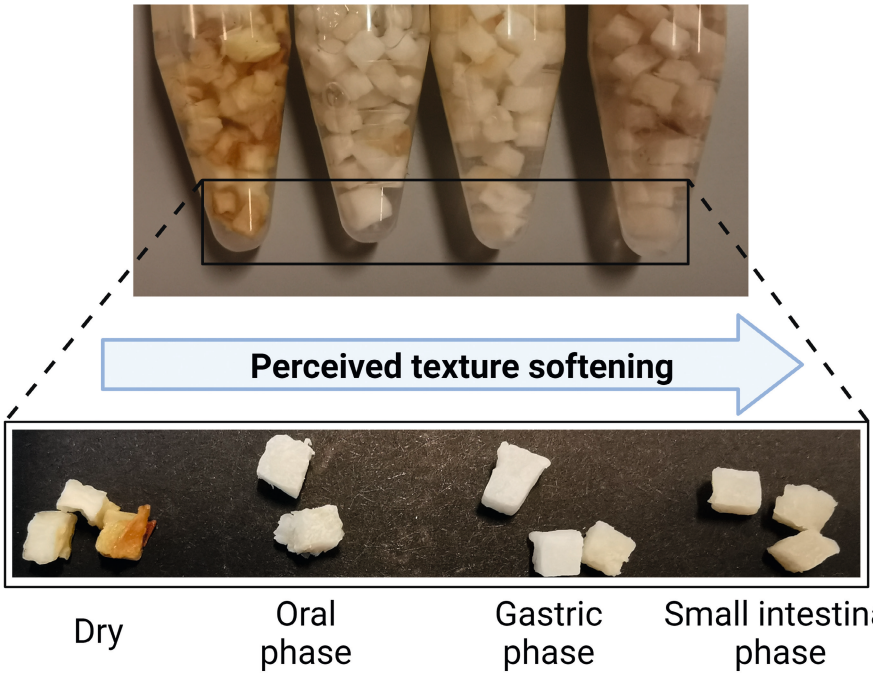
SUPPLEMENTARY MATERIAL

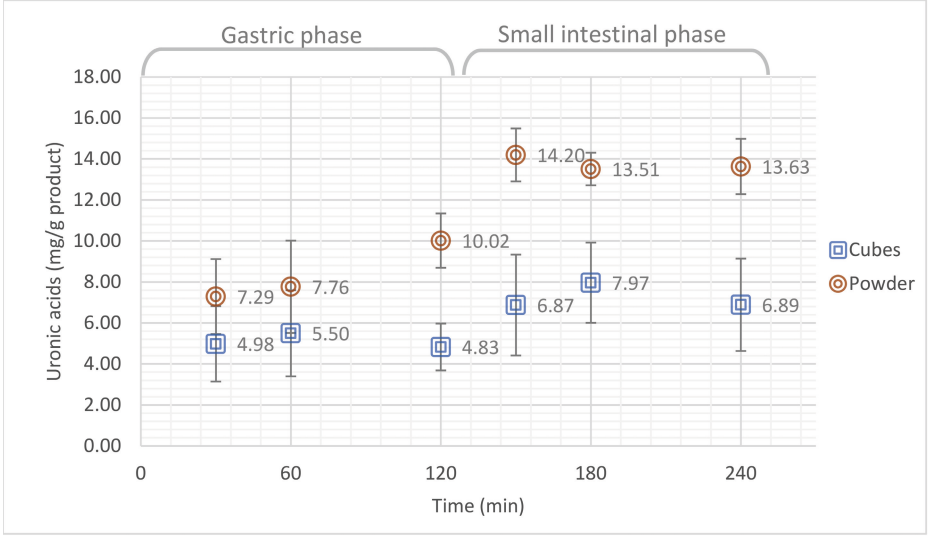
The complete supplementary material can be accessed online at the journal: mrr3004-SupplementaryMaterials.pdf (oaepublishstorage.blob.core.windows.net) or by scanning the QR code below.



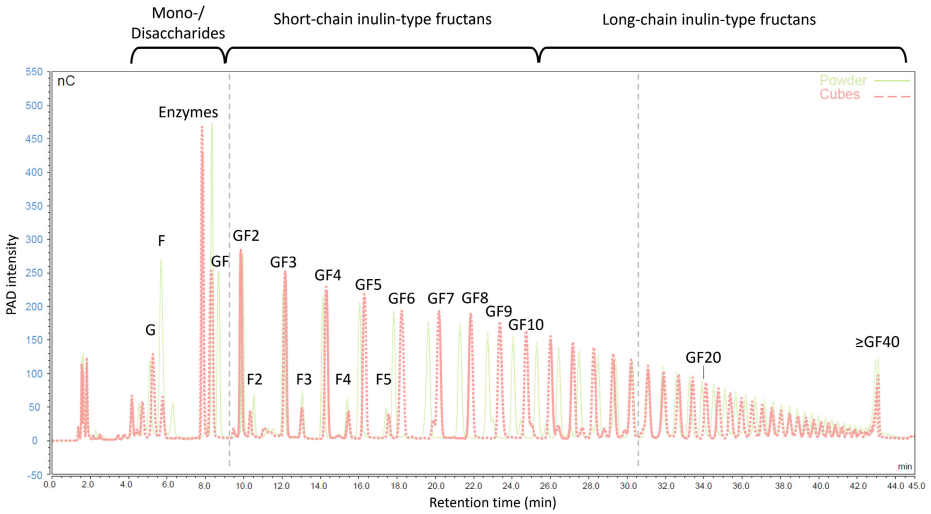
Supplementary Figure 1. Light Microscopy micrographs of the plant cell matrix of fresh and *in vitro* digested dried chicory root cubes before the oral phase (rehydrated), at the end of the gastric (120 min) and small intestinal phase (240 min). Ruthenium red (0.05 %w/v) was added to increase the contrast between plant cell and intracellular components. Each picture is shown at three magnification with the scale bar representing 200 µm for 10x magnification, 100 µm for 20x and 50 µm for 40x magnification.



Supplementary Figure 2. Macrostructure of dried chicory root cubes before and during upper gastrointestinal *in vitro* digestion. Created with Biorender.com

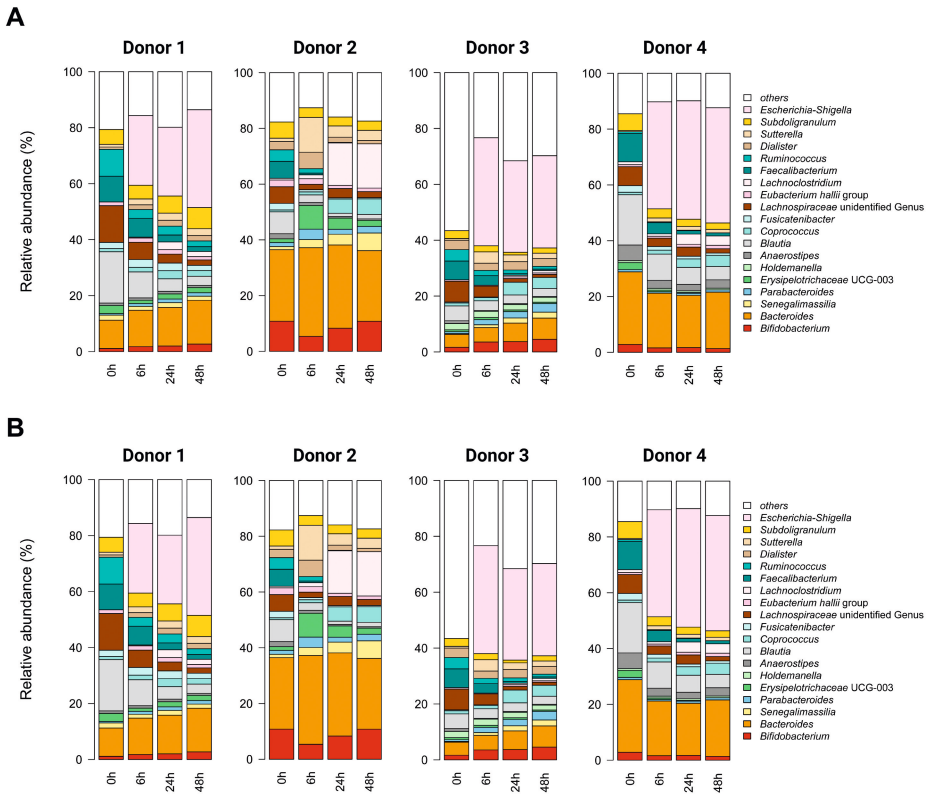


Supplementary Figure 3. Release of uronic acids as a measure of pectin leakage from dried chicory roots cubes (□) and powder (○) during *in vitro* digestion in the gastric phase (at t = 30 min, 60 min and 120 min (end)) and small intestinal phase (30 min at t = 150 min, 60 min at t = 180 min and 60 min at t = 240 min (end)). For each data point the corresponding value is given on its respective right side.

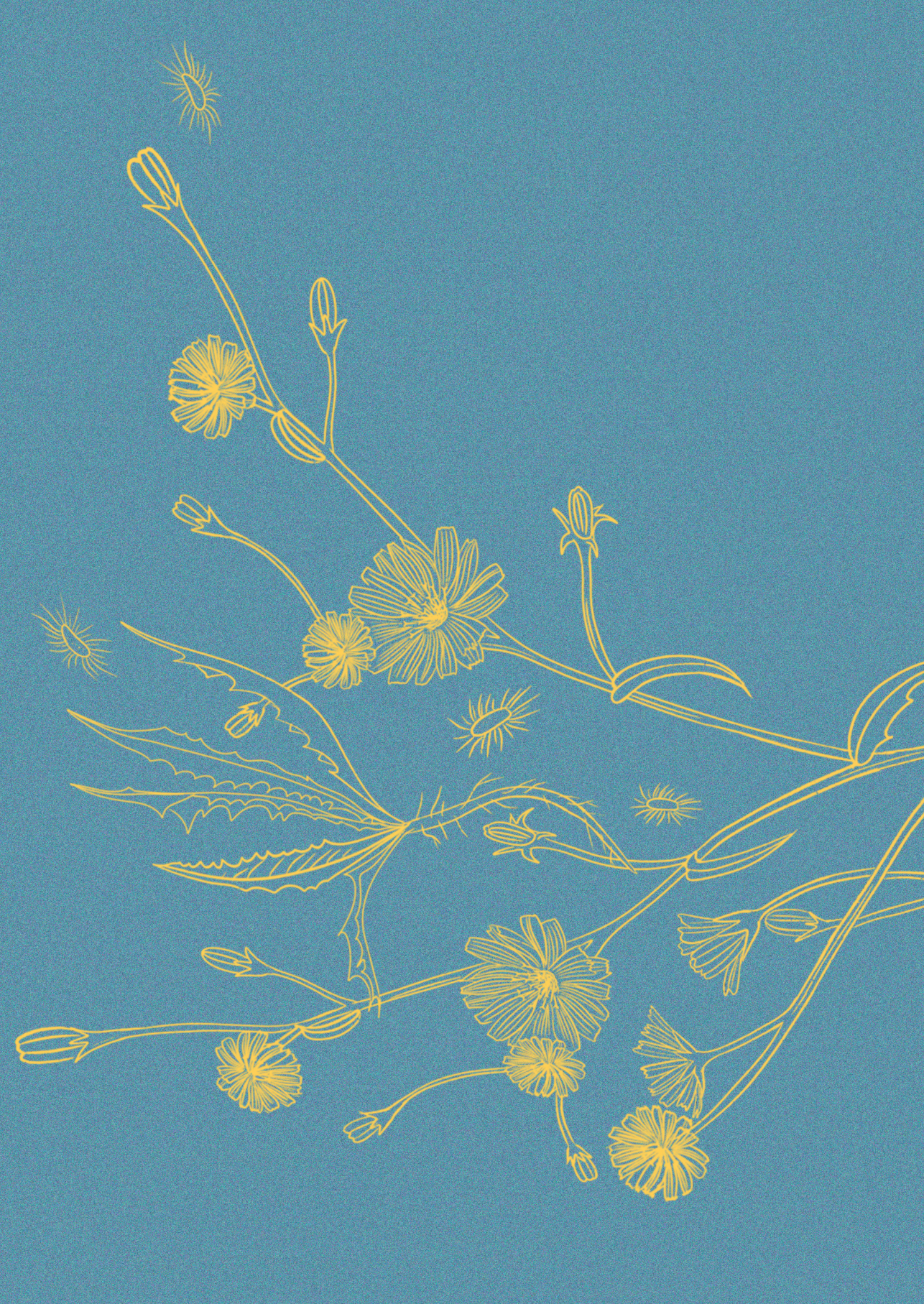


Supplementary Figure 4. High Performance Anion Exchange Chromatography (HPAEC) chromatograms of the liquid digesta retrieved at the end of the upper gastrointestinal *in vitro* digestion (end of the small intestinal phase) for dried chicory root powder (green) compared to cubes (red). Native inulin contains so-called inulin-type fructans, which are fructose-polymers linked by β(2—1)-bonds and an have varying chain lengths up to 60, which are described as degree of polymerization. Native inulin contains oligomers with and without glucose as end unit as well as glucose and fructose monomers and sucrose (di-saccharide of glucose and fructose). Different chain lengths are represented by individual peaks in the chromatogram obtained from the signal of each detected mono-, oligo- and polymer. G: glucose; F: fructose; Enzymes: signal obtained from the added pancreatin (containing digestive enzymes) in the digesta; GF: Sucrose (containing a glucose and a fructose monomer); GF2-GF40: fructose-chains of increasing chain lengths with glucose as end unit (DP is equal to the amount of fructose monomers); F2-5: fructose-chains of increasing chain lengths without glucose as end unit (DP is equal to the amount of fructose monomers).

Native inulin as present in the chicory root plant cells contains fructans with a wide range of molecular size (DP 2 – 60; average of DP 12), that decrease in solubility with increasing chain length (Mensink et al., 2015). Consequently, we expected that longer fructan-polymers (DP>10) leak less easily from the intact plant cells compared to smaller fructo-oligosaccharides (DP 2-10), but that damage might advance their release. We observed indeed higher amounts of fructan-oligomers as well as some longer-chain fructose polymers for dried chicory root powder, which we hypothesize to represent and relate to the higher content of plant cell damage. Thus, it is likely that dried chicory root cubes transport inulin mainly encapsulated inside the intact plant cells into the lower (distal) gut.



Supplementary Figure 11. Analysis of individual gut microbiota composition. Common genera present with a mean relative abundance of at least 1% and mean prevalence of 50% in the whole dataset at each timepoint in each of the *in vitro f*ermentation. (A) genera present in each donor during *in vitro* fermentation of dried chicory root cubes. (B) genera present in each donor during in the control fermentation.



CHAPTER 5

Dried chicory root improves bowel function, benefits intestinal microbial trophic chains and increases fecal and circulating short-chain fatty acids in subjects at risk for type 2 diabetes

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ABSTRACT

We investigated the impact of dried chicory root in a randomized, placebo-controlled trial with 55 subjects at risk for type 2 diabetes on bowel function, gut microbiota and its products, and glucose homeostasis. The treatment increased stool softness (+1.1 ± 0.3 units; $p = 0.034$) and frequency (+0.6 ± 0.2 defecations/day; $p < 0.001$), strongly modulated gut microbiota composition (7% variation; $p = 0.001$), and dramatically increased relative levels (3-4-fold) of *Anaerostipes* and *Bifidobacterium* spp., in a dose-dependent, reversible manner. A synthetic community, including selected members of these genera and a *Bacteroides* strain, generated a butyrogenic trophic chain from the product. Fecal acetate, propionate and butyrate increased by 25.8% (+13.02 ± 0.2 mmol/kg; $p = 0.023$) as did their fasting circulating levels by 15.7% (+7.7 ± 3.9 μM; $p = 0.057$). In the treatment group the glycemic coefficient of variation decreased from 21.3 ± 0.94% to 18.3 ± 0.84% ($p = 0.004$), whereas fasting glucose and HOMA-ir decreased in subjects with low baseline *Blautia* levels (-0.3 ± 0.1 mmol/L fasting glucose; $p = 0.0187$; - 0.14 ± 0.1 HOMA-ir; $p = 0.045$). Dried chicory root intake rapidly and reversibly affects bowel function, benefits butyrogenic trophic chains, and promotes glycemic control.

Key words chicory root, intrinsic fiber, gut microbiota, short-chain fatty acids, type 2 diabetes, continuous glucose monitoring, *Bifidobacterium, Anaerostipes, Blautia*

INTRODUCTION

Fiber intake has increasingly been recognized as a major factor in the maintenance of human health and contributes to lowering risk for metabolic diseases like type 2 diabetes (T2D) (Reynolds et al., 2019, 2020). However, only a small percentage of the general population consumes the recommended fiber amount, a problem known as the fiber gap (Jones, 2014). Since most fibers reach the colon in an undigested form, they impact the composition and activity of the gut microbiota. This is highly relevant since specific microbiota signatures are associated with various stages of T2D (De Vos & Nieuwdorp, 2013) mirroring increased insulin resistance in prediabetes and T2D by shifts towards reduced relative abundance of butyrate-producing bacteria (Wu et al., 2020). A study revisiting several dozens of T2D cohorts revealed a decrease in relative levels of butyrate-producing bacteria and an increase of *Blautia, Ruminococcus* and *Fusobacterium* spp. (Gurung et al., 2020). Moreover, intake of a complex fiber mixture in Chinese T2D patients improved glucose homeostasis concomitantly with increased levels of butyrate-producing bacteria (Zhao et al., 2018).

The gut microbiota uses fibers as carbon and energy source, resulting in the production of short-chain fatty acids (SCFA), such as acetate, propionate and butyrate. These SCFA not only exert local effects by serving as fuel for colonocytes and interacting with G-coupled protein receptors (GPRs) but might also enter the systemic circulation, directly interacting with peripheral tissue function and thereby affecting metabolism (Canfora et al., 2015). Prediabetic subjects receiving colonic infusions of SCFA mixtures at concentrations and ratios reached after fiber intake improved metabolic health parameters particularly when SCFA were administered in the distal colon (Canfora et al., 2017; van der Beek et al., 2016). Such a distal shift in fermentation might also benefit treatment of functional bowel diseases (So et al., 2021). Consequently, modulating the gut microbiota towards increased SCFA production might offer new therapeutic avenues for metabolic diseases (Canfora et al., 2015; Fan & Pedersen, 2020; Gurung et al., 2020).

Especially well-studied fibers are inulin-type fructans (ITF) (Swanson et al., 2020), including isolated and purified native inulin from chicory roots. Consumption of ITF is known to stimulate fecal levels of *Bifidobacterium* spp. (Le Bastard et al., 2019) as well as to contribute to maintaining bowel function, which has resulted in a validated health claim (EFSA, 2015). However, ITF are reported to have a rather limited effect on modulating gut microbiota (Dewulf et al., 2013; Kiewiet et al., 2021) and a moderate to no effect on fecal and circulating SCFA and insulin resistance markers (Chambers et al., 2015, 2019). A recent study reviewing interventions with ITF, other single fibers and whole grain foods concluded that total fecal SCFA were not impacted (So et al., 2018). Of note, it has been suggested that consumption of plant and fiber-rich foods would increase fiber-degrading and SCFA-producing gut bacteria (Koponen et al., 2021). While studying the effect of single or purified dietary components is the conventional way to obtain mechanistic insights, this reductionist approach does not take into account that

dietary fibers do not exist in isolation in minimally and unprocessed products. They are either part of the plant cell wall (pectin, hemicellulose, cellulose) or encapsulated by it (storage carbohydrates such as inulin), hence termed intrinsic fibers (Augustin et al., 2020). This intrinsic structure of fibers is likely to result in a gradual release of fermentable carbohydrates and hence a rather distal intestinal location where SCFA are produced and absorbed (Hansen & Sams, 2018; So et al., 2021). We therefore hypothesized that intrinsic fibers substantially increase fecal SCFA levels in the distal colon and possibly affect health differently from purified fibers (Dagbasi et al., 2020; Grundy et al., 2016; Hansen & Sams, 2018).

Previously, we showed that chicory roots have a long history of consumption and contain the highest dry fiber content of edible vegetables, nuts, seeds, and fruits (Puhlmann & de Vos, 2020). In the present study we aimed to explore the health effects of dried chicory roots that contain approximately 85% fiber, consisting of native inulin enclosed in plant cell wall fibers (pectin, cellulose and hemicellulose). We assessed the effect of daily dried chicory root intake on bowel function, gut microbiota composition, fecal and circulating SCFA levels, as well as glucose homeostasis markers in subjects at risk of T2D in a randomized, parallel, placebo-controlled, and investigator-blinded trial.

METHODS

PARTICIPANTS

Participants were locally recruited in the surroundings of Wageningen University & Research in Wageningen, the Netherlands, between May and September 2018. Participants were eligible if they were between the age of 40-75 years and had either a fasting blood glucose level between 5.0 and 5.6 mmol/L with a high risk to develop type 2 diabetes (T2D) later in life as assessed by a diabetes risk score ≥ 9 (Alssema et al., 2008; Lindström & Tuomilehto, 2003), or a fasting blood glucose level of 5.6 and 6.9 mmol/L (classified as prediabetes by the American Diabetes Association (American Diabetes Association, 2016)). Randomization was performed by a researcher not involved in the study. Participants were randomly assigned by a third researcher to either the treatment or the placebo group within strata based on sex and fasting glucose level inclusion criteria. History of medical or surgical events that may significantly affect the study outcome, medical drug use for T2D or chronic use of antacids resulted in exclusion from the study. Other exclusion criteria included the use of antibiotics during the three months prior to screening and consumption of pre- or probiotics or any fiber supplement during the last month prior to screening. Participants were also excluded if they reported any unexplained weight loss or weight gain of more than 5 kg in the one month prior to the screening, or if they followed a slimming or medically prescribed diet, or a vegan or macrobiotic lifestyle. Finally, any known sensitivity to medical skin adhesives also led to exclusion from participation. In total 156 men and women were screened, whereof 63 participants were eligible and 60 were included in the study.

STUDY DESIGN

The study was a two-arm randomized, placebo-controlled, investigator-blinded, parallel trial. The study consisted of three study periods, a run-in of two weeks, an intervention period of three weeks and a wash-out of two weeks (Figure 1). A detailed description of the study design is given in the Supplementary Methods (available online). The Medical Ethical Review Committee Wageningen University (METC-WU nr. 17/25) approved this study and registered at the ISRCTN registry (ISRCTN39985847). The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Primary outcomes were defined as changes in the static glycemic markers HOMA-ir, fasting insulin and fasting glucose after the study period (30g/day treatment intake; T2) against baseline (T0). Secondary outcomes were differences in changes over time between intervention groups in bowel function assessed by stool softness (Bristol Stool Scale, BSS) (Lewis & Heaton, 1997) and stool frequency (defecations per day), gut microbiota composition, fecal and fasting circulating SCFA, and glycemic variability metrics captured by Continuous Glucose Monitoring (CGM) after the study period (T2) and in addition after the run-in (T1) and the wash-out period (T3).

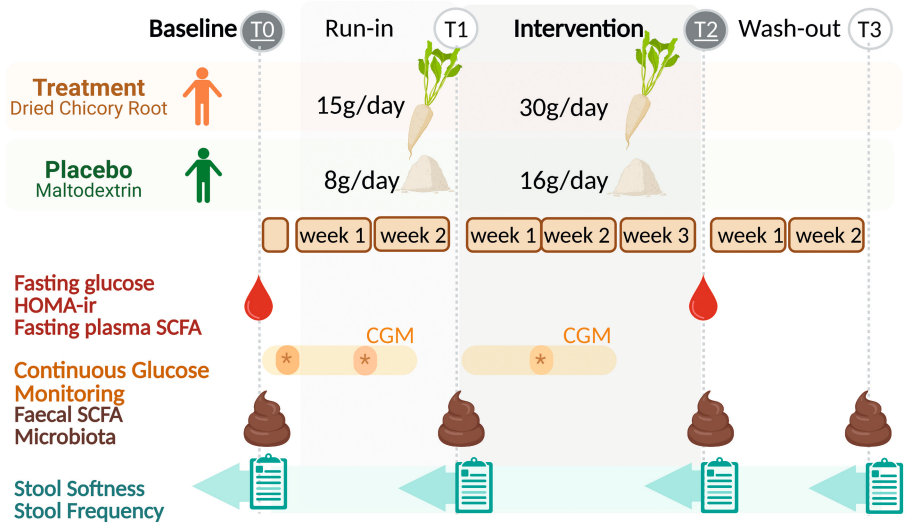

Figure 1. Trial design. Trial design of the randomized controlled parallel trial comparing dried chicory root intake with maltodextrin placebo. * indicates three-day period (same weekdays) used for the calculation of continuous glucose monitoring metrics for baseline (T0), run-in (T1) and intervention period (T2). Created with BioRender.com

INTERVENTION PRODUCTS

The treatment product consisted of WholeFiber™ (provided by WholeFiber BV, The Netherlands), an intrinsic multifiber product made from chicory root that are washed,

cut and dried. The dried chicory root product has a dry weight of 93%(w/w), including 85%(w/w) fiber, whereof 70%(w/w) is native inulin, 10%(w/w) pectin and 5%(w/w) hemicellulose and cellulose. This treatment product was taken at a daily dose of 30 g for three weeks in the treatment group, while the placebo group consumed a daily dose of an iso-caloric amount of 16 g maltodextrin (Body&Fit, The Netherlands). During the run-in period, both groups received half the amount, 15 g of the treatment product and 8 g of the placebo, respectively.

MICROBIOTA COMPOSITION, IN VITRO EXPERIMENTS, BIOCHEMICAL ANALYSES AND BOWEL FUNCTION DIARIES

Details about the analysis of gut microbiota composition using 16S rRNA amplicon sequencing, synthetic co- and tri-culture experiments, assessment of bowel function (stool softness and frequency), fecal and fasting circulating SCFA, static (fasting insulin, fasting glucose and HOMA-ir), as well as dynamic (CGM) glycemic markers can be found in the (online) Supplemental Methods. Gut microbiota data has been submitted to the European Nucleotide Archive (ENA) under accession number PRJEB47230.

STATISTICAL ANALYSIS AND SUBGROUP SEGMENTATION

Data is presented as mean ± SEM if not indicated differently. For the purpose of comparison with other studies, changes were also expressed as percentage change (absolute change divided by baseline value). GraphPad Prism 9.1 and R version 4.0.3 were used to generate corresponding graphics and for statistical analysis applying $\alpha = 0.05$. Gut microbiota data was analyzed using the R package mare (Korpela, 2016). Differences in changes in fecal SCFA, bowel function and CGM metrices were analyzed with repeated-measures analyses using the lme4 R package (Bates et al., 2015) or the rstatix R package (Kassambara, 2023). Post-intervention differences in plasma biomarkers and others were assessed by analysis of covariance. Sample size calculation, further details on the statistical analyses, on responder analyses and on subgroup segmentation are given in the Supplemental Methods.

RESULTS

BASELINE CHARACTERISTICS

In total 58 participants completed the study as two participants dropped out for personal reasons and another three were excluded due to lifestyle changes, protocol violation or missing samples (Supplemental Figure S2). Product intake compliance, assessed by returned empty and leftover sachets, was excellent (96.3%). At baseline, we observed that the placebo and treatment group were well randomized, but the placebo group had slightly softer stools (Table 1). While fasting plasma glucose levels were elevated ($f\text{-}glu_{all}$ = 6.0 ± 0.6 mmol/L) according to prediabetes criteria (American

Diabetes Association, 2016), overall insulin resistance as assessed by HOMA-ir was relatively low in the study population (HOMA-ir_{all} = 1.31 ± 0.61).

Table 1. Baseline characteristics of all subjects included in the data analysis.

	Treatment (n = 28)	Placebo (n = 27)	*p*-value
Sex			
Female, n	14	14	
Male, n	14	13	
Age, years, median (IQR)	69 (52-75)	69 (48-75)	0.76
Fiber intake (g/day), mean (±SD)	23.6 (7.6)	22.0 (8.2)	0.44
BMI (kg/m^2) , mean (±SD)	28.6 (3.7)	28.3 (3.1)	0.75
Weight (kg), mean (±SD)	85.0 (17.7)	84.7 (14.6)	0.94
Waist (cm), mean (±SD)	102.7 (12.2)	103.3 (10.0)	0.86
Glucose (mmol/L), mean (±SD)	6.1 (0.6)	5.9 (0.6)	0.20
Insulin (µU/mL), mean (±SD)	8.05 (3.86)	8.57 (3.99)	0.63
HOMA-ir, median (IQR)	1.1 (0.4 - 2.7)	1.2 (0.4-3.6)	0.52
CV (%), mean (±SD)	21.4 (4.81)	20.6 (5.57)	0.55
Stool Frequency (x/day), median (IQR)	1.0 (0.9 -3.0)	1.0 (0.3-2.5)	0.31
Stool Softness (BSS), mean (±SD)	3.3 (1.3)	4.1 (1.2)	0.02

BSS, Bristol Stool Score; CV, coefficient of variation assessed by continuous glucose measurement

BOWEL FUNCTION

First, we wanted to understand how dried chicory root would affect gut function by assessing bowel habits recorded as stool softness and stool frequency. We observed that the treatment had a pronounced effect over time on stool softness (repeated-measures ANOVA interaction: $F(3, 159) = 2.952$, $p = 0.034$) and stool frequency (Friedman test: $\chi^2(3) = 22.3$, $p < 0.001$). Stool softness increased by +1.1 ± 0.3 units (BSS; $p = 0.004$) from 3.3 ± 0.3 to 4.4 ± 0.2 after three weeks of 30 g/day treatment (T2), a change that already started after 15 g/day (T1: +0.6 ± 0.3 BSS, $p = 0.072$). Of note, stool frequency increased with +0.6 ± 0.2 from 1.3 ± 0.1 to 1.9 ± 0.1 defecations per day after 30 g/day treatment (T2; $p = 0.004$), an increase that also started at 15 g/day (T1: +0.2 ± 0.1 x/day, $p = 0.038$). In contrast, in the placebo stool softness remained constant with 4.1 ± 0.2 at baseline, 4.3 ± 0.2 after run-in (T1; $p = 0.69$) and 4.2 ± 0.2 after study period (T2; $p = 0.69$). Similar, stool frequency remained constant with 1.2 ± 0.1 defecations/day at baseline and 1.3 ± 0.2 after run-in (T1; $p = 0.347$) and 1.3 ± 0.1 study period (T2; $p = 0.582$). For both groups, stool softness and frequency returned to baseline levels after two weeks wash-out (T3 treatment: BSS = 3.5 ± 0.2, stool frequency = 1.2 ± 0.1 and placebo: BSS = 4.1 ± 0.2, stool frequency = 1.3 ± 0.1). These increasing changes in bowel function over time and related fiber dosages indicated a substantial modulatory potential of dried chicory roots on the colonic environment.

GUT MICROBIOTA COMPOSITION

To understand the effect of dried chicory root intake on the gut microbes, we then assessed the overall fecal microbiota composition as well as changes in the relative abundance of individual genera. We observed a strong modulatory effect of the treatment product on overall gut microbiota composition assessed by Bray-Curtis β-diversity (Figure 2A-D), which in this context reflects the between-person differences in gut microbiota composition. Already after two weeks of 15 g/day treatment (T1; Figure 2B), overall gut microbiota composition of the treatment and placebo shifted apart, finally explaining 7% (Permutational multivariate analysis of variance (PERMANOVA), $p = 0.001$) of the observed variation in microbial composition after three weeks of 30 g/day treatment (T2; Figure 2C). Interestingly, this effect was fully reversible as overall gut microbiota composition returned to baseline once subjects had stopped for two weeks with the treatment (wash-out; T3, Figure 2D). The overall compositional changes by the treatment were mirrored in the change of several taxa at genus level (Figure 2E). We observed the most pronounced changes in the stimulation of relative levels of *Anaerostipes* and *Bifidobacterium* spp. (Figure 2E, online Supplemental Figure S3). Of these genera, the most abundant species were identified as *A. hadrus* and *A. butyraticus* as well as *B. longum* and an unclassified *Bifidobacterium* species. After two weeks of 15 g/day treatment (T1) the relative abundance of *Anaerostipes* spp. increased already by 2.82-fold ($q < 0.001$, Supplemental Table S2) reaching levels twice as high as placebo (T1: treatment = 3.7% versus placebo = 1.6%, $q < 0.001$; online Supplemental Table S3). After 30 g/day treatment *Anaerostipes* spp. relative abundance increased 3.24-fold (T2, $q < 0.001$; Supplemental Table S2) to levels three times higher than placebo (treatment = 4.3% vs placebo = 1.4%, $q < 0.001$; online Supplemental Table S3). In addition, *Bifidobacterium* spp. relative abundance increased 3.17-fold after 15 g/day treatment (T1, $q < 0.001$) and 4.09-fold after 30 g/day treatment (T2, $q < 0.001$; Supplemental Table S2) to a relative level of 13.0%, which was also three times higher than placebo (T2: placebo = 4.5%, $q = 0.002$; online Supplemental Table S3). We also observed several taxa to decrease in relative abundance after treatment intake (Figure 2E; Supplemental Table S2), notably *Blautia* spp. of which the most abundant species were identified as *B. hominis*, *B. luti*, and *B. obeum*. None of these changes were observed in the placebo group after run-in or study ($q > 0.05$, online Supplemental Table S4) and no taxa differed between the groups after the washout (T3; online Supplemental Table S3). In summary, the modulation of the overall gut microbiota composition and concomitant changes in relative abundance of specific taxa were dose-dependent and reversible.

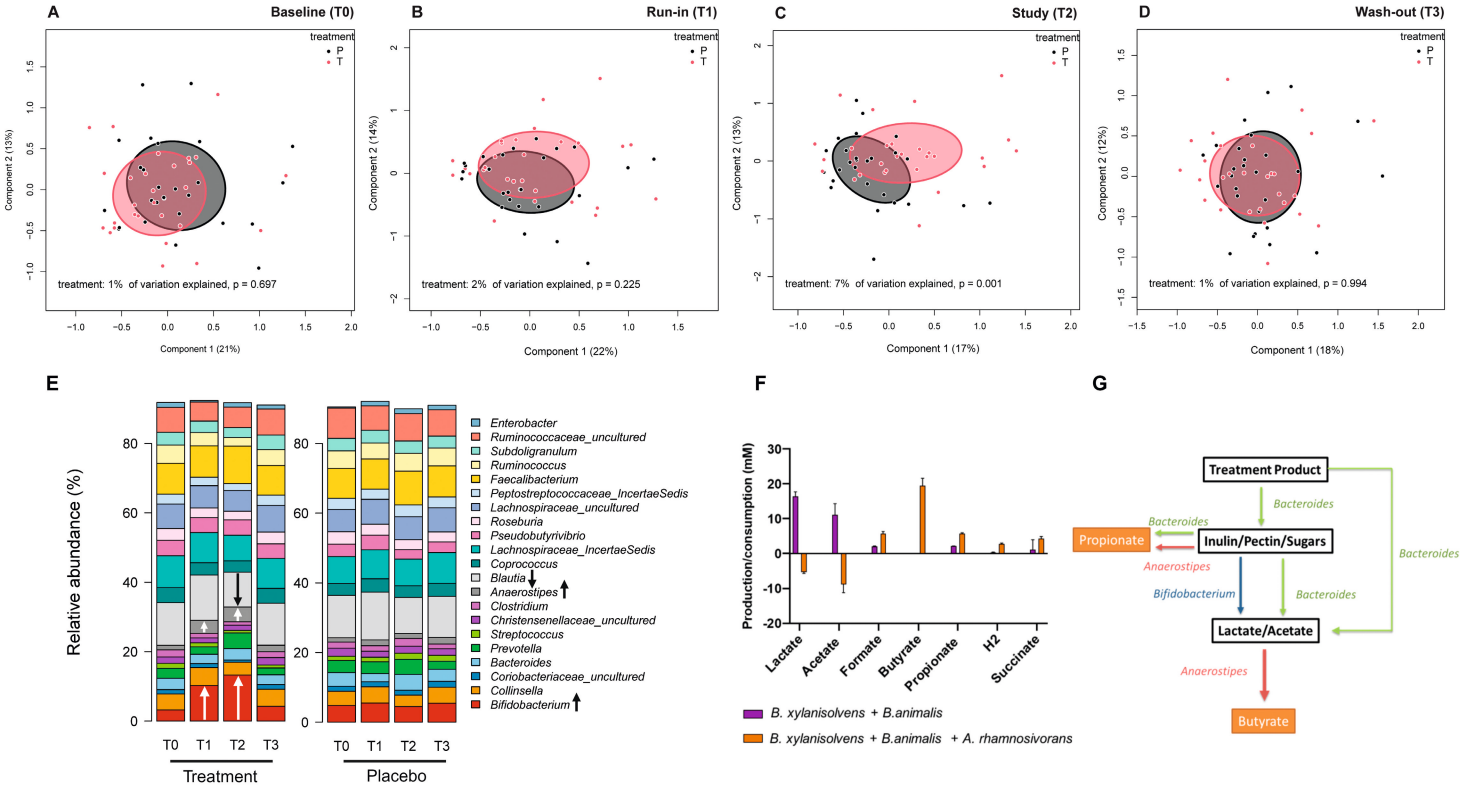

Figure 2. Changes in gut microbiota composition and trophic chain *in vitro* experiment. (A-D) Effect of dried chicory root consumption on overall gut microbiota composition (β-diversity, assessed by Principal Coordinates Analysis (PCoA) with Bray-Curtis dissimilarity) at baseline (T0) after two weeks of 15 g/day treatment or iso-caloric placebo (T2), three weeks of 30 g/day treatment or iso-caloric placebo (T2) and after two weeks of wash-out (T3). No changes in α-diversity measures were observed, which reflects the within-person variation in microbiota composition by measuring the number

(richness) of different bacterial taxa and their distribution (evenness) within a sample. (E) Gut microbiota composition at baseline (T0), 15 g/day treatment or iso-caloric placebo (T1), 30 g/day treatment or iso-caloric placebo (T2) and washout (T3). We observed a significant increase (↑) of *Bifidobacterium* spp. and *Anaerostipes* spp. at T1 and T2 in the treatment group and a significant decrease (↓) of *Blautia* spp. at T2. None of the taxa in the placebo group changed significantly between any of the timepoints. (F) Synthetic co- and tri-culture experiments. Metabolite production and consumption in a tri-culture containing *Bacteroides xylanisolvens* (*B. xylanisolvens*), *Bifidobacterium animalis* subsp. *lactis* BLC1 (*B. animalis* BLC1) and *Anaerostipes rhamnosivorans* (*A. rhamnosivorans*) incubated with the treatment product consisting of dried chicory roots (5 g/L in YCFA) (Duncan et al., 2009). Mean values are shown with standard deviation after seven days incubation at 37 °C but 80% of the conversion was already completed after three days of incubation (not shown). **(G)** Postulated microbial and metabolic interaction network involved in the colonic fermentation of the dried chicory roots (predominantly consisting of inulin, pectin and hemicellulose and cellulose). Proposed conversion by *Bacteroides* spp. is presented in green, by *Bifidobacterium* spp. in blue and by *Anaerostipes* spp. in red. The treatment product, dried chicory roots, consists of plant cells, which are envisaged to be degraded and liberate the intermediate products inulin, pectin and small sugars (like fructo-oligosaccharides) that are further converted into the end products (highlighted in orange) including propionate or butyrate via the intermediate products lactate and acetate.

Table 2. Levels and changes of fecal short-chain fatty acid levels at baseline (T0), after two weeks of 15 g/day dried chicory root or 8 g/day iso-caloric placebo (T1), after three weeks of 30 g/day dried chicory root or 16 g/day iso-caloric placebo (T2) product intake, and after two weeks of wash-out (T3).[a]

	Treatment				Placebo				*p*-value Intervention*Period		
SCFA (mmol/kg)	T0	T1	T2	T3	T0	T1	T2	T3	T1 vs T0	T2 vs T0	T3 vs T0
Total SCFA	50.65 (4.71)	60.21 (4.96)	63.67 (5.12)	48.91 (6.77)	56.50 (5.95)	59.62 (6.64)	50.00 (5.48)	51.49 (5.53)	0.415	0.023	0.690
Δ Total SCFA		9.56 (5.74)	13.02 (6.26)	1.76 (6.32)		2.75 (4.61)	-6.51 (6.19)	-4.75 (5.77)			
Acetate	32.67 (3.27)	38.34 (3.33)	41.92 (3.40)	31.42 (4.42)	37.49 (4.10)	39.90 (4.88)	33.05 (3.71)	33.99 (3.67)	0.544	0.022	0.637
Δ Acetate		5.67 (4.03)	9.25 (4.43)	1.51 (4.37)		2.16 (3.29)	-4.44 (4.35)	-3.68 (3.91)			
Propionate	9.07 (0.82)	10.66 (0.89)	11.09 (1.04)	9.13 (1.49)	9.28 (0.81)	10.11 (0.87)	8.83 (0.79)	9.10 (0.82)	0.553	0.065	0.955
Δ Propionate		1.60 (0.81)	2.02 (0.90)	0.15 (1.08)		0.85 (0.75)	-0.45 (0.90)	-0.01 (0.96)			
Butyrate	8.91 (1.02)	11.21 (1.17)	10.66 (1.08)	8.36 (1.16)	9.74 (1.29)	9.62 (1.21)	8.12 (1.13)	8.40 (1.27)	0.140	0.052	0.717
Δ Butyrate		2.30 (1.25)	1.75 (1.18)	0.09 (1.17)		-0.26 (1.12)	-1.62 (1.21)	-1.06 (1.22)			

[a] Data is presented as mean (SEM). Analysis was done by linear mixed model that assessed whether the change (Δ) in SCFA over intervention periods by the treatment was different from placebo (represented by the interaction term: intervention*period). All estimated model fixed effects are provided in online Supplemental Table S6. Analysis of post-intervention differences between groups at T2 only are provided in online Supplemental Table S7. SCFA, short-chain fatty acids

TROPHIC CHAIN IN VITRO EXPERIMENT

To demonstrate that the observed changes in relative fecal abundances of both *Bifidobacterium* and *Anaerostipes* spp. after chicory root intake were associated with a trophic chain involving these two functional bacterial groups, we performed *in vitro* experiments with synthetic co- and tri-cultures as a proof-of-concept (Figure 2F). For this purpose, we selected strains with the canonical functionality to convert inulin or ITF into lactate and acetate (*Bifidobacterium* spp.) and the capacity to convert these further into butyrate (*Anaerostipes* spp.). We incubated the treatment product at a concentration of 5 g/L with the well-studied *B. animalis* BLC1 (Bottacini et al., 2011) and *A. rhamnosivorans* 1Y2^T (Bui et al., 2014) and found a modest but reproducible increase of butyrate (1.19 ± 0.39 mM). Hence, we anticipated the need for the degradation of the plant cell-wall fibers present in the treatment product, which is a known metabolic feature of the abundant gut bacteria *Bacteroides* and *Prevotella* spp. (Martens et al., 2011). Therefore, we also included *Bacteroides xylanisolvens* HMP, a known pectin degrader (Despres et al., 2016) in a co-culture experiment with only *B. animalis* BLC1 and in a tri-culture experiment with also *A. rhamnosivorans* 1Y2^T. Only when using this latter tri-culture, we observed the production of high amounts of butyrate (19.5 ± 2.0 mM; Figure 2F), representing over half of the amount that dried chicory roots potentially may provide. Hence, this proof-of-concept experiment showed that assisted by a pectin-degrader, such as the selected *Bacteroides* strain, a butyrogenic trophic chain was formed from the dried chicory root involving representative members of *Bifidobacterium* and *Anaerostipes* spp.

FECAL SCFA

We then assessed the impact of dried chicory root on fecal SCFA levels as a measure of gut microbiota activity. Total fecal SCFA levels increased by 13.02 ± 02 mmol/kg after 30 g/day treatment for three weeks, a relative increase of +25.7% which was reflected in all three SCFA (T2; Table 2). Using linear mixed modelling we confirmed that fecal SCFA levels pronouncedly changed from placebo (p = 0.023, interaction term intervention*period) during 30 g/day treatment (T2, Table 2; estimated model fixed effects are provided in online Supplemental Table S6). The increase in acetate levels (p = 0.022) was highest with a relative change of +28.3% followed by increased propionate levels by +22.3% and butyrate levels by +19.6% (T2: p = 0.065 and p = 0.052, respectively; Table 2). The increase in fecal SCFA levels started already after 15 g/day treatment (T1; Table 2). The mean levels of fecal butyrate in the treatment group increased most at T1 by +25.8% compared to 19.6% at T2 (Table 2). However, the proportion of subjects with an increase in fecal butyrate levels (minimal observed increase > 1mM) was actually highest at T2 (18 out of 28) and differed from that in the placebo (9 out of 27; Fisher's exact: p = 0.015, online Supplemental Figure S4). Overall, fecal SCFA levels in the placebo slightly decreased (Table 2). Once treatment intake stopped, fecal SCFA levels returned to baseline (T3, Table 2). Hence, dried chicory root also modulated gut microbiota activity and increased fecal SCFA levels in a reversible way.

FASTING CIRCULATING SCFA

To determine the effect of the increased fecal SCFA on the systemic availability of these metabolites, we also measured fasting circulating SCFA at the end of the study period with 30g/day treatment intake. We observed an increase after 30 g/day treatment in total fasting circulating SCFA of 7.70 ± 3.88 μM (T2; $p = 0.057$), which represents a relative change over baseline of +15.7% and was a result of both increased fasting acetate (+16.4%; $p = 0.057$) and propionate levels (11.1%; $p = 0.431$, Table 3). The placebo remained largely unchanged, with total fasting circulating SCFA slightly decreasing (-4.1%; $p = 0.630$, Table 3), while the changes did not differ between groups (Table 3). Fasting butyrate levels decreased in the placebo group about five times more than in the treatment group, where the levels remained virtually unchanged (Table 3). Comparing adjusted post-intervention levels we found an increase in total SCFA by the treatment against placebo of +8.56 ± 4.97 μM ($p = 0.091$), for acetate of +7.47 ± 4.85 μM ($p = 0.129$), for propionate of +0.85 ± 0.76 μM ($p = 0.266$) and for butyrate of+ 0.06 ± 0.08μM ($p = 0.451$; see also online Supplemental Table S7). Hence, the treatment with dried chicory root also resulted in systemic effects.

STATIC AND DYNAMIC MARKERS OF GLUCOSE HOMEOSTASIS

Finally, we wanted to understand whether metabolic markers of glucose homeostasis were affected by the intake of dried chicory root. First, we assessed the static markers HOMA-ir, fasting insulin and fasting glucose at the end of the 30g/day treatment compared to baseline. HOMA-ir decreased from 1.28 ± 0.12 to 1.24 ± 0.09 after 30 g/day treatment ($p = 0.566$), a relative decrease over baseline by -3.1%. Similarly, insulin decrease slightly by -2.4% (-0.19 ± 0.50 μU/mL, $p = 0.710$) and fasting glucose by -2.1% (-0.13 ± 0.08 mmol/L, $p = 0.637$). Yet, none of these changes differed from those in placebo (online Supplemental Table S8). Comparing adjusted post-intervention levels between groups, we found a decrease in HOMA-ir against placebo of -0.05 ± 0.09 ($p = 0.570$), which was mirrored in fasting insulin (-0.32 ± 0.6 μU/mL, $p = 0.597$) but absent in fasting glucose (-0.02 ± 0.12 mmol/L, $p = 0.877$; online Supplemental Table S7).

In recent years continuous glucose monitoring (CGM) has been developed to provide clinically relevant and dynamic insights into glucose profiles next to static biomarkers (Danne et al., 2017). Hence, we also monitored continuous glucose levels at baseline, during run-in and during the study period on three consecutive days to understand the development of glycemic control over the intervention, as assessed by the coefficient of variation (CV) of glucose levels (Danne et al., 2017; Peyser et al., 2018). We observed that the CV decreased from 21.3 ± 0.94% at baseline to 18.1 ± 0.97% during run-in ($p = 0.001$) and 18.3 ± 0.84% in the study period ($p = 0.004$) in the treatment group. The CV also decreased to a lower extent in the placebo group from 20.6 ± 1.07% to 18.7 ± 1.00% (run-in, $p = 0.026$) and 18.9 ± 0.86% (study, $p = 0.065$; online Supplemental Figure S5), yet following a sensitivity analysis only the decreases in the treatment group was sustained (run-in $p = 0.018$ and study $p = 0.024$; Figure 3A and Supplemental Methods). These observations indicated that dried chicory root intake had the capacity to improve the dynamics of glucose levels over time.

EFFECT OF BASELINE *BLAUTIA* SPP. RELATIVE ABUNDANCE ON T2D BIOMARKERS

Besides the general effect of dried chicory root on glucose homeostasis in subjects at risk of T2D, we wanted to understand how the gut microbiota potentially impacts changes in the biomarkers of glycemic control. Hence, we further investigated the glucose homeostasis response by dividing the treatment group into HOMA-ir responders (>10% decrease HOMA-ir, n = 8) and non-responders (>10% increase HOMA-ir, n = 12). The most discriminative genus included acetogenic *Blautia* spp. and at baseline HOMA-ir responders had significantly lower relative *Blautia* spp. amounts (1.4-fold lower; p = 0.01, Figure 3B). Consequently, we segmented the intervention group based on median baseline abundance of *Blautia* spp. into low (n = 14) and high abundance (n = 14). Remarkably, we observed a decrease in fasting glucose levels of -5.1% (-0.31 ± 0.11 mmol/L, p = 0.013) in the low *Blautia* group, while the high *Blautia* group remained unchanged (0.06 ± 0.10 mmol/L, p = 0.546) leading to a difference between groups of 0.38 ± 0.15 mmol/L (p = 0.019; Figure 3D). Following up on this observation, we also observed HOMA-ir decreased more pronouncedly with -10.8% (-0.14 ± 0.10 HOMA-ir, p = 0.289) compared to the high *Blautia* group (+0.07 ± 0.12 HOMA-ir, p = 0.055), resulting in a difference of 0.21 ± 0.16 between groups (p = 0.045; Figure 3E). Similarly, fasting insulin levels decreased with –9.9% (-0.82 ± 0.65 low *Blautia* versus 0.45 ± 0.75 µU/mL high *Blautia*; p = 0.210; online Supplemental Table S9).

Of note, segmenting subjects into those with low *Blautia* spp. baseline levels also led to even more pronounced effects on glucose variability and fasting circulating SCFA. Glucose variability decreased in the low *Blautia* group from 21.3 ± 1.3% to 17.1 ± 1.0% in run-in (p = 0.002) and 17.7 ± 0.8% during the study (p = 0.018), while in the high *Blautia* group this decrease was more gradual from 21.4 ± 1.4% to 19.0 ± 1.6% in run-in (p = 0.127) and 18.3 ± 1.4% during the study period (p = 0.061; Figure 3C). Total fasting circulating SCFA increased by +22.7% nearly three-times more in the low *Blautia* group (+11.66 ± 5.64 µM; p = 0.059) compared to +8.0% in the high *Blautia* group (+3.75 ± 5.32 µM; p = 0.493), which was associated with a higher increase in fasting acetate levels in the low *Blautia* (+10.26 ± 5.00 µM, p = 0.061) versus the high *Blautia* group (+4.13 ± 5.30 µM, p = 0.450; Supplemental Figure S6 A-B). Interestingly, fasting propionate levels increased only in the low *Blautia* group by +27.0% (+1.39 ± 1.16 µM, p = 0.252) and fasting butyrate levels remained unchanged (- 0.01 ± 0.9µM, p = 0.688) while in the high *Blautia* group fasting propionate (- 0.29 ± 0.73 µM, p = 0.702) and butyrate levels slightly decreased (-0.08 ± 0.19µM, p = 0.813; Supplemental Figure S6 C-D). Hence, modulation of metabolic markers by the dried chicory root intake was observed to relate to differences in the baseline abundance of gut bacteria.

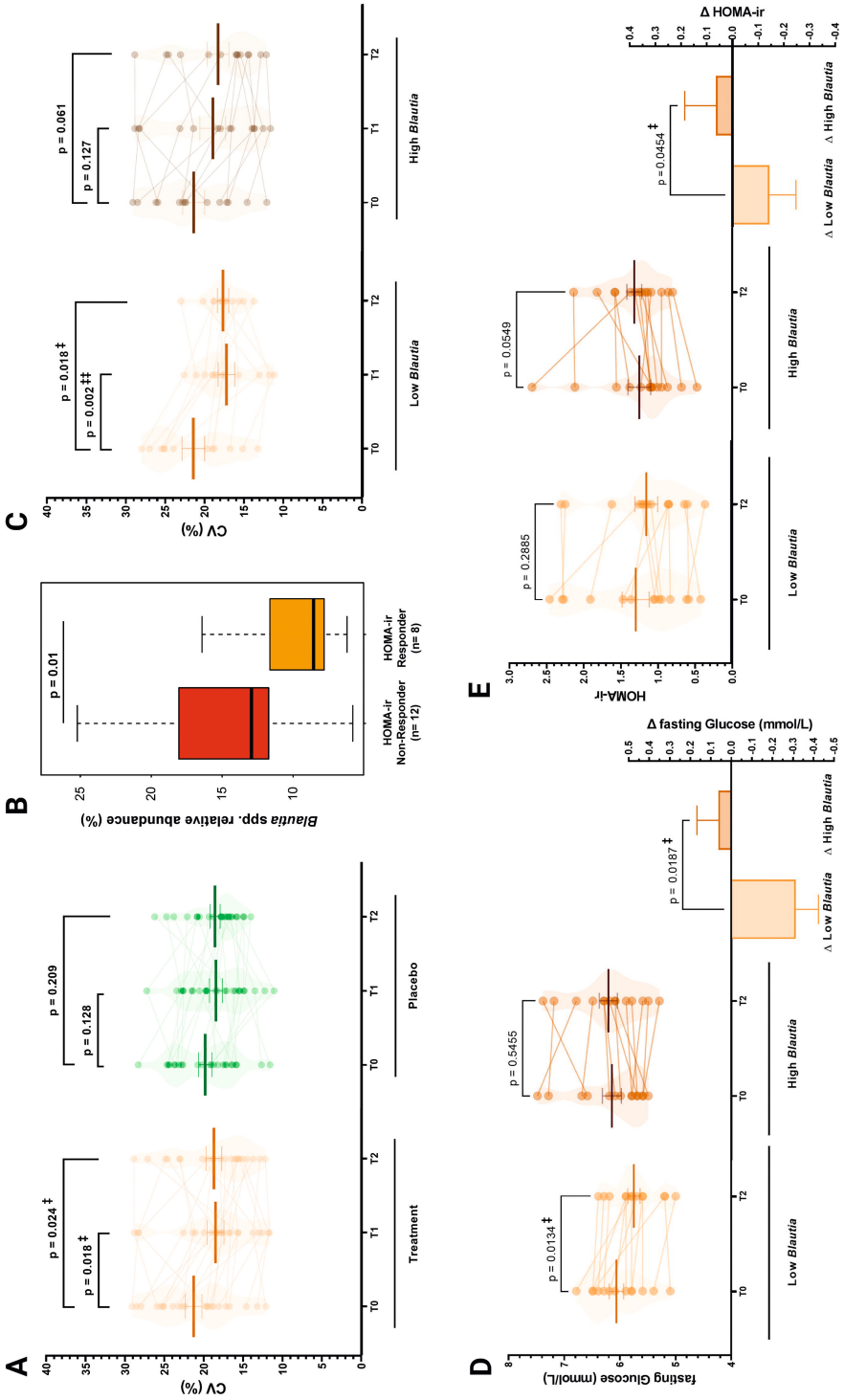
A
CV (%)
p = 0.024 ‡
p = 0.018 ‡
p = 0.209
p = 0.128
T0
T1
T2
Treatment
Placebo
B
Blautia spp. relative abundance (%)
p = 0.01
HOMA-ir Non-Responder (n= 12)
HOMA-ir Responder (n= 8)
C
CV (%)
p = 0.018 ‡
p = 0.002 ‡‡
p = 0.127
p = 0.061
Low Blautia
High Blautia
D
fasting Glucose (mmol/L)
p = 0.0134 ‡
p = 0.5455
Δ fasting Glucose (mmol/L)
p = 0.0187 ‡
Δ Low Blautia
Δ High Blautia
E
HOMA-IR
p = 0.2885
p = 0.0549
Δ HOMA-ir
p = 0.0454 ‡

Figure 3. Changes in static and dynamic markers of glucose homeostasis. (A) Coefficient of variation (CV%) as a measure of glycemic control assessed using continuous glucose measurement on three same consecutive weekdays during baseline (T0), the run-in period (T1) with 15 g/day treatment or 8 g/day iso-caloric placebo, and during the study period (T2) with 30 g/day treatment or 16 g/day isocaloric placebo (repeated-measures ANOVA with main effect of period p=0.001, post-hoc tests with FDR-adjustment). Data is presented after sensitivity analysis excluding datasets with more than 20% missing data and an extreme outlier (3x IQR) for treatment (n=22) and placebo group (n=24). No difference between groups in baseline CV was observed before (p = 0.55) and after sensitivity analysis (p = 0.25). (B) Difference in relative abundance of *Blautia* spp. at baseline (T0) between HOMA-ir Responders (>10% decrease, n = 8) and Non-Responders (>10% increase) (C-E) Differences in changes in subjects with low (n = 14) or high (n = 14) baseline *Blautia* relative abundance in the treatment group in (C) CV% as a measure of glycemic control (low n= 13, high n = 14; repeated-measures ANOVA with main effect of period p<0.001, post-hoc tests with FDR-adjustment), (D) fasting glucose levels and (E) HOMA-ir as glucose homeostasis markers (assessed using non-parametric testing). *‡ p <0.05, ‡‡ p <0.01*

Table 3. Fasting circulating SCFA levels at baseline (T0), after three weeks of treatment or placebo (T2) and change (Δ) over baseline.[a]

	Treatment				Placebo				
Circulating SCFA (μM)	T0	T2	Δ_T	*p*-value	T0	T2	Δ_P	*p*-value	*p*-value Δ_T - Δ_P
Fasting Total SCFA	49.10 (3.60)	56.80 (4.02)	7.70 (3.88)	0.057	51.86 (4.40)	49.53 (3.85)	-2.33 (4.29)	0.591	0.088
Fasting Acetate	43.93 (3.50)	51.13 (3.92)	7.20 (3.62)	0.057	45.89 (4.43)	44.60 (3.85)	-1.29 (4.33)	0.768	0.137
Fasting Propionate	4.98 (0.65)	5.53 (0.57)	0.55 (0.69)	0.431	5.65 (0.54)	4.85 (0.53)	-0.80 (0.68)	0.251	0.170
Fasting Butyrate[b]	0.19 (0.09)	0.15 (0.06)	-0.04 (0.10)	0.946	0.31 (0.12)	0.08 (0.06)	-0.23 (0.14)	0.092	0.094

SCFA, short-chain fatty acids
[a] Data is presented as mean (SEM).
[b] Butyrate levels were analyzed using nonparametric testing.

DISCUSSION

We examined the effect of an intrinsic, high-fiber product based on dried chicory roots, consisting of native inulin, pectin, hemicellulose and cellulose embedded in plant cells, in adults at risk of T2D. We observed a significant and strong dose-dependent and reversible effect of the treatment on improved bowel function, microbiota composition and activity as well as noted improvement of dynamic glucose levels measured by CGM.

Purified native inulin is thought to stimulate bacterial growth in the colon, which leads to increased bacterial mass and fecal bulk, and is hence well-known for its positive effect on bowel function maintenance (EFSA, 2015). Here we observed that both stool softness and stool frequency increased after 15g/day and further after 30g/day dried chicory root treatment and returned to baseline levels after the wash-out, indicating a time- and dose-dependent effect of the treatment on the colonic environment by the intrinsic fibers, that by definition include native inulin within the plant cell walls.

Modulation of the gut microbiota towards the stimulation of taxa that are associated with health benefits forms the basis for prebiotic interventions (Gibson et al., 2017). Two well-known genera in this context are *Bifidobacterium* and *Anaerostipes* spp., which have been reported to be slightly increased (approximately 1.2 - 1.4-fold) following intake of isolated, purified ITF (Vandeputte et al., 2017). However, the strong overall modulation of the gut microbiota (Figure 2A-D) and the more than three-fold increase in both *Bifidobacterium* and *Anaerostipes* spp. we observed here (Figure 2E), is remarkable and much higher compared to earlier ITF studies. Especially in subjects with low baseline abundance of these genera, we observed an increase by up to 11.6-fold for *Bifidobacterium* and 6.12-fold for *Anaerostipes* spp. after 30 g/day treatment (Supplemental Table S5). One study in healthy subjects consuming inulin-rich foods instead of purified ITF for two weeks also observed a four-fold increase in *Bifidobacterium* spp. but no changes in *Anaerostipes* spp. (Hiel et al., 2019). The high co-occurring fold-changes observed with the dried chicory roots point towards an improvement of the trophic chain involving bacteria of these two genera. Several *Bifidobacterium* spp., including the here identified *B. longum*, are known to use inulin and ITF, generating acetate and lactate (Falony et al., 2009; Flint et al., 2012). These metabolites are proven substrates for butyrate production by all known *Anaerostipes* species such as the here detected *A. hadrus* and *A. butyraticus* (Allen-Vercoe et al., 2012; Bui et al., 2019, 2021). Butyrate production is a desired feature of microbial fiber degradation since this SCFA is known to maintain gut homeostasis, *e.g.* by serving as energy source for colonocytes (Canfora et al., 2015). However, the plant cell walls might shield inulin from utilization by these two genera. To assess the possibility of a trophic chain from the dried chicory root we performed a proof-of-concept experiment using a minimal set of strains with the canonical functionality of the genera of interest. We needed to include a pectin-utilizer like *Bacteroides xylanisolvens* HMP (Despres et al., 2016) to fully unlock the potential of the dried chicory roots *in vitro. Bacteroides* spp. as well as *Prevotella* spp. are known degraders of plant cell-wall fibers (Martens et al., 2011)

that may partially degrade and thereby open up the plant cells, and both genera were are found to be abundantly present in fecal samples (Figure 2E, online Supplemental Figure S3). Using the synthetic tri-culture consisting of selected strains of *Bacteroides, Bifidobacterium* and *Anaerostipe*s spp., we indeed observed a substantial increase in butyrate (Figure 2F), for which the presence of *A. rhamnosivorans* was essential as it consumed lactate and acetate generated by *Bacteroides* spp. and *Bifidobacterium* spp. and in small amounts present in the YCFA medium (Duncan et al., 2009). It is possible that *Anaerostipes* spp. may even form propionate from a small amount of inositol in chicory (Hernández-Hernández et al., 2011) that is converted via the recently discovered metabolic pathway in *A. rhamnosivorans* and some other *Anaerostipes* spp. (Bui et al., 2021). Based on these proof-of-concept *in vitro* simulations, we propose a multi-species microbial network, operating in the colon of the subjects consuming dried chicory roots, that generates butyrate and propionate (Figure 2G).

Fiber-derived colonic SCFA that subsequently enters the systemic circulation is believed to contribute to the preventive effect of fermentable fibers on metabolic diseases (Canfora et al., 2015). Here, we observed that intake of dried chicory root can increase all three SCFA, i.e. acetate, propionate and butyrate, by more than a quarter of their baseline levels (Table 2), which is much higher than previously reported in studies with purified ITF. Most ITF studies detected small or no differences (Baxter et al., 2019; Kiewiet et al., 2021; Vandeputte et al., 2017) or even a decrease in fecal SCFA (Salazar et al., 2015). One trial reported an increase in all three SCFA after six weeks of 16 g/day ITF supplementation by a total of about one sixth over baseline levels (Birkeland et al., 2020). However, the reported mean increase in butyrate was 15%, which is lower than the here found increase by more than 19% (Table 2) and the higher proportion of subjects with fecal butyrate increase ($p = 0.015$). Interestingly, the subjects in the trial already had a high baseline fiber intake of 32.2 g/day in contrast to that in our study (22.8 ± 7.9 g fiber/day, Table 1). Another trial using mainly whole foods instead of purified fibers to increase fiber intake did not detect changes in fecal SCFA levels after two weeks of 40-50 g/day fiber intake (Oliver et al., 2021). In the placebo group fecal SCFA levels slightly decreased (Table 2), an observation reported also in other fiber studies (Birkeland et al., 2020; Chambers et al., 2019; Deroover et al., 2021). Fecal SCFA levels are a reflection of the balance between SCFA production, uptake and potential use by colonocytes (Canfora et al., 2015). Isolated, purified inulin is a chemically and physically simple polymer, readily fermentable in the proximal colon (Flint et al., 2012). In contrast, in dried chicory roots the microbial degradation of native inulin and other fibers into SCFA is potentially slowed down due to the enclosure within plant cells, resulting in a gradual release (Puhlmann & de Vos, 2020). Therefore, we hypothesize that dried chicory root fermentation was shifted towards a more distal location leading to a higher recovery of fecal SCFA as compared to purified ITF (Dagbasi et al., 2020; Hansen & Sams, 2018). The physical location of SCFA uptake in the colon appears to be critical for metabolic health markers favoring a distal above a proximal SCFA uptake (van der Beek et al., 2016). Hence, such a shift in location of SCFA production could

impact circulating SCFA levels and might be a distinguishing and desired therapeutic feature of intrinsic fiber products (Canfora et al., 2015; Müller et al., 2019; So et al., 2021; van der Beek et al., 2016).

The effect of SCFA reaching the systemic circulation is not often reported in fiber studies. Systemically available SCFA are ligands to GPR41 and GPR43 expressed on various organs involved in T2D etiology (Canfora et al., 2015). Intriguingly, circulating rather than fecal SCFA have been related to markers of insulin sensitivity (Müller et al., 2019) pointing towards a potential benefit of increasing their levels. We observed that besides fecal SCFA, the intake of dried chicory root also increased fasting circulating SCFA by more than 14% compared to placebo (Table 3). In contrast, a seven-week cross-over trial in overweight subjects with inulin and inulin-propionate reported lower total fasting SCFA (-9.0%), acetate (-9.9%) and butyrate (-9.1%) levels than the cellulose-control, while propionate (+6.7%) levels were higher (Chambers et al., 2019). Interestingly, a recent four-week parallel trial with reduced sized wheat bran particles instead of purified fiber in subjects with obesity only showed a normalization of circulating SCFA to levels of normal weight subjects but no effects on fecal microbiota or health parameters (Deroover et al., 2021). This contrasts strongly with our observations and although the nature of the subjects, fiber type and structure differ between the trials, it suggests that gut microbiota changes are prerequisite for SCFA-mediated health-promoting effects of dietary fiber modulations.

In view of the pronounced changes in fecal microbiota and metabolites, we observed only subtle changes in static glucose homeostasis markers (online Supplemental Table S8). In another parallel, but longer, 24-week trial in overweight subjects, HOMA-ir decreased over baseline by -14.8% (from 2.7 to 2.3), fasting insulin by -7.8%, and fasting glucose by -2.0% after ITF consumption (Chambers et al., 2015). However, these levels did also not differ from baseline, and no non-fermentable placebo was included. A seven week cross-over design in overweight and obese patients found a post-intervention difference between ITF and cellulose-control of 1.17 versus 1.59 in HOMA-ir and 9.0 µU/mL versus 12.3 µU/mL in fasting insulin without differences in fasting glucose levels (Chambers et al., 2019). In comparison to the static plasma glucose and insulin concentrations that represent rather a snapshot than a dynamic response, we observed that the CV as a measure of glucose variability decreased during run-in and the study period below 20% (Figure 3A). Glucose variability assessed by CGM has developed into an important clinical variable besides traditional glycemic markers (Danne et al., 2017) and CV's below 20% are reported for non-diabetic adults (Peyser et al., 2018) and considered to reflect stable glucose control in diabetes treatment (Monnier et al., 2017). In contrast, a similar trial in length and design using 30g/day purified ITF did not observe changes in glucose variability assessed by CGM, even while subjects were more insulin resistant than in our study (Guess et al., 2016). A decrease in dynamic glucose variability below 20% after dried chicory root intake might point towards an improvement in glucose control, potentially mediated by circulating SCFA and not yet manifested in static glycemic markers.

It has been reported in early studies that the baseline gut microbiota composition may affect the response to fiber interventions, allowing the stratification in responders and non-responders (Korpela et al., 2014; Salonen et al., 2014). This was also found in a recent ITF intervention on weight loss in an obesity cohort (Rodriguez et al., 2020). Similarly, a recent trial with wheat-bran arabinoxylan oligosaccharides indicated that baseline levels of *Prevotella* spp. affected the fecal microbiota response to the fiber intervention (Chung et al., 2020). However, we could not confirm a similar impact in the present intervention with the dried chicory root. In contrast, we observed here *Blautia* spp. to discriminate between subjects that responded to the treatment with a decrease in HOMA-ir versus those who did not (Figure 3B). This is noteworthy, since studies have found that *Blautia* spp. levels are increased in T2D and also T1D patients compared to healthy controls (Gurung et al., 2020; Qi et al., 2016). *Blautia* spp. has been implied as heritable risk factor for visceral fat mass predisposing to metabolic disease (Le Roy et al., 2017) and associated with long-term consumption of processed foods (Bolte et al., 2021). Some studies attributed beneficial properties to *Blautia* spp. (Benítez-Páez et al., 2020), which may be caused by the incorrect assumption that these species produce butyrate (Louis & Flint, 2017) – this is not the case and this group of intestinal acetogens might be undesired in the context of insulin sensitivity. Consequently, we segmented the treatment based on low (n = 14) and high (n = 14) relative abundance of *Blautia* spp. and remarkedly observed that static glycemic markers pronouncedly decreased in the low *Blautia* group, but not in the high *Blautia* group (Figure 3D-E). Moreover, a low *Blautia* spp. baseline relative abundance also appeared to impact more pronouncedly the other metabolic markers as CV of glucose levels decreased faster (Figure 3C) and circulating SCFA were higher in the low *Blautia* group compared to the high *Blautia* group (Supplemental Figure S6). We identified as major human *Blautia* species in this study *B. hominis*, *B. luti*, and *B. obeum*. Several of these species are abundant members in the human gut and utilize different sugars and starch to produce acetate (Liu et al., 2008; Shin et al., 2018; Touyama et al., 2015). It has been reported that subjects consuming processed foods have increased levels of *Blautia* spp. (Bolte et al., 2021; Koponen et al., 2021). Hence, it is possible that subjects with these dietary habits are initially less responsive to the intrinsic fiber intake. We observed a decrease in *Blautia* spp. levels after 30 g/day dried chicory root treatment (Figure 2E) and also an ITF-induced decrease in this genus has been reported earlier (Chambers et al., 2019; Hiel et al., 2020). Extrapolation of the intervention-induced decrease in relative amounts of *Blautia* spp. suggested the high *Blautia* group might reach levels of the low *Blautia* group after an additional six to eight weeks. This is relevant since a recent meta-analysis concluded that ITF interventions of six weeks or longer are needed to sufficiently decrease T2D markers in diabetic subjects (Wang et al., 2019). This also addresses the most important limitations of this study, which includes the short intervention duration that precludes meaningful measuring of Hb1Ac levels and the rather low level of insulin resistance.

In conclusion, this study shows the rapid effect of intrinsic fiber intake on bowel function, gut microbiota composition, fecal and fasting circulating SCFA and glucose variability in subjects at risk of T2D. The evidence for a trophic chain including *Bifidobacterium* and *Anaerostipes* spp. was recapitulated by *in vitro* incubations that resulted in high levels of butyrate and propionate production from the treatment product. Moreover, we observed a simultaneous increase in fecal and circulating SCFA levels and a marked improvement in dynamic markers of glucose control (CGM). In subjects with a low relative abundance of *Blautia* spp. – a genus that previously has been associated with T2D – also static glycemic markers decreased pronouncedly. Since the chicory root treatment decreased levels of *Blautia* spp. (Figure 2E), increasing the intervention time is expected to provide glucose homeostasis improvement for all subjects at risk for T2D. Our results demonstrate a strong modulatory potential on gut health and microbial metabolism by native inulin and cell wall fibers pectin, cellulose and hemicellulose in the intrinsic form of dried chicory roots. Incorporating these minimally-processed, intrinsic fibers into long-term dietary therapeutic interventions could greatly impact the management of metabolic health via the increased levels of fecal and circulating SCFA.

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CONFLICT OF INTEREST

WMdV provided scientific advice to WholeFiber BV.

AUTHOR CONTRIBUTIONS

Conceptualization, W.M.d.V. and E.J.F.; Methodology, M-L.P., R.J., K.C.W.v.D., T.P.N.B. and R.W.J.H., W.M.d.V. and E.J.F.; Investigation, M-L.P., R.J., K.C.W.v.D. and T.P.N.B.; Data Curation, M-L.P., R.J., K.C.W.v.D., and R.W.J.H.; Formal Analysis, M-L.P., R.J., K.C.W.v.D., T.P.N.B. and R.W.J.H.; Software, R.J. and R.W.J.H.; Visualization, M-L.P., T.P.N.B. and W.M.d.V.; Writing Original Draft, M-L.P., T.P.N.B. and W.M.d.V.; Writing Review and Editing, M-L.P., R.J., K.C.W.v.D., T.P.N.B., R.W.J.H., H.S., W.M.d.V. and E.J.F.; Project administration, M-L.P., W.M.d.V. and E.J.F.; Resources, K.C.W.v.D., H.S., W.M.d.V. and E.J.F.; Supervision, H.S., W.M.d.V. and E.J.F.; Funding Acquisition, W.M.d.V. and E.J.F.

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SUPPLEMENTAL MATERIAL

The complete supplementary material can be accessed online at the journal: S2632289722000044sup001.pdf (cambridge.org) or by scanning the QR code below.

TRIAL DESIGN

Participants arrived at the first study visit after an overnight fast (minimum eight hours fasted), where the pre-intervention (T0) blood sample was drawn, and anthropometric measures were taken. Then the first sensor for the Continuous Glucose Monitoring (CGM) was placed. The study procedure was explained along with handing out the diaries for the bowel function and (gastrointestinal) well-being and sachets for the run-in period. At home, participants filled in the study diary for the past week, collected the first fecal sample (stored directly in-home freezer) and measured their glucose levels using the CGM. Three days after the first study visit, participants started consuming the treatment or placebo. At the end of the run-in period (T1), prior to the second study visit, participants collected the second fecal sample. On the second study visit, participants returned the fecal samples in frozen form and the diaries of the baseline and run-in period along with the empty leftover sachets of the run-in period. Anthropometric measures were taken again, the CGM sensor was replaced and new diaries along with the sachets for the intervention period were handed out. At the end of the intervention period (T2) participants collected the third fecal sample. At the third study visit participants arrived again fasted and the post-intervention blood sample was taken as well as anthropometric measures. The second CGM was removed, and the diaries of the intervention period and the empty and leftover sachets were returned. Participants received the diaries of the wash-out period. At the end of the wash-out period (T3) participants collected the fourth fecal sample, after which the third and fourth fecal sample were transported to the university together with the diaries of the wash-out period.

The products were provided as single, daily dosage packed in transparent sachets stored in nontransparent bags for each week of the study. Intervention products were provided by a third, independent researcher. Neither the researchers nor the participants were told which intervention product they received. As the placebo product could not fully match the treatment product in color, taste and shape, implying the possibility of participants to being not fully blinded, the study was considered investigator-blinded. Participants were instructed to consume the content of one sachet per day, preferably

in the morning with their breakfast or morning snack. Compliance was assessed by counting empty and leftover sachets. All participants were instructed to maintain their normal diet and level of physical activity throughout the whole study.

DIARIES FOR BOWEL FUNCTION AND WELL-BEING

Participants were asked to record their bowel function and (gastrointestinal) well-being using weekly questionnaires. Bowel function was assessed as average stool softness over the last week using the Bristol Stool Scale and stool frequency as average number of stools per day during the last week. Gastrointestinal symptoms and satiety feelings were recorded using a 0 - 100 visual analogue scale.

GUT MICROBIOTA ANALYSIS.

Bacterial DNA was extracted from frozen fecal samples that were thawed on ice, using the repeated bead-beating method as described before (Salonen et al., 2010) and purified using the Maxwell® 16 Tissue LEV DNA Purification Kit. The quantity of the extracted DNA was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Microbiota composition was analyzed by Illumina MiSeq sequencing of the 16S rRNA gene hypervariable V3-V4 region using primers 341F/785R as previously described (Korpela et al., 2018). Sequences were further processed using the mare package in R (Korpela, 2016) that relies on USEARCH (Edgar, 2010). In short, only forward reads were used, primers were cut from the 5'-end of each read, and reads were quality filtered with quality score 2 and truncated to a length of 150 bp. The rarest reads, likely sequencing errors, were removed based on a minimum read abundance of 0.005%. After quality filtering, we obtained on average 34952 reads per sample, ranging from 3164 to 78916. The reads were taxonomically annotated using USEARCH (Edgar, 2010) to map to the SILVA 16S rRNA reference database version 115 (Quast et al., 2013). OTUs (operational taxonomic unit), with clustering at 97% identity, were used to calculate the diversity and richness measures. Further analyses were performed using the mare package. The data has been submitted to the European Nucleotide Archive (ENA) under the accession number PRJEB47230. To attain species level annotations, the same reads were annotated using the BLAST function (Camacho et al., 2009) in the mare package.

TROPHIC CHAIN IN VITRO EXPERIMENT - SYNTHETIC CO- AND TRI-CULTURES

As a proof-of-concept a trophic chain experiment with dried chicory root (WholeFiber™) was performed using synthetic co- and tri-cultures with strains having the canonical functionality of the genera of interest. For this purpose, *Bifidobacterium animalis subsp. lactis* BLC1 (obtained from Dr L Morelli Sacco SRL), *Anaerostipes rhamnosivorans* 1Y2^T (Laboratory of Microbiology, Wageningen University & Research) and *Bacteroides xylanisolvens* HMP (obtained from the Human Microbiome Program as HMP 2_1_22) were grown in co- or tricultures. Bacteria were routinely maintained in a modified yeast extract, casitone fatty acid (YCFA) medium (Duncan et al., 2009) supplemented with 20 mM xylose and 20 mM galactose for *Bacteroides xylanisolvens* HMP or 20 mM glucose

for *Bifidobacterium animalis subsp. lactis* BLC1 and *Anaerostipes rhamnosivorans* 1Y2^T. All growth experiments were performed in duplicate in the modified YCFA medium (Duncan et al., 2009) containing 5 g/L WholeFiber™. Equal amounts of the overnight preculture of the three individual bacteria (5%, v/v) were simultaneously added to the media to complete the tri-culture. In parallel, same amounts of *Bacteroides xylanisolvens* HMP and *Bifidobacterium animalis subsp. lactis* BLC1 were added in the modified YCFA medium with dried chicory roots. All cultures were subsequently incubated at 37°C up to seven days. Gas and liquid samples were collected at different time intervals for H_2 and organic acid analysis, respectively, as previously described (Bui et al., 2019).

GLUCOSE HOMEOSTASIS MARKERS

Fasting blood samples were collected after antecubital venipuncture into BD® sodium fluoride vacutainers (2 mL) for glucose measurement and into BD® EDTA K2E vacutainers (4 mL) for insulin. Samples were centrifuged for 10 min at 4 °C and 12,000 x g and subsequently stored at -80 °C until further analysis. Fasting glucose concentrations were analyzed in the automated systems of the local hospital "De Gelderse Vallei" (Ede, The Netherlands). Fasting insulin concentrations were measured with enzyme-linked immunosorbent assay (ELISA) (Mercodia Ultrasensitive Insulin ELISA, Uppsala, Sweden). HOMA-ir values were calculated from fasting glucose and fasting insulin values using the HOMA2 calculator (https://www.dtu.ox.ac.uk/homacalculator) (Levy et al., 1998; Matthews et al., 1985).

CONTINUOUS GLUCOSE MONITORING

Abbott's FreeStyle Libre Flash was used for Continuous Glucose Monitoring (CGM; Abbott, Marne-la-Vallée, France). The system used in this study measured for up to 14 days every 15 min the glucose profiles in the interstitial fluid using a sensor and a reader. The CGM sensor was placed on the upper back of the arm preferred by the participant and following the manufacturer instructions. If necessary, the sensors were additionally secured by covering them with medical tape (leaving a hole in the middle on top of the sensor for moisture removal). When a sensor was lost before the end of the 14-day period, it was replaced with a new one. Every participant received an accompanying reader and was instructed to read out the sensor every eight hours. The screen of the reader was covered to ensure that participants were blinded to their own glucose read-outs. Each participant wore a CGM twice; the first covering baseline and the run-in period and second during the first 14 days of the intervention period. At the end of each period data was transferred from the reader to a computer using the available Freestyle Libre App.

CGM readouts were analyzed by an in-house developed open-source R script, CGM Shiny (Hangelbroek, 2021). CGM metrics were calculated for periods of three consecutive days covering the same weekdays (Tuesday, Wednesday, Thursday) in the baseline, the run-in and the intervention period. We omitted the first hours until midnight from analysis,

also when sensors were replaced due to sensor loss. The first day of sensor readings was omitted from analysis as recommended (Bailey et al., 2015). Calculations of CGM metrics started at midnight (12 am). CGM Shiny can calculate metrics using either data from all 24 h of a day (starting at midnight), or from the period subjects are awake (6:00 am to 12:00 am) or asleep (12:00 am to 6:00 am) as recommended (Danne et al., 2017). Despite precise instructions, it proved difficult for participants to scan the sensor every 8 h to transfer data to the reader. Data gaps of less than an hour (<4 datapoints) were linearly interpolated. Larger gaps were left as missing data. One subject was excluded from data analysis due to sensor failure. From the CGM metrics available we selected the coefficient of variation as a measure of glucose variability expressed as percentage, since it is generally well understood (Danne et al., 2017) and as we observed that it strongly correlated with other glucose variability CGM metrics. To understand the effect of missing data on the outcomes of the CV we conducted a sensitivity analysis. We assessed the percentages of missing data and determined a threshold at 20% missing data. Consequently, the subjects that had more than 20% missing data were excluded from the analysis (n=4 for the placebo and n=6 for the treatment group). Furthermore, we identified and extreme outlier in the placebo group (more than three times interquartile range above third quartile or below first quartile), which appeared to substantially influence the result and, hence, was excluded during the sensitivity analysis. Similarly, an outlier was excluded from analysis when investigating differences in CV after *Blautia* baseline segmentation. Following data exclusion, baseline CV was 21.3% for treatment versus 19.7% for placebo with ($p = 0.25$).

STATISTICAL ANALYSIS

The study samples size was based on the detection of a mean within-individuals reduction in fasting insulin levels of 29 pmol/L with a two-sided 5% significance level and a power of 80% requiring 27 subjects per study arm. This sample size is sufficient to detect a mean decrease of 29 pmol/L, which is lower than the 34 pmol/L found earlier with ITF (Guess et al., 2016). To account for a potential dropout rate of 10%, a final sample size of 60 patients (30 per study arm) was used. Normality of the outcome variables was assessed by inspecting Q-Q-plots. Depending on normality corresponding parametric or nonparametric testing was applied. For baseline characteristics data was expressed as mean and SD or median and IQR depending on normality to indicate spread in the study population. Changes in biochemical markers, anthropometric measures, and bowel function were expressed as absolute change and as percentage change (relative change). Statistical inference was performed on absolute changes only. The R package mare (Korpela, 2016) was used for gut microbiota analysis uses tools from the R packages vegan (Oksanen et al., 2022), nlme (Pinheiro et al., 2020), MASS (Venables & Ripley, 2002) and glmmADMB (Fournier et al., 2012; Skaug et al., 2016). Multivariate analysis for microbial composition was done using implemented Principle Coordinates Analysis with Bray-Curtis dissimilarities (Korpela, 2016). Changes in levels of individual taxa were assessed by implemented repeated measures analysis,

for which the mare package uses subjects as random factor and also automatically performs false-discovery rate correction. The package checks model assumptions and consecutively fits alternative models, which in case of assumption violation by all fitted models leads to the production of no *P*-values and estimates (Korpela, 2016). To assesses differences in changes in levels of fecal SCFA over the intervention periods between the treatment groups we used using linear mixed models, as implemented in the lme4 R package (Bates et al., 2015) that uses by default an unstructured variance-covariance matrix. We included SCFA as dependent variable and as fixed effect intervention group and intervention period as well as their interaction term and as random effect the subject. Statistical significance levels were calculated using the lmerTest R package (Kuznetsova et al., 2017), which estimated degrees of freedom and *P*-values based on the Satterthwaite's method. Changes over time in stool softness and glycemic variability were analyzed by repeated measures mixed ANOVA (RM ANOVA) with intervention group as between-subject factor, intervention period as within-subject factor, intervention × study period as interaction term and pairwise t-test as post-hoc test with FDR correction. Changes in stool frequency were assessed by Friedman's test with Wilcoxon signed rank-test as post-hoc test with FDR correction. RM ANOVA and Friedman test were performed using the rstatix R package (Kassambara, 2023). Static glycemic markers and circulating SCFA levels were assessed for differences after the study period between treatment groups by ANCOVA (using baseline as covariate) and for changes over baseline and comparison between groups by paired or unpaired t-test to explore the modulatory potential of the treatment on biomarkers.

RESPONDER AND FURTHER SUBGROUP ANALYSIS

To further explore gut microbiota differences in relation to metabolic responses, we divided the treatment group based on the relative change in HOMA-ir into responders and non-responders. Subjects with a reduction in HOMA-ir larger than 10% were defined as responders (n = 8), subjects with an increase in HOMA-ir larger than 10% were defined as non-responders (n = 12). Subjects with relatively unchanged HOMA-ir (eight subjects) were excluded (Supplemental Figure S1). Analysis of differences in baseline microbiota highlighted *Blautia* spp. as major differentiating factor between responders and non-responders ($p = 0.01$). This finding was also sustained ($p = 0.02$) when we set the responder definition threshold less stringent to 0% (responder = more than 0% reduction and n = 10; non-responder = no reduction or more than 10% increase and n = 18). The *Blautia* spp. median relative abundance was subsequently used to divide the treatment group into high (n = 14) and low (n = 14) baseline abundance groups. This division was further used to analyze changes in static and dynamic glycemic biomarkers and fasting circulating SCFA in the treatment group.

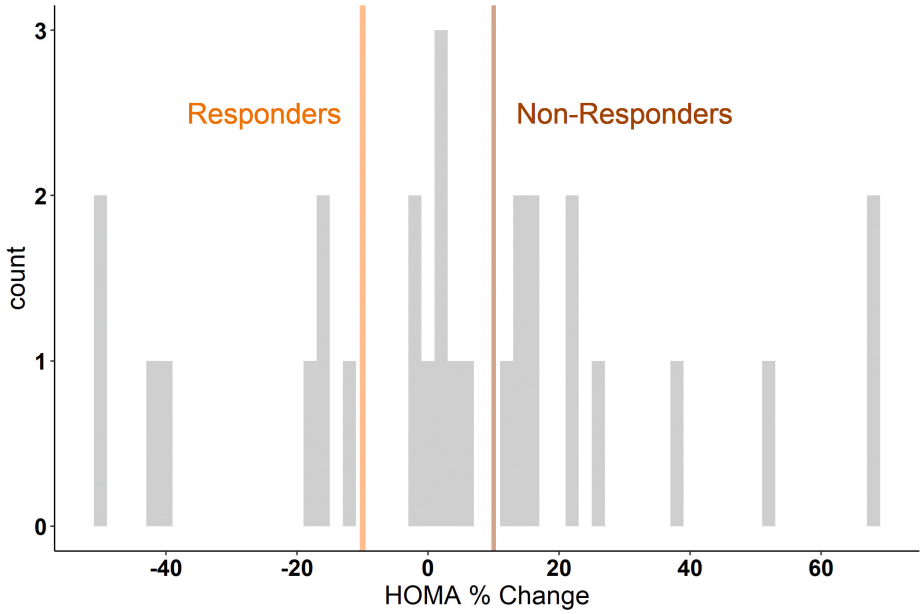


Figure S1 Division of HOMA-responder and non-responders of the treatment group using a 10% cut-off (vertical lines) and excluding subjects with relatively unchanged HOMA-ir (n=8).

PARTICIPANTS BASELINE INFORMATION

In the Placebo group one participant dropped out during the run-in period due to chest pain, another was excluded from analysis due to missing samples and a third due to medically advised lifestyle changes during the intervention period. In the treatment group one participant dropped out during the run-in period due to an inflammation of the inner ear, another was excluded from analysis due to early discontinuation of the treatment (protocol violation). One participant in the treatment group developed an eczema one week into the study. As the symptoms worsened after the end of the intervention and no earlier allergic reactions to chicory were reported, a possible causal relation with the treatment was excluded.

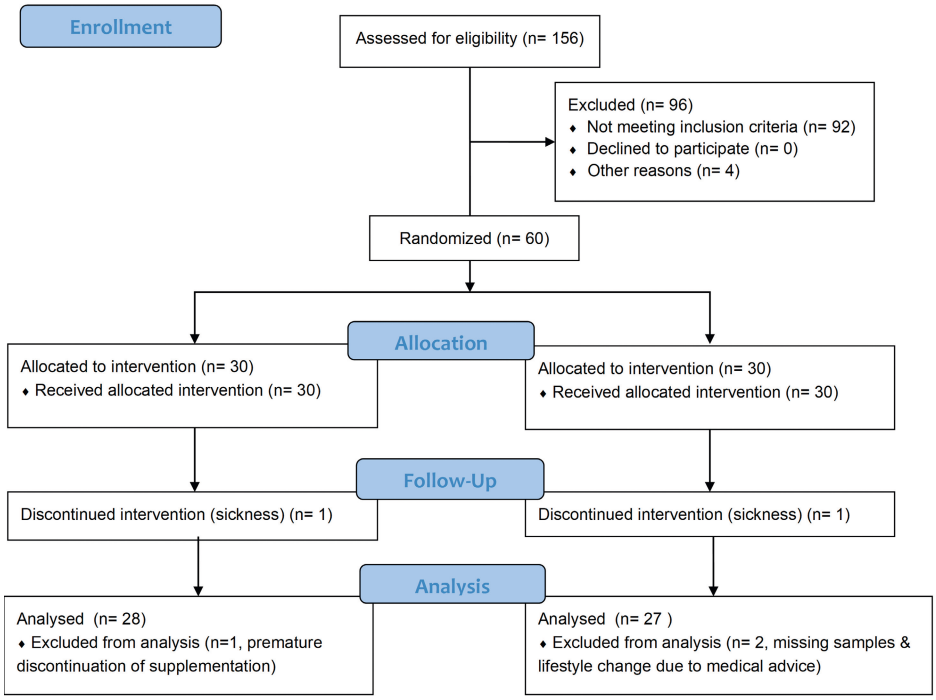


Figure S2. Consort Statement Flow Diagram

Table S2. Changes in mean relative abundances of abundant taxa[1] in the treatment group from Baseline (T0) to 15 g/day treatment intake for two weeks (T1) to 30 g/day treatment intake for three weeks (T2) and differences in those changes from the placebo group.[2]

taxon	T0	T1	T2	ΔT1	ΔT2	Fold ΔT1	Fold ΔT2	T1-T0		T2-T0		ΔT1 vs Placebo		ΔT2 vs Placebo	
								p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value
Bifidobacterium	3.2%	10.1%	13.1%	6.9%	9.9%	3.17	4.09	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.006	0.050	**<0.001**	**<0.001**
Collinsella	4.4%	5.1%	3.7%	0.7%	-0.7%	1.16	0.83	0.069	0.154	0.365	0.425	0.789	0.876	0.450	0.495
Coriobacteriaceae uncultured	1.2%	1.2%	0.6%	-0.1%	-0.6%	0.93	0.51	0.400	0.514	**<0.001**	**<0.001**	0.825	0.876	-	-
Bacteroides	3.2%	2.7%	3.3%	-0.5%	0.1%	0.83	1.02	-	-	-	-	-	-	0.270	0.393
Prevotella	2.7%	2.1%	3.8%	-0.7%	1.1%	0.76	1.41	0.756	0.756	0.008	0.015	-	-	-	-
Streptococcus	1.1%	1.2%	0.7%	0.1%	-0.3%	1.08	0.68	0.343	0.514	0.377	0.425	0.575	0.876	-	-
Christensenellaceae uncultured	1.8%	1.4%	1.5%	-0.4%	-0.3%	0.79	0.84	0.046	0.118	0.104	0.133	0.941	0.941	0.464	0.495
Clostridium	1.8%	1.3%	1.0%	-0.5%	-0.9%	0.71	0.53	0.170	0.277	0.017	0.026	0.461	0.872	-	-
Anaerostipes	1.3%	3.7%	4.3%	2.4%	3.0%	2.82	3.24	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Blautia	12.3%	13.0%	10.0%	0.7%	-2.3%	1.06	0.81	0.547	0.616	**0.003**	**0.007**	0.573	0.876	0.682	0.682
Coprococcus	4.3%	3.5%	3.3%	-0.8%	-1.0%	0.82	0.76	0.004	0.023	**<0.001**	**<0.001**	0.053	0.226	0.207	0.332
Lachnospiraceae IncertaeSedis	9.1%	8.7%	7.4%	-0.4%	-1.8%	0.95	0.81	0.459	0.550	**<0.001**	**0.001**	0.664	0.876	0.176	0.332
Pseudobutyrivibrio	4.4%	4.3%	4.4%	-0.2%	0.0%	0.96	1.00	0.666	0.705	0.420	0.444	-	-	0.187	0.332
Roseburia	3.4%	2.7%	2.5%	-0.7%	-0.9%	0.80	0.72	0.012	0.043	**0.001**	**0.002**	0.447	0.872	0.340	0.453
Lachnospiraceae uncultured	7.0%	6.4%	6.0%	-0.6%	-1.1%	0.91	0.85	0.141	0.254	0.016	0.026	0.028	0.157	0.140	0.332
Peptostreptococcaceae IncertaeSedis	2.8%	2.4%	2.0%	-0.4%	-0.8%	0.86	0.71	-	-	-	-	0.680	0.876	-	-
Faecalibacterium	8.9%	9.1%	10.8%	0.2%	1.9%	1.02	1.21	-	-	-	-	0.788	0.876	0.463	0.495
Ruminococcus	5.1%	3.8%	2.5%	-1.3%	-2.6%	0.75	0.48	0.027	0.082	**<0.001**	**<0.001**	0.176	0.500	**<0.001**	**<0.001**
Subdoligranulum	3.7%	3.3%	2.8%	-0.4%	-0.8%	0.89	0.78	0.386	0.514	0.020	0.027	0.308	0.749	0.149	0.332
Ruminococcaceae uncultured	7.2%	5.4%	5.9%	-1.7%	-1.3%	0.76	0.81	0.009	0.042	0.012	0.021	0.131	0.445	0.181	0.332
Enterobacter	1.1%	0.5%	0.9%	-0.6%	-0.2%	0.44	0.85	0.141	0.254	0.465	0.465	-	-	0.165	0.332

[1] Abundant taxa represent those with a mean relative abundance of at least 1% in the whole dataset.

[2] Fold ΔT1 = Fold change after 15 g/day treatment; Fold ΔT2 change after 30 g/day of treatment; T1-T0 = comparison between 15 g/day treatment (T1) and baseline (T0) within the treatment group; T2-T0 = comparison between 30 g/day treatment (T2) and baseline (T0) within the treatment group; ΔT1 vs Placebo = comparison of change over baseline after 15 g/day treatment between the treatment group and the placebo group; ΔT2 vs Placebo = comparison of change over baseline after 30 g/day treatment between the treatment group and the placebo group, - represents not calculable estimates and p- and q-values (Korpela, 2016))

Table S5. Differences in fold-change between subjects of the treatment group with high or low baseline relative abundance (%) of *Bifidobacterium* and *Anaerostipes* spp. based on baseline median division.[1]

Group		Relative abundance (%) T0	Relative abundance (%) T2	Fold-change T2
Bifidobacterium spp.	High (n=14)	5.67 ± 1.21	18.67 ± 2.98	4.23
	Low (n=14)	0.72 ± 0.18	7.84 ± 1.72	11.55
Anaerostipes spp.	High (n=14)	2.04 ± 0.32	5.31 ± 0.78	2.74
	Low (n=14)	0.60 ± 0.05	3.24 ± 0.55	6.12

[1] Data is presented as mean with SEM in parentheses. T0 = baseline; T2 = 30 g/day treatment; Fold-change T2 = fold change after 30 g/day treatment over baseline.

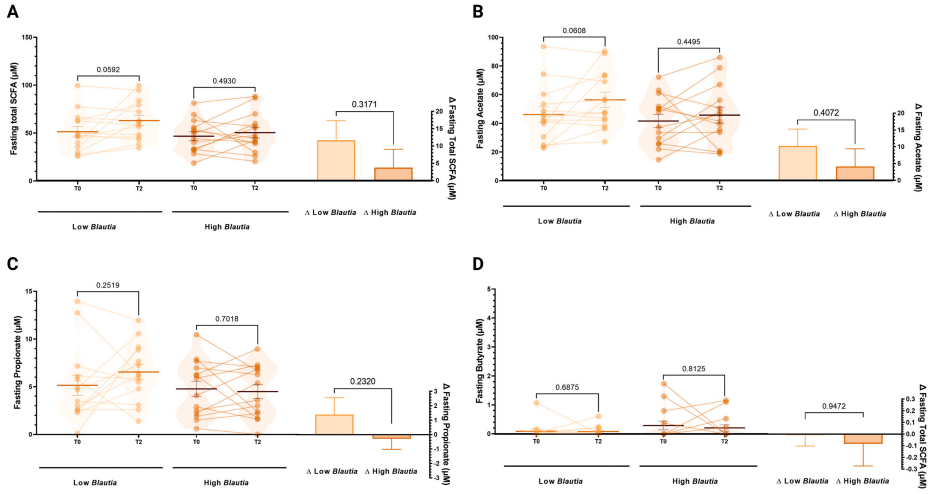
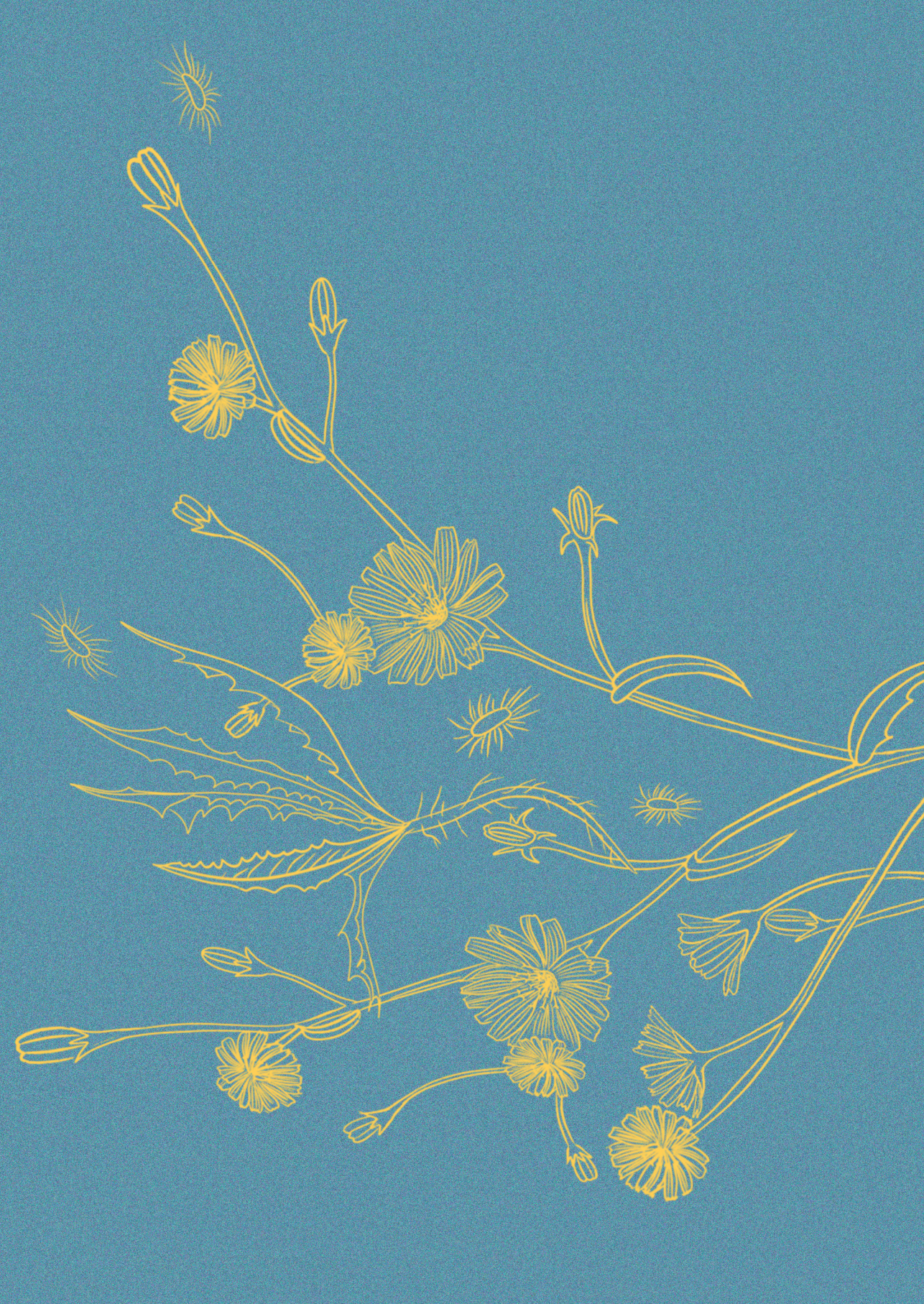


Figure S6. Effect of *Blautia* baseline abundance on changes in fasting circulating short-chain fatty acids (SCFA) after 30 g/day treatment in subjects of the treatment group with either low (n=14) or high (n=14) baseline *Blautia* spp. relative abundance. Butyrate levels were analyzed using nonparametric testing. (A) fasting circulating total SCFA, (B) fasting circulating acetate levels, (C) fasting circulating propionate levels, (D) fasting circulating butyrate levels.



CHAPTER 6

Prolonged intake of dried chicory root reproducibly modulates the gut microbiome and improves metabolic health in individuals with obesity and at risk for type 2 diabetes: Responder signatures indicative of colonic butyrate production

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ABSTRACT

Gut microbiota modulation appears to fundamentally facilitate fiber-mediated interventions aimed at improving metabolic health. Previously, we demonstrated that a three-week daily 30 g intake of dried chicory root, an intrinsic fiber product, rapidly and effectively modulated the gut microbiota towards butyrogenic trophic chains and promoted glucose control in individuals at risk of type 2 diabetes. Here, we aimed to investigate how prolonged intake of 30 g/day dried chicory root cubes during ten-weeks in individuals with obesity who are at risk for type 2 diabetes affects the gut microbiome and markers of metabolic health. In this randomized, placebo-controlled trial in 35 individuals, we assessed gut microbiome modulation using 16S rRNA gene amplicon sequencing combined with in-depth taxonomic and functional profiling using shotgun metagenomics. In addition, we related these outcomes to microbial and metabolic health markers, including fecal short-chain fatty acids, fasting plasma triglyceride concentrations, as well as tissue-specific insulin sensitivity assessed by a two-step hyperinsulinemic-euglycemic clamp. In line with previous findings, we observed a rapid 4- to 5-fold increase in relative abundances of *Bifidobacterium* spp. and *Anaerostipes* spp. by the dried chicory root product using 16S rRNA gene profiling. Metagenome analysis revealed that these changes were mainly attributed to species-level modulations in *B. adolescentis, B. bifidum, B. longum*, and *A. hadrus*. Individuals who metabolically responded to the dried chicory root by a large improvement of insulin-mediated glucose disposal (> 15% improvement, high responders, n = 9) had lower levels of potential pectin-degrading bacteria and pectate lyase genes throughout the study compared to those who had a low response (≤ 15% improvement; low responders, n = 8). Moreover, high responders had a more rapid increase in relative levels of *Bifidobacterium* spp. and associated bifid shunt pathway encoding genes and higher final levels of known propionate- and butyrate-producers, notably *Anaerobutyricum* spp. that, like *Anaerostipes* spp. can convert lactate and acetate into butyrate. These compositional and functional changes aligned with higher average fecal butyrate, propionate, and plasma acetate levels in high responders compared to low responders. For all individuals in the treatment group, increases in fecal butyrate levels were related to decreased fasting triglyceride levels, increased proportion of small adipocytes well as increased plasma fasting acetate levels, suggesting a potential mediating role of fiber-derived colonic butyrate on systemic metabolic health outcomes. In conclusion, dried chicory root reproducibly modulated the gut microbiota towards increased levels of bacteria known to be involved in butyrogenic trophic chains, with prolonged intake benefitting fiber-modulated metabolic health outcomes in individuals with overweight and at risk for type 2 diabetes.

Key words: intrinsic dietary fiber, insulin sensitivity, overweight, microbial pectin-degradation, human gut microbiota, butyrate

INTRODUCTION

Over the past 50 years, humanity has seen remarkable technological progress, but at the same time, a worrying increase in the prevalence of obesity and related non-communicable diseases, along with a decline in metabolic health. This decline has reached such proportions that poor metabolic health is now considered a global health epidemic (Saklayen, 2018; The Lancet Diabetes & Endocrinology, 2021). According to the World Health Organization, more than four out of eight people are currently overweight (defined as a body mass index (BMI) ≥25 kg/m^2) and one out of eight has obesity (BMI ≥ 30kg/m^2) (World Health Organization, 2024). These numbers are on a steep rise (Jaacks et al., 2019), and weight gain due to fat deposition has been shown to relate to the development of other metabolic impairments, such as insulin resistance (Buscemi et al., 2024; World Health Organization, 2024). Insulin resistance is a condition in which the body's ability to respond to insulin is reduced. As insulin regulates hepatic glucose and lipid output, peripheral glucose uptake, as well as intra- and extracellular lipolysis in adipose tissue (Saltiel & Kahn, 2001), impaired insulin sensitivity can lead to other metabolic disturbances, such as dysglycemia and dyslipidemia (Ginsberg et al., 2005). Together with obesity, high blood pressure, and low high-density cholesterol levels, these metabolic disturbances are called metabolic syndrome (Alberti et al., 2009; Huang, 2009; National Institute of Health, 2022). Metabolic syndrome precedes the development of cardiovascular diseases and type 2 diabetes mellitus (T2D), and the co-existence of metabolic syndrome traits in these diseases exacerbates their complications (National Institute of Health, 2022; Shang et al., 2024).

The rise of obesity and metabolic syndrome together with the ongoing global decline in metabolic health requires urgent countermeasures, notably as there appears to be a certain window of opportunity to reverse the impaired metabolic processes (Grundy et al., 2005; Vickers & Sloboda, 2012). While food intake is a key driver for the development of obesity and metabolic syndrome, it can also be used as a treatment or prevention strategy (Venkatesan, 2024). Indeed, dietary intervention may be effective in preventing impaired metabolic health, but often long-term outcomes are poor (Blaak & Goossens, 2023; Shannon et al., 2023). A characteristic food component that plays an essential role herein is dietary fiber (Deehan et al., 2024). Dietary fibers are polysaccharides and lignin compounds that form the backbone of plant foods and are indigestible to human endogenous enzymes. A lack of fiber intake has been linked to all-cause mortality, and notably, levels below 25 g/day are associated with T2D incidence (Reynolds et al., 2019). The health effects mediated by dietary fiber intake are partly explained by their physicochemical properties, which affect energy intake (gastrointestinal transit and satiety) and lipid metabolism (cholesterol and fat-binding). However, a considerable contribution to fiber-mediated health effects is attributed to the conversion of fibers by the gut microbiota, communities of microorganisms residing in the human digestive tract, predominantly composed of anaerobic bacteria (De Vos et al., 2022). Due to the fibers' indigestibility in the upper gastrointestinal tract, the plant polysaccharides reach

the lower gastrointestinal tract, where they serve as substrates for energy and growth for bacteria, fundamentally shaping gut microbiota composition and activity. Low fiber intake has hence been associated with alterations in the abundance of specific gut microbiota taxa (Deehan & Walter, 2016) and the gut microbiota, in turn, has emerged as an important player in metabolic health (Cox et al., 2022; Fan & Pedersen, 2020; Fava et al., 2019).

Altered gut microbiota profiles have been associated with obesity and T2D, and these profiles were characterized by decreased levels of putative butyrate-producing bacteria in affected individuals (Fan & Pedersen, 2020). A primary metabolic function of the gut microbiota is the production of fiber-derived short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate. While most gut bacteria can produce acetate and many also propionate, butyrate is only produced by a subset of specialist bacteria through cross-feeding on acetate and lactate generated by other gut bacteria, comprising butyrogenic trophic chains (Louis & Flint, 2017). Locally, butyrate serves as an energy source for colonocytes, strengthening the gut lining. Systemically, it acts as a histone deacetylase (HDAC) inhibitor, impacting gene expression, and binds to G protein-coupled receptors (GPR), influencing pathways involved in insulin sensitivity including the production of the gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Bourassa et al., 2016; Coppola et al., 2021; van Deuren et al., 2022).

These insights have led to the hypothesis that butyrate may have both protective as well as therapeutic properties against obesity, T2D, and metabolic syndrome (Arora & Tremaroli, 2021; Bridgeman et al., 2020; Mayorga-Ramos et al., 2022; Peng et al., 2023). Consequently, the effect of butyrate on adipose tissue functioning and insulin signaling has been extensively studied, however, our current understanding remains mainly based on animal and *in vitro* models, limiting its generalizability to the human *in vivo* situation (van Deuren et al., 2022). Available data from human trials suggests that the route of delivery impacts the benefits of butyrate on metabolic impairment, as oral butyrate supplementation failed to improve insulin sensitivity in individuals with metabolic syndrome or T2D (Bouter et al., 2018; Hartstra et al., 2020; Khosravi et al., 2022). In contrast to oral deliveries, rectally infused SCFA mixtures containing butyrate have been demonstrated to improve energy expenditure, lipid oxidation and increase circulating PYY levels (Canfora et al., 2017), and distinctly, a distal location appears to accentuate the metabolic effect (van der Beek et al., 2016). Collectively, these findings indicate that colon-derived butyrate has the potential to improve metabolic health. Thus, targeting the gut microbiota by using dietary fiber to promote the production of butyrate, particularly in the distal colon, appears to be a promising strategy for improving metabolic health. Having said that, fiber-based human intervention studies have been shown to exhibit heterogeneity in the observed metabolic response (Deehan et al., 2024). This has been linked to the distinct composition of an individual's gut microbiota present at baseline, as well as the gut microbiota's responsiveness to the intervention of interest (Korpela et al., 2014a; Salonen et al., 2014). Consequently, the concept of categorizing individuals as responders or non-responders has emerged,

emphasizing the need to identify factors that enhance treatment success (Korpela et al., 2014a; Salonen et al., 2014), notably by assessing gut microbiota composition before fiber interventions (Deehan et al., 2024).

Dietary fiber interventions targeting metabolic health have predominantly focused on specific fiber types either derived from plant materials through extraction, purification, or isolation or through synthetic production (Armet et al., 2020). However, studies have revealed that such isolated single fibers resulted in improved metabolic health markers in only half of the interventions, with certain metabolic markers affected in less than one out of five trials (Armet et al., 2020). Maintaining fiber as part of the original plant cell matrix of whole foods has, therefore, been recognized as a potential contributor to fiber-mediated metabolic health outcomes (Armet et al., 2020; Augustin et al., 2020). These fibers, which remain unextracted and within their original plant cell matrix, are termed 'intrinsic fiber' (Augustin et al., 2020). Such intrinsic fibers fundamentally differ in their three-dimensional organization from isolated single fibers (Puhlmann & de Vos, 2022). One such dietary fiber product with a preserved original plant cell matrix is dried chicory root, known for its high intrinsic fiber content (85%), particularly rich in inulin (70% inulin, 10% pectin, 5% hemi-/cellulose) (Puhlmann et al., 2024; Puhlmann & de Vos, 2020). We previously found that short-term, three-week intake 30 g/day dried chicory root particles in individuals at risk of T2D rapidly modulated the gut microbiota by increasing relative abundances of known inulin-degrading *Bifidobacterium* spp. and butyrate-producing *Anaerostipes* spp. up to three to four-fold. This was accompanied by higher levels of fecal SCFAs acetate, propionate, and, notably, butyrate (Puhlmann et al., 2022). We then demonstrated that a butyrogenic trophic chain could be reconstituted by a synthetic triculture consisting of *Bacteroides xylanisolvens, Bifidobacterium animalis* and *Anaerostipes rhamnosivorans*. Here, *B. xylanisolvens* liberated inulin from the dried chicory root plant cells, *B. animalis* converted this into lactate and acetate that subsequently was converted into butyrate by *A. rhamnosivorans* (Puhlmann et al., 2022). Recently, using fecal *in vitro* batch fermentations, we confirmed high levels of butyrate production from dried chicory root, notably at a later stage during the fermentation process, which coincided with the presence of pectin-degrading and butyrate-producing bacteria (Puhlmann et al., 2024). We hypothesize that these *in vitro* observations translate *in vivo* into a prolonged fermentation, spreading butyrate production from the proximal throughout the distal colon and resulting in improvements in insulin sensitivity and other metabolic health parameters.

Following short-term intake of dried chicory root, we observed previously that the glycemic coefficient of variation as a measure of glucose control was notably improved in the treatment group (Puhlmann et al., 2022). However, observed metabolic improvements were modest, which was possibly related to the short study duration (three-week 30 g/day preceded by two-week 15 g/day of dried chicory root intake). In addition, participants were selected based on elevated fasting blood glucose levels and a high risk of developing T2D according to diabetes risk scores (Alssema et al., 2008; Lindström & Tuomilehto, 2003), with their HOMA-IR values (HOMA1-IR = 2.2) suggesting

they were not markedly metabolically impaired. Inulin, in its isolated form, has been studied for its potential benefits on glucose control, and collective data suggest that periods of more than six weeks are required to have a prominent effect on markers of T2D (Wang et al., 2019). Consequently, we aimed to understand how a long-term intake of the dried chicory product in individuals with marked metabolic impairment would affect the gut microbiota and metabolic response in individuals who are metabolically compromised. We report here the effect of a prolonged ten-week intake of 30 g/day dried chicory root cubes on the gut microbiota of individuals with obesity and at risk of T2D in a randomized placebo-controlled trial. Substantial metabolic improvements were observed in response to the chicory root intervention, and these were related to specific changes in gut microbiota composition and functionality as determined by 16S rRNA gene amplicon and metagenomic sequence analysis as well as fecal and plasma SCFA levels, indicating an important role for distally produced butyrate.

MATERIALS AND METHODS

STUDY DESIGN AND OUTCOME ASSESSMENTS

This study was a 12-week randomized, placebo-controlled, parallel trial in individuals with obesity and insulin resistance and/ or prediabetes executed at Maastricht University between January 2021 to March 2023 under the supervision of Prof. Dr Ellen Blaak and Dr Emmanuel Canfora. The study is registered at ClinicalTrials.gov under NCT04714944. The study period consisted of a two-week run-in period followed by a ten-week intervention period (Figure 1). The study was approved by the Medical Ethical Committee of Maastricht University Medical Center+ and was conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul, South Korea). Monitoring was performed by the independent Clinical Trial Center Maastricht (CTCM), Maastricht, The Netherlands. Written informed consent was obtained from all volunteers. The details on the study procedures, sample size calculation, outcome measurements, and data analysis and interpretation of the metabolic, bowel function, fecal, and plasma SCFAs, and other (anthropometrics, dietary intake, physical activity) outcomes will be published elsewhere (Omary et al., 2024, in preparation).

The primary outcome was the change in insulin-mediated glucose disposal as a measure of peripheral insulin sensitivity assessed by the gold standard hyperinsulinemic-euglycemic clamp method (DeFronzo et al., 1979). In short, the study population consisted of 35 individuals with overweight or obesity, defined as a BMI above 28 and below 35 kg/m^2, who had either a HOMA-IR above 2.2 (impaired insulin sensitivity), fasting glucose above 5.6 and below 7.0 mmol/L (impaired fasting glucose), or impaired glucose tolerance indicated by a two-hour 75 g oral glucose tolerance test value above 7.0 and below 11.0 mmol/L.

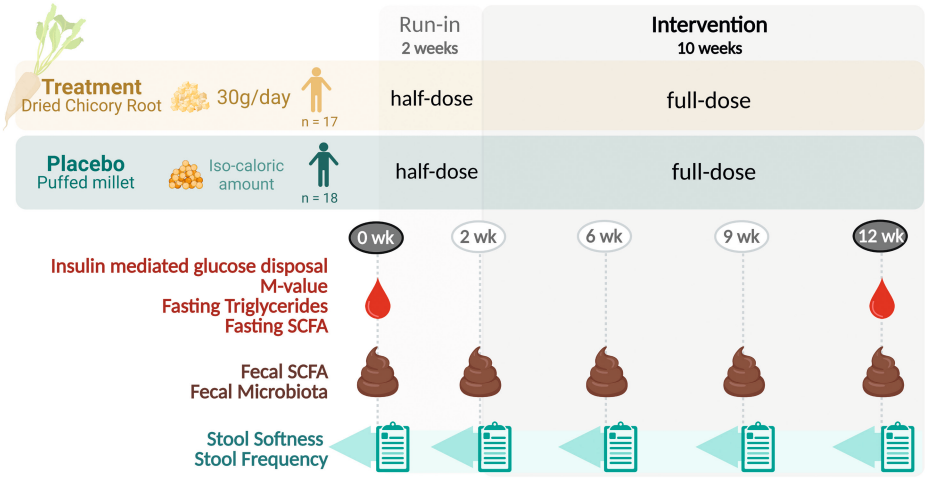

Figure 1. Study design. Study design of the 12-week (wk) randomized placebo-controlled parallel trial in individuals with overweight to assess prolonged intake of dried chicory root compared to placebo in 35 individuals with overweight or obesity at risk of type 2 diabetes. Created with BioRender.com.

None of the individuals took antibiotics, prebiotics, or probiotics during the previous 3 months, used laxatives regularly, had a gastrointestinal disease or previous abdominal surgery, or followed a weight loss or vegetarian diet. Eligible individuals were allocated to one of the two intervention groups consuming either dried chicory root cube-like particles (provided by WholeFiber BV, The Netherlands) as treatment (treatment group, n = 17) or puffed millet (provided by DO-IT BV, The Netherlands) as an isocaloric placebo (placebo group, n = 18). The dried chicory root product was consumed in two daily portions at a dosage of 15 g/day during the first two week run-in weeks and then at 30 g/day during the remaining 10 intervention weeks (Figure 1). Iso-caloric amounts of the placebo were provided in the same manner. Participating individuals and researchers were both blinded to the intervention product allocation. Metabolic health outcomes were measured at baseline (week 0) and endpoint (week 12). Those included commonly assessed fasting blood markers, among which glucose, insulin, and triglyceride levels, and HOMA-IR, as well as markers derived from the hyperinsulinemic-euglycemic clamp, among which whole-body insulin sensitivity (M-value) and insulin-mediated glucose disposal (Rdstim) (DeFronzo et al., 1982; Gijbels et al., 2021; Müller et al., 2019). Bowel function included stool frequency (number of defecations per week), stool softness based on the Bristol Stool Form Scale (BSFS) (Lewis & Heaton, 1997a), and gastrointestinal symptoms (bloating, rumbling, cramping, regurgitation, flatulence) which were scored on a scale from 1 to 10 using a weekly diary. Fecal SCFAs were measured in feces collected at baseline, 6 weeks, and endpoint, and plasma fasted SCFAs at baseline and endpoint (Müller et al., 2019; van Eijk et al., 2009; Ziemons et al., 2023). Here, we summarize the metabolic health outcomes and focus in detail on the gut microbiota analysis.

GUT MICROBIOME ANALYSIS USING V4-16S RRNA GENE AMPLICON AND SHOTGUN METAGENOMIC SEQUENCING

Fecal samples were collected at home using a provided sampling kit and stored in a home freezer. Samples were transported frozen to the research facility, where there were stored at -80 °C until further processing. DNA was extracted by weighing 0.25 g of frozen feces into sterile 2.0 mL screw-cap tubes filled with 0.5 g of 0.1 mm zirconia bead and 5 glass beads of 2.5 mm diameter and adding 700 µL Stool Transport and Recovery (STAR) buffer (Roche Diagnostics, Almere, The Netherlands). Samples were subjected to repeated (3 x 1 min at 5.5 ms) bead-beating in a FastPrep-24™ 5G Instrument (MP Biomedicals, The Netherlands) followed by heating for 15 min at 95 °C at 300 rpm and centrifuging for 5 min at 4 °C at 16,100 x g. The supernatant was transferred into a sterile Eppendorf tube, the pellet was resuspended in 700 µL STAR and the cycle of bead-beating, heating and centrifuging was repeated. Supernatants were pooled and DNA was purified using a customized Maxwell® 16 Tissue LEV Total RNA purification Kit (XAS1220) on the Maxwell® 16 LEV Instrument (Promega, The Netherlands) and eluted in 50 µL nuclease free water (Qiagen, Hilden, Germany). DNA concentration was measured using a Qubit™ dsDNA Quantification BR Assay Kit on a Qubit Fluorometer (ThermoFisher Scientific, The Netherlands) and adjusted to 20 ng/µL with nuclease-free water. The V4 region of the 16S rRNA gene was amplified in duplicate using the barcoded primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015). Each 50 µL PCR reaction contained 10 µL 5x Phusion Green HF buffer (ThermoFisher Scientific), 1 µL 10mM dNT's (Promega, Madison, WI, United States), 0.5 µL Phusion Hot start II DNA 2 U/µL polymerase (ThermoFisher Scientific, The Netherlands), 1 µL of each barcoded forward and reverse 10 µM primer, 1 µL of 20 ng/µL DNA template and 36.5 µL nuclease-free water. The PCR program consisted of an initial 30 s denaturation at 98 °C for 10 min, followed by 25 cycles of 10 s denaturation at 98 °C, 10 s annealing at 50 °C, 10 s elongation at 72 °C, and final extension for 420 s at 72 °C. To verify the presence and size of each PCR product 2 µL were loaded onto a 2.2% agarose gel (Lonza Benelux B.V., Breda, The Netherlands) and run for 5 min at 200 V. The PCR products were pooled, further purified using the CleanPCR kit (CleanNA, Waddinxveen, The Netherlands) and the DNA concentration was again measured using Qubit. For 16S rRNA gene amplicon sequencing, a library with an equimolar mix of purified PCR product, no-template negative control PCR, and DNA extraction controls, as well as positive control mock communities (MC3 and MC4; (Ramiro-Garcia et al., 2018)) was prepared and sent for Illumina Hiseq sequencing to Novogene (Novogene, The Netherlands). Raw amplicon sequences were processed using NG-Tax 2.0 with default settings but trimmed to 100 bp (Poncheewin et al., 2020) and resulting amplicon sequence variants (ASVs) were taxonomically annotated using the SILVA 138.1 database (Quast et al., 2013). On average, we obtained 86,302 reads per sample, ranging from 10,746 to 200,239 reads. For shotgun metagenomic sequencing, total DNA was diluted to > 400 ng in 25 µL of nuclease-free DNA water to provide a concentration of >20 ng/µL and sent for Illumina

NovaSeq 6000 sequencing (paired-end 150 bp) to Novogene (Novogene, Cambridge, United Kingdom). Quality of raw metagenomic reads was checked using FastQC (version 0.12.1) (Andrews, 2023). All sequences passed quality control with an average Phred score above 35 indicating high quality and suitability for downstream analysis. On average, we obtained 50,232,230 reads per sample, ranging from 30,610,398 to 203,879,552 reads. Read-based taxonomic profiling was done using MetaPhlAn4 (version 4.0.6) with the mpa_vJun23_CHOCOPhlAnSGB_202307 database (Blanco-Míguez, Beghini, et al., 2023), and functional profiling using HUMAnN (version 3.0) (Beghini et al., 2021).

STATISTICAL ANALYSIS

Metabolic, bowel function, fecal and plasma SCFAs, and gut microbiota taxonomic and functional outcomes were analyzed using R version 4.2.3 (R Core Team, 2023). For metabolic, bowel function, fecal and plasma SCFA outcomes, normality was checked by inspecting QQ-plots using base R and the ggplot2 (Wickham, 2016) and patchwork packages (Pedersen, 2022). Descriptive statistics were calculated using the tidyverse (Wickham et al., 2019) and rstatix packages (Kassambara, 2023b) and visualized using the ggstatsplot package (Patil, 2021), the ggpubr package (Kassambara, 2023a) and the ggsignif package (Ahlmann-Eltze & Patil, 2021). Metabolic and plasma SCFA outcomes at baseline (week 0) and endpoint (week 12) were assessed using linear mixed modeling as implemented in the lme4 (Bates et al., 2015) and lmerTest (Bates et al., 2015; Kuznetsova et al., 2017) packages. The intervention group (treatment or placebo), time (pre-or post-intervention), and their interaction (representing the change over time) were included as fixed effects and subject as a random effect. Fecal SCFA outcomes at baseline and endpoint were analyzed using the same linear mixed model approach. Estimated marginal means were calculated using the emmeans package (Lenth, 2023). Stool softness at baseline, 2 weeks, 6 weeks, 9 weeks, and endpoint was also analyzed using the same linear mixed model approach. All other bowel function outcomes were analyzed using the Friedman test due to assumption violation for linear repeated measures testing, and if applicable, pairwise comparisons were calculated using paired Wilcoxon test with false-discovery rate (FDR) correction as implemented in the rstatix package. For this purpose, missings in bowel function outcomes were imputed using the median of the respective intervention group's time period. Associations between changes in significantly affected fecal and metabolic health markers at the intervention group level (treatment versus placebo) were assessed using Pearson correlation as implemented in the ggpubr package (Kassambara, 2023a). Gut microbiota outcomes at baseline, 2 weeks, 6 weeks, 9 weeks, and end-point based on 16S rRNA gene amplicon sequencing were analyzed as described previously (Puhlmann et al., 2022) using the R packages mare (Korpela, 2016) as well as vegan (Oksanen et al., 2022), phyloseq (McMurdie & Holmes, 2013), microbiome (Lahti & Shetty, 2019) and microViz (Barnett et al., 2021). Multivariate community analysis was done using Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity (β-diversity). For univariate analysis

at the genus level, taxa counts were converted into relative abundances (%), and differential abundance testing with FDR correction was performed as implemented in mare. Shotgun metagenomics-based taxonomic and functional profiles from baseline, week 6, and endpoint obtained from MetaPhlAn as relative abundances (Blanco-Míguez, Beghini, et al., 2023) were used to assess species-level information of taxa and pathways/genes of interest and processed using linear mixed modeling as described above. Further responder analysis was performed by dividing the treatment group based on the observed improvement in the primary study outcomes (insulin-mediated glucose disposal) of more than 15% (high responders) or less than/equal to 15% (low responders). Baseline and endpoint differences in gut microbiota composition were then assessed using the same data analytical approaches as described above.

RESULTS AND DISCUSSION

Here, we aimed to investigate the effect of prolonged dried chicory root intake, on the gut microbiome to understand the effect of prolonged intake on gut microbiota composition, metabolites, and metabolic health in individuals with overweight/obesity at risk for T2D. Thirty-five individuals were included, of which 20 were males, and 15 were females, with a mean age of 63 years and a mean BMI of 32.4 ± 3.5 kg/m^2 (Table 1). No baseline differences in anthropometrics, blood pressure, fasting glucose, glucose tolerance, or HOMA-IR were observed between the intervention groups. Individuals had a median (range) stool frequency of 1.0 (1.0, 4.0) defecations per day and a mean (SD) stool softness of BSFS score 4.0 (1.4). Over the course of 12 weeks of dried chicory root intake, changes in metabolic health markers differed statistically significantly from placebo with improvements in whole-body insulin sensitivity (M-value; $p = 0.016$), attributed to an average increase in peripheral insulin sensitivity by +18.9% (insulin-mediated glucose disposal; $p = 0.085$). Furthermore, dried chicory root intake decreased fasting plasma triglyceride ($p = 0.049$) and glucose levels ($p = 0.04$; Supplementary Table 1). In addition, the proportion of small adipocytes (<50 µm) increased at the expense of very large adipose tissue cells (> 90 µm; Supplementary Table 1). Finally, all fecal SCFAs increased statistically significantly after dried chicory root intake, with acetate showing a 56.8% increase post intervention compared to baseline levels ($p < 0.001$) and propionate and butyrate increasing by about a fifth over their baseline levels (propionate: + 18.2%, $p = 0.002$; butyrate: +17.4% $p = 0.015$; Supplementary Table 1).

Table 1. Baseline characteristics of all individuals included in the data analysis. Averages are represented as mean and standard deviation (mean (SD)), count and proportion (n (%)), or median and inter-quartile range (median [IQR]).

	Placebo group	Treatment group	p-value
n	18	17	
Age (years)	63.11 (5.75)	61.82 (5.91)	0.518
Sex (%)			
female n (%)	8 (44.4)	7 (41.2)	
male n (%)	10 (55.6)	10 (58.8)	
Height (cm)	173.18 (8.92)	173.59 (8.78)	0.892
Weight (kg)	95.44 (14.84)	97.35 (12.95)	0.688
BMI (kg/m^2)	31.66 (2.52)	32.34 (4.17)	0.561
Glucose (mmol/L)	5.91 (0.59)	5.99 (0.56)	0.672
Insulin (mU/L)	13.25 (6.92)	11.98 (5.91)	0.563
HOMA-IR	3.56 (2.01)	3.23 (1.78)	0.615
M-value	4.64 (1.97)	4.53 (1.66)	0.869
Peripheral insulin sensitivity (Rdstim)	14.05 (10.52)	14.31 (8.21)	0.936
Total Cholesterol (mmol/L)	5.04 (0.67)	4.99 (0.93)	0.860
HDL Cholesterol (mmol/L)	1.15 (0.24)	1.03 (0.28)	0.214
Triglycerides (mmol/L)	1.74 (0.66)	2.00 (1.01)	0.366
Fat body mass (kg)	32.63 (7.87)	33.35 (8.74)	0.798
Lean body mass (kg)	61.41 (12.22)	59.30 (18.10)	0.687
Fiber (g/day)	21.81 (6.88)	22.60 (11.08)	0.802
Fiber (g/1000kcal)	11.46 (3.55)	10.28 (3.11)	0.301
Stool Frequency (defecations/day)	1.00 [1.00, 2.00]	1.00 [0.00, 4.00]	0.239
Stool softness (BSFS)	4.08 (1.30)	3.97 (1.48)	0.812

BMI, Body Mass Index; BSFS, Bristol Stool Form Score, HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance

DRIED CHICORY ROOT MODULATES BOWEL FUNCTION

One of the most immediate indicators of modulation in the gut microbiota environment is a change in stool frequency and consistency. In our previous study, chicory root positively impacted bowel function by improving stool softness (BSFS) from 3.3 to 4.4 units and increasing stool frequency from one to two defecations per day (Puhlmann et al., 2022). In our current study, individuals consuming dried chicory root had a softer baseline stool softness of 4.0 but increased to similarly high BSFS scores of up to 4.5, accounting for an overall smaller increase in softness. Stool softness did not increase within the first two weeks of 15 g/day dried chicory root intake but did increase after the first four weeks of 30 g/day intake (week 6) by 0.53 (0.42, p = 0.392) to 4.50 (0.37) BSFS units and remained increased by 0.19 (0.43) at week 9 (p = 0.488) and by 0.38 (0.42) at week 12 (p = 0.678; Supplementary Table 2). In contrast to stool

softness, baseline stool frequency and changes were highly comparable in our current compared to our previous study, increasing from one to two defecations per week. However, this increase in stool frequency was more rapid, with one extra defecation per day already after the first two weeks of 15 g/day intake, compared to our previous study's increase of 0.2 defecations per day during the run-in with the same dosage. This increase to two defecations per day persisted throughout the remaining 10 weeks of 30 g daily chicory root intake, despite not being statistically significant (Friedman test: $\chi2(4) = 5.09$, $p = 0.278$; Supplementary Table 3), possibly due to the smaller sample size compared to our previous study (35 vs. 55 participants). Nevertheless, no changes in stool frequency, consistency, or gastrointestinal symptoms were observed in the placebo group. Both BSFS scores 3 and 4 are considered normal stool consistencies, with 3 being slightly more solid than 4 (Lewis & Heaton, 1997a). Stools that are harder (indicated by lower scores) may become softer after consuming dried chicory root, while stools that are already soft may not necessarily get even softer. Instead, they could potentially increase in fecal mass, for example due to an increase in microbial mass or the production of microbial metabolites that together lead to bulking and stimulate motility, consequently affecting stool frequency. Evaluating other stool-related aspects, such as fecal weight and bacterial load, may provide further insights into these dynamics in future studies (Daniel, 2022). Generally, the dried chicory root product was well tolerated, with minor changes in gastrointestinal symptoms and an expected increase in flatulence (Friedman test: $\chi2(4) = 20.66$, $p < 0.001$; Supplementary Table 3), pointing towards increased microbial metabolism.

MODULATION OF GUT MICROBIOTA COMPOSITION BY DRIED CHICORY ROOT

Previously, we used 16S rRNA gene amplicon sequencing of DNA isolated from fecal samples to assess changes in the composition of the gut microbiota and individual taxa at the genus level after dried chicory root intake (Puhlmann et al., 2022). In our current study, we employed the same method to evaluate time-dependent changes over the longer course of dried chicory root intake. Species-level microbiota changes and changes in functional profiling of microbial pathways and genes were subsequently determined using metagenomic sequence analysis at baseline and endpoint. At baseline, both groups had a similar gut microbiota composition assessed by using β-diversity based on Bray-Curtis dissimilarity (Figure 2A). Consistent with our previous findings, this rapidly changed within two weeks of 15 g/day dried chicory root intake with gut microbiota composition being statistically significantly different from week 6 onward, explaining 6% of the variation (PERMANOVA $p = 0.014$). These changes persisted over time at week 9 (PERMANOVA $p = 0.065$) and diminished at week 12 (Figure 2A). Similarly, α-diversity, representing within-sample characteristics, decreased as expected over time, with the most prominent differences observed at week 6. This was reflected in lower Shannon diversity, observed bacterial richness,

and phylogenetic diversity in individuals consuming dried chicory root compared to the placebo group (Supplementary Figure 2).

MODULATION OF INDIVIDUAL GENERA BY DRIED CHICORY ROOT INTAKE

Further analysis of individual taxa at the genus level confirmed that above-mentioned changes in gut microbiota composition were, in line with previous findings, primarily driven by differences in changes in *Bifidobacterium* spp. and *Anaerostipes* spp. (Figure 2B and Supplementary Figure 3). Dried chicory root intake was associated with a rapid increase in *Bifidobacterium* spp. mean relative abundances by 2.2-fold at two weeks ($p = 0.013$, $q = 0.224$), further increasing to 3.1-fold at week six ($p < 0.001$, $q < 0.001$), and 3.5-fold at week nine ($p < 0.001$, $q < 0.001$), resulting in a 4.1-fold increase at week 12 ($p < 0.001$, $q < 0.001$) compared to baseline (Supplementary Table 4). Similarly, *Anaerostipes* spp. mean relative abundances increased rapidly within two weeks by 2.5-fold ($p < 0.001$, $q < 0.001$), peaking at week six with 4.8-fold higher levels ($p < 0.001$, $q < 0.001$) and remaining elevated at week nine with a 4.4-fold increase ($p < 0.001$, $q < 0.001$), before slightly decreasing to 3.7-fold elevation in relative levels at 12 weeks ($p < 0.001$, $q < 0.001$; Supplementary Table 4). In contrast, changes in taxa at the genus level in the placebo group were minimal and inconsistent over time (Supplementary Table 5). In our previous work, using 16S rRNA gene-based taxonomic profiling, the detected *Bifidobacterium* spp. were predominantly *Bifidobacterium longum*, while *Anaerostipes* spp. mainly consisted of *Anaerostipes hadrus*, the most abundant in adults (Bui et al., 2021; Puhlmann et al., 2022). Using the metagenomic taxonomic profiling with the MetaPhlAn4 workflow, we confirmed the presence of *Bifidobacterium longum* alongside *Bifidobacterium bifidum* and *Bifidobacterium adolescentis* (Figure 2C) as well as the exclusive presence of *Anaerostipes hadrus*. Several other genera, such as *Blautia* spp. and *Coprococcus* spp., decreased over time in the treatment group, although none of these changes were statistically significant after multiple-testing correction. Overall, despite a smaller sample size and different geographic location as compared to our previous study, our findings reported here highlight the consistent and reproducible effects of dried chicory root intake on gut microbiota composition, particularly the increase in four- to five-fold increase in *Bifidobacterium* spp. and *Anaerostipes* spp. relative abundances.

MODULATION OF FUNCTIONAL PROFILES BY DRIED CHICORY ROOT INTAKE

Bifidobacterium and *Anaerostipes* species have been shown *in vitro* to form butyrogenic trophic chains through the degradation of inulin by *Bifidobacterium* spp., with subsequent cross-feeding on produced lactate and acetate by *Anaerostipes* spp. leading to butyrate production (Belenguer et al., 2006; Falony et al., 2006). A fundamental difference, between inulin and dried chicory root, however, is the presence of the plant cell wall, which shields inulin from immediate use by gut bacteria (Puhlmann & de Vos, 2022). Consequently, gut bacteria need to break down the plant cell matrix before accessing the intracellularly stored inulin, which most likely occurs through the degradation of

pectin in the plant cell wall. We previously demonstrated using tri-culture synthetic communities that butyrate production is possible from dried chicory root, including a known pectin degrader, such as *Bacteroides xylanisolves* (Puhlmann et al., 2022). Moreover, by using *in vitro* fecal batch fermentations, we demonstrated that butyrate production from dried chicory root occurred later during fermentation compared to inulin, which we attributed to a slower release of fiber from the dried chicory root cubes, prolonging fiber fermentation (Puhlmann et al., 2024). Here, through metagenomic functional profiling based on the presence of genes known to encode proteins involved in specific metabolic pathways, we confirmed that both the unique degradation of sugars into lactate and acetate by *Bifidobacterium* spp., known as the bifid shunt pathway (Scardovi & Trovatelli, 1965) (week 6: $p = 0.016$; week 12: $p = 0.014$) and the acetyl-CoA fermentation to butanoate II pathway (butyryl-Co:acetateCoA-transferase pathway; week 6: $p < 0.001$; week 12: $p < 0.001$), were statistically significantly increased in the treatment group compared to the placebo group (Supplementary Figure 4). Moreover, *Anaerostipes hadrus* had the largest contribution to the acetyl-CoA fermentation to butanoate II pathway (Figure 2D). These findings underscore our previous findings of a butyrogenic trophic chain being formed from the dried chicory root product *in vivo*.

DIFFERENCES IN GUT MICROBIOTA COMPOSITION AND FUNCTIONALITY IN LOW COMPARED TO HIGH RESPONDERS

The response of the microbiota to dietary stimuli and the conversion of fiber into SCFAs by the gut microbiota are highly individualized processes, influenced by the individual's gut microbiota signatures (Korpela et al., 2014b; Salonen et al., 2014). Consequently, utilizing baseline gut microbiota signatures to predict treatment success has emerged as a crucial strategy for employing fiber interventions in metabolic disease management (Deehan et al., 2024). Previously, we showed that dividing individuals into responders and non-responders based on the primary outcome revealed that improvements in glucose control, fasting glucose, and HOMA-IR were linked to low baseline levels of *Blautia* spp. that have been positively associated with visceral fat accumulation and intake of highly processed foods (Bolte et al., 2021; Le Roy et al., 2017; Ozato et al., 2019). Here, we adopted a similar approach and categorized individuals based on an improvement of more than 15% in the primary outcome, insulin-mediated glucose disposal (Rdstim). Individuals with a larger improvement in insulin-mediated glucose disposal (> 15% improvement) were categorized as high responders ($n = 9$), and those with a lower improvement (≤ 15% improvement) were categorized as low responders ($n = 8$). High responders had statistically significantly lower relative abundance of *Bacteroides* spp., *Monoglobus* spp., and statistically significantly higher relative abundance of *Erysipelotrichaceae* UCG-003 spp. compared to low responders (Figure 3). These differences persisted throughout the study to week 12, although the relative abundances of these taxa decreased over time.

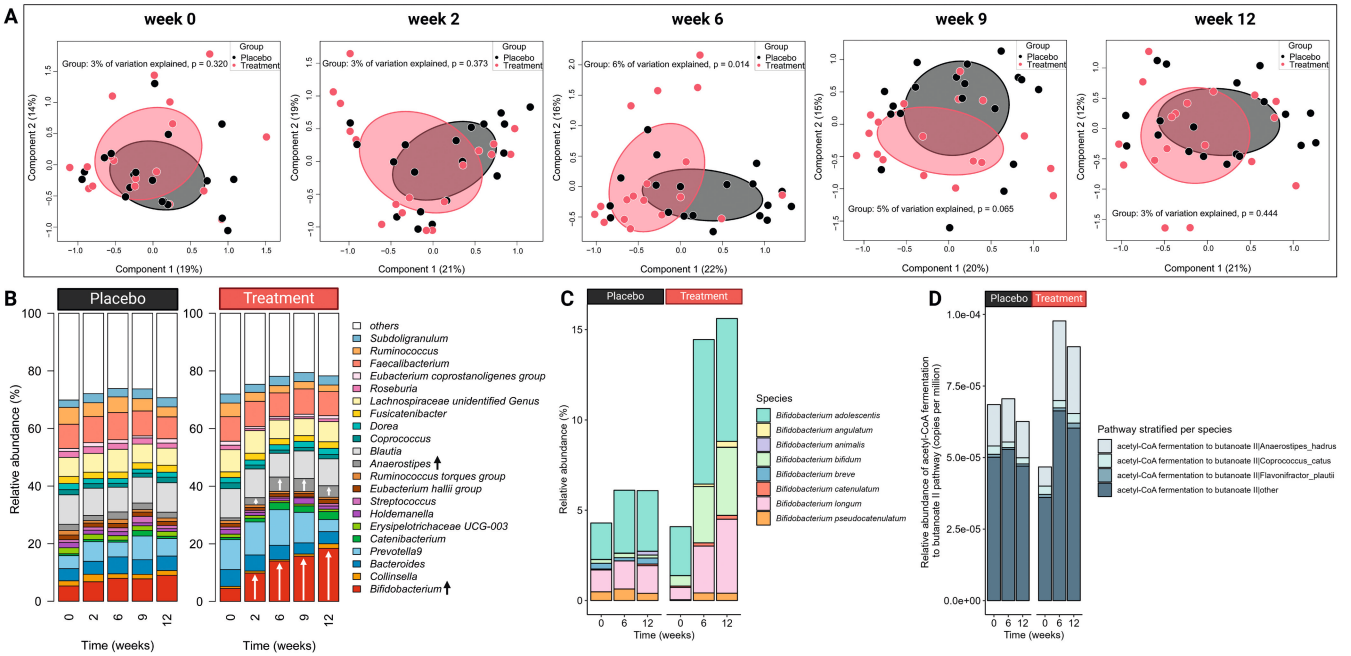

Figure 2. Modulation of the gut microbiota communities over time by dried chicory root intake compared to placebo. (**A-D**) Differences in gut microbiota composition based on 16S rRNA gene amplicon sequencing assessed by β-diversity using Bray-Curtis dissimilarity (**A**), representing here the between-sample differences over time (week 0, 2, 6, 9 and 12). Differences in β-diversity were calculated using Permutational multivariate analysis of variance (PERMANOVA). (**B**) Common taxa (mean relative abundance of at least 1% and mean relative prevalence in at least 50% of all samples) based on 16S rRNA gene amplicon sequencing in the treatment and the placebo group following dried chicory root intake with statistically significant increases in relative abundance of *Bifidobacterium* spp. and *Anaerostipes* spp. over time. (**C**) Changes in *Bifidobacterium* species relative abundances (%) based on taxonomic profiling using metagenomic analysis in the placebo and treatment group at weeks 0, 6, and 12. (**D**) Changes in the relative abundance (copies per million) of the butyrate production pathway based on functional profiling using metagenomic analysis, notably attributed to increases in *Anaerostipes hadrus*, the most abundant *Anaerostipes* species. Created with BioRender.com.

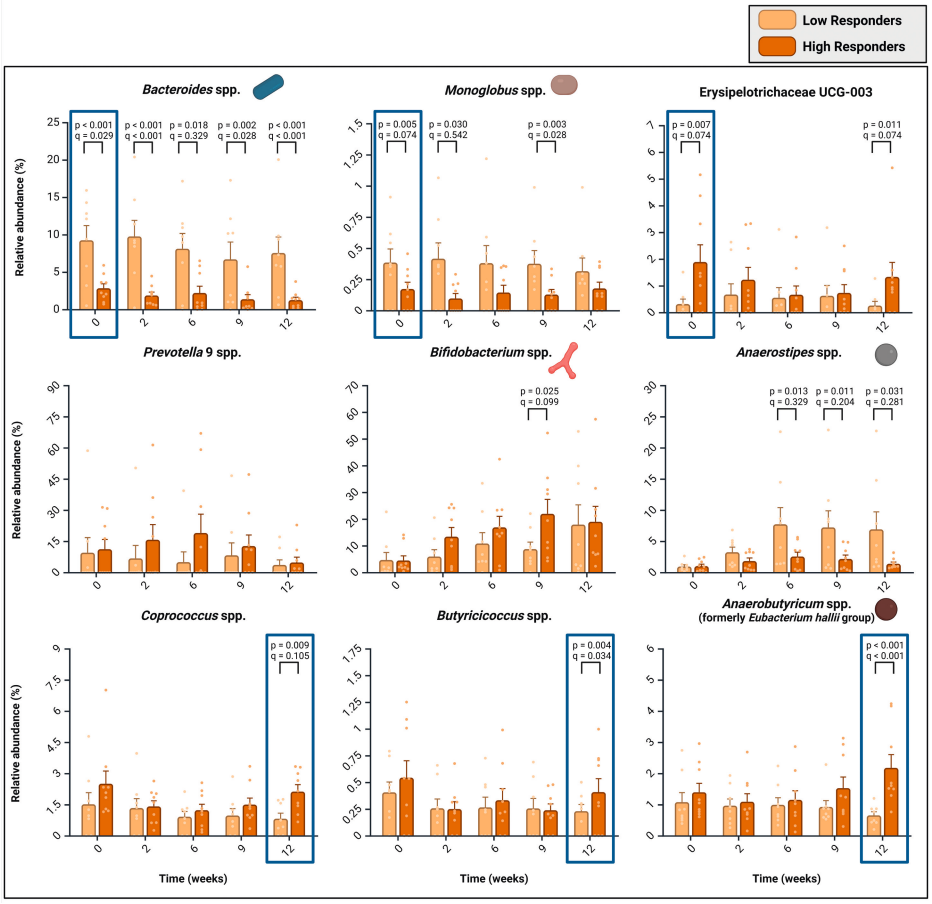

Figure 3. Responder differences in gut microbiota composition. Gut microbiota differences between low and high responders in the treatment group at baseline (week 0) and over the course of the study (week 2, 6, 9 and 12). Created with BioRender.com.

Metagenomic profiling revealed that the most abundant *Bacteroides* species included *B. uniformis* and *B. xylanisolvens,* both known pectin degraders (Despres et al., 2016; Feng et al., 2022) and that *Monoglobus pectinilyticus* was detected at species level, which is also a dedicated pectin degrader (Kim et al., 2019). Aside from these baseline differences, we observed that, notably, relative abundances of *Prevotella* 9 spp. (primarily composed of *Segatella*) and *Bifidobacterium* spp. increased rapidly in high responders, with the latter mainly attributed to higher relative abundances of *Bifidobacterium adolescentis* at species level (Supplementary Figure 5). In contrast, *Anaerostipes* spp. relative abundance increased more rapidly in low responders. Finally, several butyrate and propionate producers, including *Butyricicoccus* spp., *Coprococcus* spp., and *Anaerobutyricum* spp., formerly known as *Eubacterium hallii* group (Shetty et al., 2018), reached statistically significantly higher final relative abundances in high

responders compared to low responders (Figure 3). Of note, *Anaerostipes* spp. and *Anaerobutyricum* spp. are related bacteria that have the unique capacity to convert lactate and acetate into butyrate in a unique pathway (Shetty et al., 2020).

CONSEQUENCE OF MICROBIAL SIGNATURES ON GUT MICROBIAL BREAKDOWN OF DRIED CHICORY ROOT CUBES

We propose that these gut microbiota distinctions between high responders and low responders, notably in their pectin-degrading capacity, may translate into differences in the gut microbial environment, impacting the kinetics and location of fiber breakdown and butyrate production in the colon (Figure 4). Previously, we demonstrated, using *in vitro* fecal batch fermentations, that butyrate production from dried chicory root occurred at a later stage during fermentation, which, *in vivo*, would translate into a distal butyrate production (Puhlmann et al., 2024). Such distal butyrate production might benefit its uptake as higher concentrations in the blood vessels draining from the distal colon have been reported (Neis et al., 2019). Experimental evidence for increased metabolic effects of distal SCFAs has been provided in individuals with obesity where distal but not proximal infusion of SCFAs was found to generate an increased fasting fat oxidation and energy expenditure (Canfora et al., 2017; van der Beek et al., 2016).

To break down the dried chicory root cubes, the gut microbial community first must degrade the plant cell wall, which consists of a cellulose skeleton that is reinforced by hemicellulose and filled with pectin (Figure 4). Degrading pectin opens up the plant cell network and facilitates the liberation of inulin for further use by other bacteria, such as *Bifidobacterium* spp., which, in turn, can cross-feed to butyrate producers (Figure 4). Hence, the high relative abundance of pectin-degrading bacteria such as *Bacteroides* spp. and *Monoglobus* spp. in low responders is expected to promote a fast degradation of the plant cell wall and liberation of inulin, leading to a fast release of fiber and subsequent rather proximal location of butyrate production. In contrast, the low abundance of pectin-degrading bacteria in high-responders may result in a slow release of fiber from the plant cell matrix and a prolonged fermentation, benefitting a more distal location of butyrate production. This hypothesis is supported by a statistically significantly lower abundance of genes encoding pectate lyase in high responders compared to low responders, throughout the study (week 6: $p = 0.044$ and week 12 $p = 0.057$; Figure 5A). Various *Bacteroides* spp. genomes are known to encode pectate lyase genes, and in an earlier study, it was found that in individuals with obesity, pectate lyase protein levels positively correlated with insulin resistance (Kolmeder et al., 2015). Additionally, the complementary observation of high relative abundances of *Bacteroides* spp. and low relative abundances of *Prevotella* spp., and vice versa, is well-documented (Gorvitovskaia et al., 2016). Notably, high responders had higher relative levels of *Prevotella* 9 over the course of the study than low responders (Figure 3).

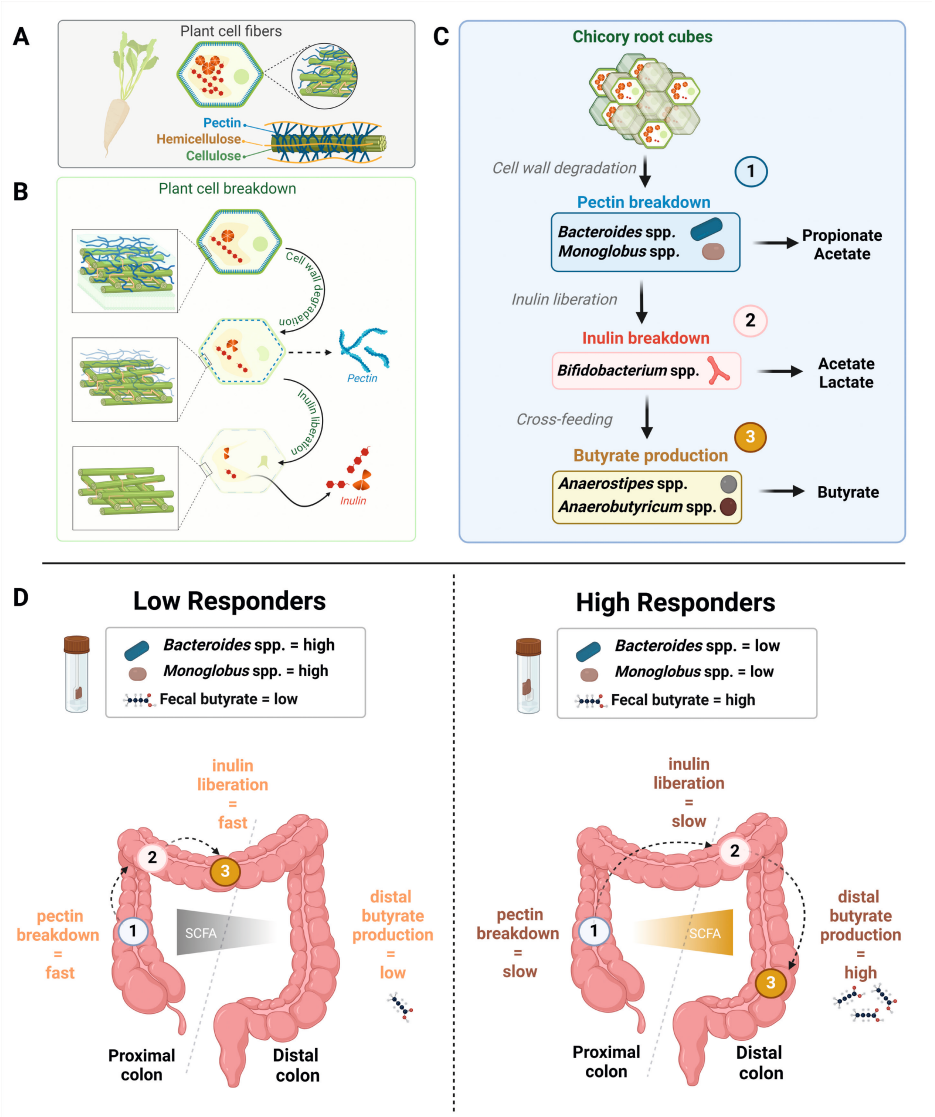

Figure 4. Hypothesized difference in the conversion of dried chicory root cubes to butyrate in low responders compared to high responders. (A-B) To break down and access intracellular inulin, the cell walls of the dried chicory roots must first be degraded. (A) These cell walls consist of a cellulose skeleton reinforced by hemicellulose and filled with pectin. (B) The degradation of pectin leads to a gradual release of cell wall fibers and the liberation of inulin from the plant cells making inulin accessible to other gut bacteria. (C) *Bacteroides* spp. and *Monoglobus* spp., known pectin-degraders, facilitate the degradation of the cell walls, which in turn aids in the breakdown of inulin by *Bifidobacterium* spp., resulting in the production of lactate and acetate. Lactate and acetate are then utilized by *Anaerostipes* spp. and *Anaerobutyricum* spp. (previously named *Eubacterium hallii*) to produce butyrate. (D) In low responders, the degradation of the plant cell wall and liberation of inulin occur more rapidly, leading to a fast release of fibers and a more proximal production of butyrate. In high responders, these processes are slow, resulting in a gradual release of fiber and a more prolonged fermentation, which shifts butyrate production to a more distal location. Ultimately, this high distal concentration of butyrate benefits metabolic health outcomes. Created with BioRender.com.

Individuals with a high *Prevotella*-to-*Bacteroides* ratio have been shown to be more responsive to high-fiber diets for weight loss (Hjorth et al., 2018) and also, high *Segatella copri* levels, formerly known as *Prevotella copri* (Hitch et al., 2022), have been linked to an improved glucose response (Blanco-Míguez, Gálvez, et al., 2023). The reduced relative abundance of *Bifidobacterium* spp. in low responders might appear contradictory in light of an expected fast degradation of inulin, but might be explained by the observation that notably *B. adolescentis* is quite fragile and when active in the proximal colon may die off and lyse during its voyage through the colon (Ben-Amor et al., 2005).

Moreover, in high responders, low *Bacteroides* spp. levels also coincided with a more rapid increase in the relative abundance of bifid shunt pathway genes compared to low responders (Figure 5B). It is possible that the metabolic activity of *Bifidobacterium* spp. could affect the growth of *Bacteroides* spp.. The production of organic acids, such as acetate and notably lactate by *Bifidobacterium* spp. is linked to a reduction in pH (Duncan, Louis, et al., 2004), to which *Bacteroides* spp. are known to be highly sensitive (Walker et al., 2005). *In vitro,* the growth of *Bifidobacterium* spp. has been demonstrated to be promoted at a more acid pH of 5.5 compared to 6 or higher, while that of *Bacteroides* spp. was reduced at pH 5.5 (Chung et al., 2016; Duncan et al., 2009; Louis & Flint, 2017; Walker et al., 2005). Also, a more acidic pH has been linked to the effective conversion of lactate to butyrate (Belenguer et al., 2007), and while acetate-to-butyrate conversion is the most common cross-feeding route, the lactate-to-butyrate conversion is used by a limited number of gut bacteria including *Anaerobutyricum* spp. (formerly *Eubacterium hallii* group (Shetty et al., 2018)) and *Anaerostipes* spp. (Belenguer et al., 2006; Duncan, Holtrop, et al., 2004; Duncan, Louis, et al., 2004; Louis et al., 2022; Morrison et al., 2006; Shetty et al., 2020). Notably, *Bifidobacterium adolescentis,* the most abundant and prevalent *Bifidobacterium* species in high responders, has been shown to tolerate lactate/acetate-related lower pH *in vitro* and to cross-feed lactate to *Anaerostipes* spp. and *Anaerobutyricum* spp. for butyrate production (Belenguer et al., 2006; Ping Wang et al., 2020). As the production of lactate and/or acetate is needed to form a butyrogenic trophic chain, it is, hence, possible that the lactate/acetate-to-butyrate conversion was more effective in high responders and potentially facilitated by a lower pH, despite higher *Anaerostipes* spp. levels in low responders. In our current study, we did not measure fecal pH, but *in vivo*, a reduced fecal pH is associated with reduced transit times, which in turn is associated with higher fecal butyrate (Lewis & Heaton, 1997b; Procházková et al., 2023). Additionally, high responders had statistically significantly higher final levels of *Anaerobutyricum* spp. (formerly *Eubacterium hallii* group (Shetty et al., 2018)) (Supplementary Figure 5). These species may have played a vital role in the metabolic response, as demonstrated in previous studies involving fecal microbiota transplantations and duodenal *Anaerobutyricum* infusions in humans (Koopen et al., 2022; Vrieze et al., 2012) as well as oral *Anaerobutyricum* supplementation in both humans and mice (Gilijamse et al., 2020; Udayappan et al., 2016). Finally, high responders also had high final levels of known butyrate-producers *Butyricicoccus*

spp. and *Coprococcus eutactus* (Louis & Flint, 2017) and other propionate-producers *Coprococcus comes* and *Coprococcus catus* (Louis et al., 2022; Sheridan et al., 2022) (Figure 3 and Supplementary Figure 5). Together, these observations suggest that the gut microbial communities in high responders favored a more distal production of SCFAs potentially driving the observed higher levels of fecal butyrate and propionate after dried chicory root intake (Supplementary Table 1).

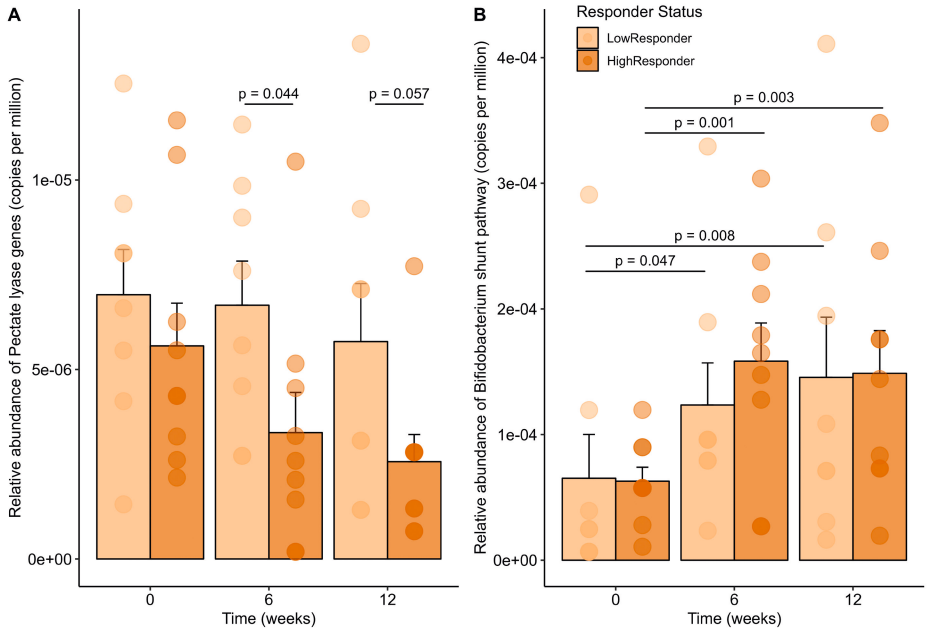

Figure 5. Responder differences in functional profiles. (**A**) Relative abundance (copies per million) of pectate lyase genes is higher in the metagenomes of low responders compared to high responders. No differences in changes over time between responders were noted, but high responders had notably lower relative abundances of pectate lyase genes at week 6 and week 12. (**B**) Relative abundance (copies per million) of the *Bifidobacterium* shunt pathway genes is lower in low responders compared to high responders. The relative abundance of *Bifidobacterium* shunt pathway genes increased statistically significantly in both groups without changes over time differing between groups.

It is worth noting that the high levels of *Anaerostipes* spp. in the low responder group were specifically driven by three of the individuals, of which two showed an improvement in insulin-mediated glucose disposal of 12% and 13%, just below our set threshold. It is likely that the biological processes underlying these differences exist more on a continuum rather than as a strict dichotomy. Also, inferring functionality based solely on metagenomic profiles, as done in this study, reflects the functional potential rather than the actual expression of the genes and the production of proteins. Transcriptomic approaches have previously demonstrated that the lactate/acetate-to-butyrate conversion can be increased in the absence of changes in the relative abundance of the butyrate-producing *Anaerobutyricum* spp. in a synthetic community (Shetty et al.,

2022). Applying such omics techniques in the future, together with the measurements of fecal pH, may offer more detailed insights into the observed responder differences, notably related to *Bacteroides* spp. and *Anaerostipes hadrus*. Similarly, more in-depth profiling of the genomic potential of *Erysipelotrichaceae* UCG-003 spp. might reveal their importance in the observed differences, as members of this genus are not well studied and have been associated with insulin resistance (Atzeni et al., 2022) but also with gut microbial networks in Dutch individuals without T2D (Nayman et al., 2024).

DIFFERENCES IN FECAL AND PLASMA METABOLITES BETWEEN HIGH AND LOW RESPONDERS

Drawing from the findings that responders had distinctly different gut microbiota profiles, we investigated how these differences were reflected in fecal SCFAs, plasma SCFAs, and other metabolic health markers. In line with the higher final relative abundances of butyrate and propionate-producing bacterial taxa in high responders, we also observed statistically significantly higher increases in fecal butyrate ($p = 0.006$) and propionate levels ($p = 0.031$) in high compared to low responders (Figure 6A-B and Table 2). Specifically, the higher increase in fecal butyrate in high responders was due to all these individuals increasing in fecal butyrate, which was not the case for the non-responders (Figure 6A). Fecal butyrate can be increased by shortening transit time (Lewis & Heaton, 1997b), and, hence, might simply be a result of faster transit. However, here, assuming higher stool softness and frequency are proxies for faster transit (Asnicar et al., 2021), we did not find indications thereof in high responders, as they did not show an increase in stool softness or frequency (Table 2). Consequently, we expect that a high amount of fecal butyrate likely reflects the more distal production of butyrate facilitated by different gut microbial communities in these individuals.

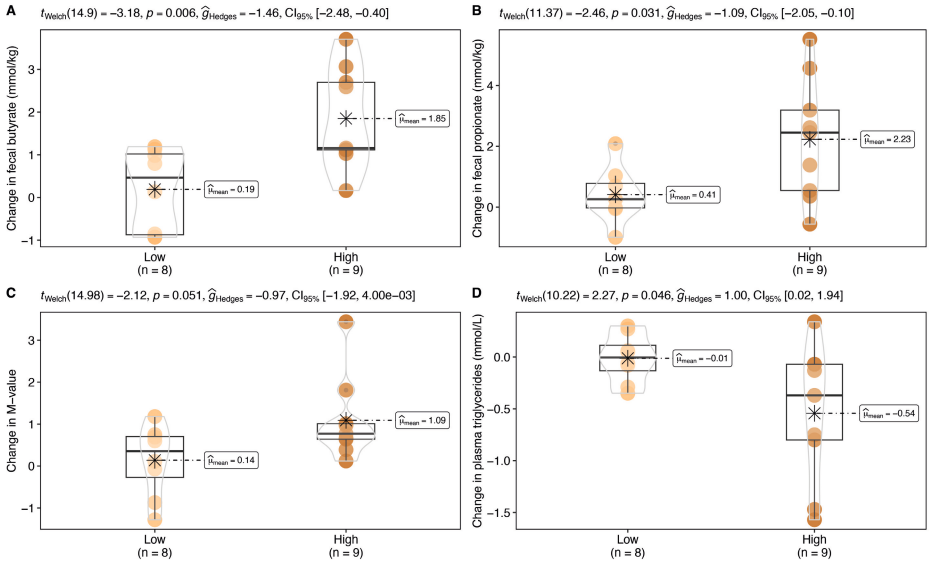

Figure 6. Responder differences in selected fecal and plasma metabolites. Differences in changes after 12 weeks in (**A**) increases in fecal butyrate levels (mmol/kg) at 12 weeks over baseline, (**B**) increases in fecal propionate levels (mmol/kg) at 12 weeks over baseline, (**C**) increases in whole body insulin sensitivity assessed by M-value at 12 weeks over baseline (**D**) decreases in plasma triglyceride levels (mmol/L) at 12 weeks over baseline.

In addition to the fecal changes, we observed marked differences in systemic metabolic health markers. As expected from the treatment effect of dried chicory root compared to placebo (Supplementary Table 1), high responders with respect to peripheral insulin sensitivity showed larger improvements in whole-body insulin sensitivity (M-value, p = 0.051; Figure 6C) and decreased fasting glucose levels (p = 0.190); changes which were not apparent in low responders (Table 2). High responders also showed a larger decrease in triglyceride levels (p = 0.046; Figure 6B) and an increase in high-density lipoprotein (HDL) cholesterol levels (p = 0.019; Table 2). Both these changes are indicative of metabolic improvement as high triglyceride and low HDL cholesterol levels are markers of dyslipidemia in T2D and metabolic syndrome (Ginsberg et al., 2005). Moreover, it became apparent that the observed increases in small adipocytes and related decreases in large adipocytes, indications of improved insulin sensitivity (Stenkula & Erlanson-Albertsson, 2018), were also driven by high responders (Table 2). Finally, high responders had a slightly greater, but not statistically significantly different, decrease in BMI and weight compared to low responders (Table 2), and also, at the intervention level, no differences between treatment placebo were observed (Supplementary Table 1). This indicates that the observed systemic changes were not caused by weight loss, known to promote insulin sensitivity (Taylor et al., 2019), but by changes in metabolic processes induced by the gut bacterial colonic conversion of dried chicory root and associated metabolite production.

Table 2. Responder differences in all fecal and plasma metabolites. Differences in changes after 12 weeks (compared to baseline, week 0) in metabolic health markers, fecal and plasma short-chain fatty acids, anthropometric, and bowel function outcomes in low compared to high responders based on peripheral insulin sensitivity.

	Low Responders	High Responders	p-value
M-value change	0.14 (0.30)	1.09 (0.33)	0.051
Fasting glucose	0.00 (0.07)	-0.14 (0.07)	0.190
HDL (mmol/L) change	-0.04 (0.01)	0.07 (0.04)	0.019
Triglycerides (mmol/L) change	-0.01 (0.08)	-0.54 (0.22)	0.046
Small adipocytes (%) change	0.84 (2.17)	7.51 (1.81)	0.033
Very large adipocytes (%) change	-0.60 (2.33)	-6.13 (2.35)	0.115
Fecal butyrate (mmol/kg) change	0.19 (0.34)	1.85 (0.40)	0.006
Fecal propionate (mmol/kg) change	0.41 (0.32)	2.23 (0.67)	0.031
Fecal acetate (mmol/kg) change	8.64 (2.39)	10.24 (2.00)	0.614
Plasma acetate (µmol/L) change	-14.12 (13)	17.94 (12.56)	0.097
Plasma propionate (µmol/L) change	0.17 (0.2)	0.51 (0.73)	0.665
Plasma butyrate (µmol/L) change	0.26 (0.15)	0 (0.14)	0.229
BMI (kg/m^2) change	-0.26 (0.16)	-0.38 (0.33)	0.759
Weight (kg) change	-0.75 (0.30)	-1.21 (0.80)	0.601
Stool softness (BSFS) week 12	4.75 (0.68)	4.00 (0.65)	0.435
Stool softness (BSFS) Change	1.19 (0.72)	-0.33 (0.99)	0.233
Stool frequency (x/day) week 12	2.00 [1.00, 3.35]	2.00 [1.00, 2.50]	0.580
Stool frequency (x/day) Change	0.50 [0.00, 1.25]	0.00 [0.00, 0.00]	0.085

BMI, Body Mass Index; HDL, high-density lipoprotein
Averages are represented as mean (SEM).

RELATION BETWEEN SIGNIFICANTLY AFFECTED FECAL AND METABOLIC HEALTH OUTCOMES IN THE TREATMENT COMPARED TO THE PLACEBO GROUP

The differences between low and high responders, notably in fecal butyrate, pointed towards a potential relation between dried chicory root-induced changes in fecal metabolites and metabolic health outcomes. Hence, we correlated changes at the intervention level (treatment versus placebo) in significantly affected fecal and metabolic health outcomes, comparing those in the whole treatment group (low and high responders together) with the placebo group (Figure 6). Indeed, at the intervention level, treatment-induced increases in fecal butyrate were moderately associated with both improvements in plasma triglyceride levels ($r = -0.54$, $p = 0.024$; Figure 6A) and small adipocytes ($r = 0.45$, $p = 0.073$; Figure 6B), and these outcomes were also related to each other (Figure 6C). However, such a relationship was not observed for changes in fecal butyrate with those in peripheral insulin sensitivity (insulin-mediated glucose disposal; $r = 0.24$, $p = 0.36$) or whole-body insulin sensitivity (M-value; $r = 0.079$, $p = 0.76$, Supplementary Figure 7A-B). In the placebo group, none of the changes were meaningfully related (Figure 6). Considering the shift towards smaller adipocytes and that fasted triglycerides originate from hepatic very low-density lipoprotein (VLDL) (Gill & Sattar, 2011), driven by free fatty acids released from adipose tissue (Barrows & Parks, 2006), we might hypothesize that dried chicory root-derived butyrate benefits insulin sensitivity indirectly through changes in adipose tissue and hepatic function. Butyrate has been shown to influence adipogenesis and hepatic lipid metabolism, although these observations are predominantly derived from animal studies (Blaak et al., 2020; van Deuren et al., 2022). In humans, butyrate uptake from the distal colon is higher than the proximal colon (Neis et al., 2019). However, fecal butyrate does reportedly not correlate with plasma butyrate (Müller et al., 2019), a relationship we observed here as well ($r = -0.20$, $p = 0.43$; Supplementary Figure 6). This is explained by the effective extraction of butyrate by colonocytes and the liver, leading to very low systemic circulating levels (Bloemen et al., 2009; Boets et al., 2015, 2017).

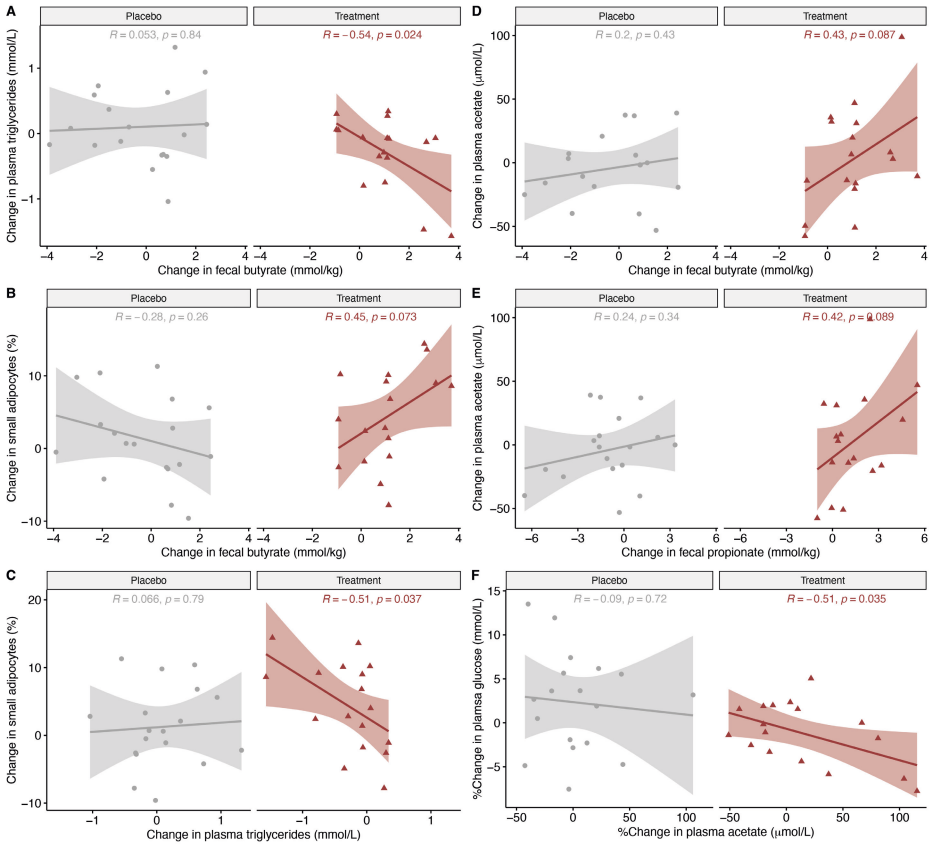

Figure 7. Pearson correlations between changes at 12 weeks in metabolic health markers, fecal and plasma short-chain fatty acids, showing moderate relations between outcome variables. (A) Correlation between increases in fecal butyrate (mmol/kg) and decreases in plasma triglycerides (mmol/L) levels. (B) Correlation between increases in fecal butyrate levels (mmol/kg) and increases in small adipocytes (% of all adipocytes). (C) Correlation between decreases in plasma triglyceride levels (mmol/L) and increases in small adipocytes (% of all adipocytes). (D) Correlation between increases in fecal butyrate (mmol/kg) and increases in plasma acetate (µmol/L) levels. (E) Correlation between increases in fecal propionate (mmol/kg) and increases in plasma acetate (µmol/L) levels. (F) Correlation between percentage changes (week 0 to week 12 change expressed as percentage of baseline) between plasma acetate (µmol/L) and plasma glucose (mmol/L) levels.

Instead, here, we found that higher changes in fecal butyrate and propionate were moderately related to higher plasma acetate levels (Figure 6D-E). Remarkably, high responders showed greater absolute increases in plasma acetate (Table 2), with a relative increase (%change) of 36.7% in high responders compared to -10.9% in low responders (%change p = 0.045). In addition, larger relative increases in plasma acetate were associated with larger relative decreases in plasma glucose (Figure 6F), suggesting that plasma acetate may relate to or mediate metabolic health outcomes. The benefits of acetate on metabolic health outcomes, particularly through modulating

adipose tissue function, have been demonstrated before. Cell line and animal models have shown that acetate may improve hepatic glucose and lipid metabolism, as well as adipose lipid metabolism, potentially through GPR43 (Blaak et al., 2020; Canfora et al., 2015). In humans, distal infusions of SCFAs have been shown to reduce whole-body lipolysis, and related changes in plasma acetate levels were linked to higher fat oxidation and resting energy expenditure (Canfora et al., 2017). Hence, colon-derived butyrate may act through changes in plasma acetate levels. It is currently unclear how increased fecal butyrate, propionate, and acetate are linked to plasma acetate and improve dysglycemia, dyslipidemia, and insulin sensitivity. However, it is clear that acute elevation of colonic SCFA levels benefits the above-mentioned metabolic markers in individuals with obesity (Canfora et al., 2017; van der Beek et al., 2016). In addition, chronically elevated fecal acetate and butyrate levels, as well as the abundance of fecal acetyl-CoA:butyrylCoA-transferase genes, have been shown to be associated with lower postprandial glucose and insulin response in healthy individuals (Wijdeveld et al., 2023). Potential mechanisms via GPR-signaling and the stimulation of gut hormonal release of GLP-1 and PYY have been proposed (Freeland & Wolever, 2010; van der Beek et al., 2016). The colonic action of SCFAs appears to be crucial for such a signaling pathway, as intravenous acetate infusions failed to promote the production of such hormones (Freeland & Wolever, 2010). Moreover, a distal location of colonic SFCA uptake clearly plays a role herein, as demonstrated by site-specific SCFA infusion studies (Canfora et al., 2017; van der Beek et al., 2016). Notably, GPR43 (Free Fatty Acid receptor 2, FFA2) and GLP-1 enteroendocrine cells are more abundant in the distal colon (Kaji et al., 2011). We expect that the larger response in peripheral insulin sensitivity observed here is linked to such a more distal production and uptake of notable butyrate due to a slower release of fiber resulting from the observed gut microbial taxonomic and functional differences. It is possible that *Anaerobutyricum* spp. play a crucial role in the distal conversion of lactate and acetate into butyrate, as their relative abundance correlated positively with fecal butyrate levels in the treatment group and with insulin-mediated glucose disposal, especially in high responders (Supplementary Figure 8).

CONCLUSION

We investigated the effect of a ten-week daily 30 g dried chicory intake on the gut microbiota to understand the effect of prolonged intake on gut microbiota composition, metabolites, and metabolic health in individuals with obesity and at risk for T2D. We found a rapid modulation in gut microbiota composition, resulting in a 4- to 5-fold increase in the relative abundance of *Bifidobacterium* spp. and *Anaerostipes* spp. These gut microbiota changes were accompanied by increased levels of fecal acetate, propionate, and butyrate, followed by a significant improvement in total body insulin sensitivity, particularly in peripheral insulin sensitivity. Individuals responding to the intervention with a larger improvement in peripheral insulin sensitivity had lower relative abundances of pectin-degrading taxa and pectate lyase genes but higher final levels of fecal butyrate and propionate and more pronounced improvements in plasma triglyceride levels and the proportion of small adipocytes, indicative of a beneficial role of colon-derived butyrate on metabolic health. Overall, our findings highlight the consistent and reproducible effects of dried chicory root intake on gut microbiota composition and metabolites. These results underscore the potential of dried chicory root as a dietary intervention for modulating gut microbiota and improving metabolic health.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Difference between the placebo and treatment groups in metabolic health markers from baseline to 12 weeks of intervention.

	Treatment		Placebo			
	Week 0	**Week 12**	**Week 0**	**Week 12**	**Change**	**p-value**
Insulin-mediated glucose disposal (µmol/kg/min)	14.31 (2.47)	17.02 (2.47)	14.05 (2.40)	13.71 (2.4)	3.05 (1.72)	0.085
M-value	4.53 (0.45)	5.18 (0.45)	4.64 (0.44)	4.33 (0.44)	0.95 (0.37)	0.016
Fasting glucose (mmol/L)	5.94 (0.14)	5.87 (0.14)	5.88 (0.14)	6 (0.14)	-0.19 (0.09)	0.040
TG (mmol/L)	2.00 (0.20)	1.71 (0.20)	1.74 (0.19)	1.84 (0.19)	-0.39 (0.19)	0.049
HDL (mmol/L)	1.03 (0.06)	1.05 (0.06)	1.15 (0.06)	1.12 (0.06)	0.05 (0.04)	0.190
Small fat cells (%)	30.18 (1.66)	34.55 (1.66)	31.19 (1.62)	32.44 (1.62)	3.12 (2.11)	0.149
Very large fat cells (%)	17.44 (2.08)	13.91 (2.08)	15.5 (2.03)	13.94 (2.03)	-1.97 (2.75)	0.477
Fecal butyrate (mmol/kg)	6.10 (0.27)	7.16 (0.27)	6.81 (0.26)	6.56 (0.26)	1.32 (0.53)	0.015
Fecal propionate (mmol/kg)	7.51 (0.46)	8.88 (0.46)	8.51 (0.45)	7.45 (0.45)	2.42 (0.73)	0.002
Fecal acetate (mmol/kg)	16.7 (1.49)	26.19 (1.49)	19.71 (1.45)	14.54 (1.45)	14.66 (2.11)	<0.001
Plasma butyrate (µmol/L)	1.00 (0.12)	1.12 (0.12)	0.92 (0.11)	0.96 (0.11)	0.08 (0.16)	0.627
Plasma propionate (µmol/L)	3.62 (0.34)	3.97 (0.34)	4.27 (0.33)	4.94 (0.33)	-0.32 (0.51)	0.543
Plasma acetate (µmol/L)	88.96 (15.45)	91.81 (15.45)	103.52 (15.02)	99.34 (15.02)	7.04 (11.39)	0.541
BMI (kg/m^2)	32.34 (0.85)	32.02 (0.85)	31.66 (0.83)	31.78 (0.83)	-0.45 (0.28)	0.116
Weight (kg)	97.35 (3.45)	96.36 (3.45)	95.44 (3.35)	94.92 (3.35)	-0.47 (0.80)	0.559

BMI, Body Mass Index; HDL, high-density lipoprotein; TG, triglycerides

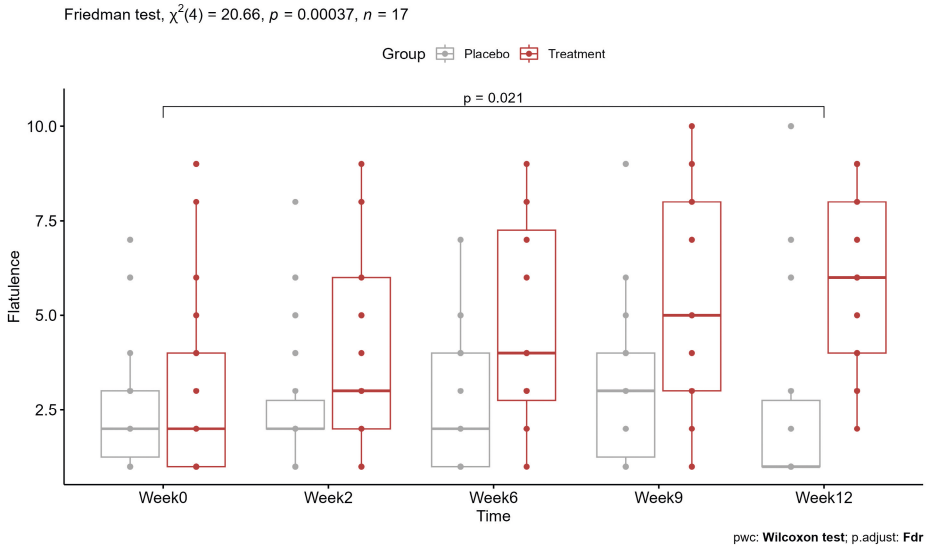
Supplementary Table 2. Stool softness outcomes measured by using the Bristol Stool Form Scale (BSFS) over time in the treatment (dried chicory root) and placebo group and the respective p-value of the difference in change between intervention groups per week as assessed using linear mixed modeling.

		Week 0	Week 2	Week 6	Week 9	Week 12	Linear mixed model Intervention*Time p-value			
							Wk2	Wk6	Wk9	Wk12
Stool Softness (BSFS)	Treatment	3.97 (0.37)	3.94 (0.37)	4.50 (0.37)	4.16 (0.38)	4.35 (0.37)	0.847	0.392	0.488	0.678
	Placebo	4.08 (0.36)	4.17 (0.36)	4.11 (0.36)	3.86 (0.36)	4.22 (0.36)				

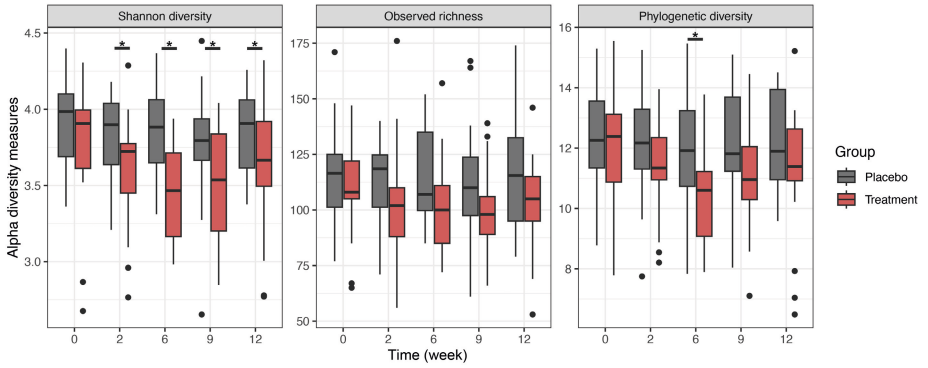
BSFS, Bristol Stool Form Scale

Supplementary Table 3. Stool frequency and gastrointestinal outcome scores over time in the treatment (dried chicory root) and placebo group and their respective test statistics (Chi-square test χ^2) as assessed using the Friedman test.

		Week 0	Week 2	Week 6	Week 9	Week 12	Friedmann test
Stool Frequency (x/day)	Treatment	1.00 [1.00, 2.00]	2.00 [1.00, 2.00]	2.00 [1.00, 2.50]	2.00 [1.00, 2.50]	2.00 [1.00, 3.00]	$\chi^2(4) = 5.09$, $p = 0.278$
	Placebo	1.00 [1.00, 1.00]	1.00 [1.00, 2.00]	1.00 [1.00, 1.00]	1.00 [1.00, 1.75]	1.00 [1.00, 2.00]	$\chi^2(4) = 2.97$, $p = 0.563$
Flatulence score	Treatment	2.00 [1.00, 4.00]	3.00 [2.00, 6.00]	4.00 [2.75, 7.25]	5.00 [3.00, 8.00]	6.00 [4.00, 8.00]	$\chi^2(4) = 20.66$, $p < 0.001$
	Placebo	2.00 [1.25, 3.00]	2.00 [2.00, 2.75]	2.00 [1.00, 4.00]	3.00 [1.25, 4.00]	1.00 [1.00, 2.75]	$\chi^2(4) = 4.48$, $p = 0.344$
Bloating score	Treatment	1.00 [1.00, 2.00]	1.00 [1.00, 3.00]	1.00 [1.00, 2.50]	1.00 [1.00, 2.00]	2.00 [1.00, 3.00]	$\chi^2(4) = 5.47$, $p = 0.242$
	Placebo	1.00 [1.00, 2.00]	1.00 [1.00, 2.00]	2.00 [1.00, 3.00]	1.00 [1.00, 2.75]	1.00 [1.00, 2.75]	$\chi^2(4) = 5.60$, $p = 0.231$
Rumbling score	Treatment	1.00 [1.00, 2.00]	2.00 [1.00, 3.00]	2.00 [1.00, 4.00]	2.00 [1.00, 3.50]	2.00 [1.00, 2.00]	$\chi^2(4) = 3.46$, $p = 0.485$
	Placebo	1.00 [1.00, 2.00]	1.00 [1.00, 2.00]	1.00 [1.00, 3.00]	1.00 [1.00, 2.00]	1.00 [1.00, 1.00]	$\chi^2(4) = 8.61$, $p = 0.072$
Cramping score	Treatment	1.00 [1.00, 2.00]	1.00 [1.00, 1.00]	1.00 [1.00, 1.25]	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	$\chi^2(4) = 0.92$, $p = 0.922$
	Placebo	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	1.00 [1.00, 1.75]	1.00 [1.00, 1.00]	$\chi^2(4) = 2.38$, $p = 0.667$
Regurgitation score	Treatment	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	1.00 [1.00, 2.00]	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	$\chi^2(4) = 6.33$, $p = 0.176$
	Placebo	1.00 [1.00, 1.38]	1.00 [1.00, 1.75]	1.00 [1.00, 2.75]	1.00 [1.00, 2.00]	1.00 [1.00, 1.75]	$\chi^2(4) = 6.17$, $p = 0.187$



Supplementary Figure 1. Statistically significant changes in flatulence between baseline (week 0) and end of the study (week 12) following dried chicory root intake (treatment) versus placebo.



Supplementary Figure 2. Alpha-diversity measures over time for the treatment (dried chicory root) and placebo group. Alpha-diversity levels differed between groups for Shannon diversity at weeks 2, 6, 9, and 12 and for phylogenetic diversity at week 6 (indicated by an asterisk *), but their changes over baseline did not differ between groups.

Supplementary Table 4. Changes in mean relative abundances of common taxa (mean relative abundance of at least 1% and mean prevalence in at least 50% of the samples) in the treatment group from baseline (wk 0) over time and their respective fold changes, p-values and fdr-corrected q-values.

taxon	mean relative abundance (%)					foldΔ over baseline				p-value				q-value			
	Wk 0	Wk 2	Wk 6	Wk 9	Wk 12	W0-2	Wk0-6	Wk0-9	Wk0-12	W0-2	Wk0-6	Wk0-9	Wk0-12	Wk0-2	Wk0-6	Wk0-9	Wk0-12
Alistipes	0.49%	0.41%	0.19%	0.25%	0.30%	0.84	0.39	0.51	0.60	0.829	0.242	0.397	0.527	0.975	0.553	0.805	0.898
Anaerostipe**s**	0.98%	2.49%	4.70%	4.27%	3.65%	2.54	4.80	4.37	3.73	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Bacteroides	5.85%	5.58%	4.99%	3.88%	4.22%	0.95	0.85	0.66	0.72	0.448	0.240	**0.035**	0.211	0.849	0.553	**0.326**	0.898
Barnesiella	0.43%	0.36%	0.31%	0.36%	0.30%	0.83	0.72	0.83	0.70	0.835	0.707	0.833	0.683	0.975	0.844	0.922	0.932
Bifidobacteriu**m**	4.54%	9.87%	14.02%	15.71%	18.39%	2.17	3.08	3.46	4.05	**0.013**	**<0.001**	**<0.001**	**<0.001**	**0.244**	**<0.001**	**<0.001**	**<0.001**
Blautia	10.13%	9.97%	8.27%	9.57%	9.36%	0.98	0.82	0.95	0.92	0.414	**0.018**	0.273	0.553	0.849	**0.108**	0.761	0.898
Butyricicoccus	0.48%	0.25%	0.30%	0.25%	0.32%	0.53	0.63	0.51	0.68	0.248	0.333	0.270	0.431	0.849	0.615	0.761	0.898
CAG-56	0.28%	0.21%	0.18%	0.18%	0.22%	0.76	0.65	0.64	0.78	0.777	0.661	0.642	0.801	0.975	0.816	0.922	0.956
Christensenellaceae **R7 group**	0.93%	0.56%	0.37%	0.77%	0.69%	0.60	0.40	0.83	0.74	0.064	**0.006**	0.144	0.931	0.547	**0.056**	0.543	0.957
Coprococcus	1.93%	1.37%	1.08%	1.25%	1.51%	0.71	0.56	0.65	0.78	0.089	**0.005**	**0.032**	0.177	0.547	**0.056**	**0.326**	0.898
Dorea	1.86%	1.73%	1.86%	2.05%	2.14%	0.93	1.00	1.10	1.15	0.518	0.397	0.827	0.569	0.857	0.699	0.922	0.898
Erysipelotrichaceae **UCG-003**	1.12%	0.96%	0.61%	0.68%	0.78%	0.86	0.54	0.61	0.70	0.808	0.188	0.341	0.926	0.975	0.553	0.788	0.957
Eubacterium coprostanoligenes **group unidentified Genus**	1.21%	0.69%	0.57%	0.89%	1.10%	0.57	0.47	0.74	0.91	**0.039**	**0.009**	0.153	0.448	**0.483**	**0.068**	0.543	0.898
Eubacterium eligens **group**	0.35%	0.29%	0.25%	0.28%	0.12%	0.83	0.74	0.82	0.35	0.835	0.739	0.831	0.250	0.975	0.844	0.922	0.898
Eubacterium hallii **group**	1.24%	1.03%	1.08%	1.24%	1.44%	0.83	0.86	1.00	1.15	0.248	0.283	0.872	0.434	0.849	0.553	0.922	0.898
Faecalibacterium	8.53%	8.77%	8.19%	8.77%	8.38%	1.03	0.96	1.03	0.98	0.955	0.447	0.972	0.463	0.975	0.720	0.983	0.898
Fusicatenibacter	1.94%	2.34%	2.12%	2.01%	2.29%	1.21	1.09	1.03	1.18	0.431	0.781	0.983	0.382	0.849	0.850	0.983	0.898
Holdemanella	1.59%	1.12%	0.94%	1.87%	1.09%	0.70	0.59	1.17	0.69	0.428	0.232	0.694	0.545	0.849	0.553	0.922	0.898
Lachnoclostridium	0.37%	0.22%	0.22%	0.15%	0.22%	0.59	0.59	0.41	0.60	0.533	0.534	0.292	0.553	0.857	0.775	0.761	0.898
Lachnospira	0.48%	0.43%	0.47%	0.39%	0.30%	0.90	0.98	0.81	0.62	0.908	0.987	0.823	0.607	0.975	0.987	0.922	0.898
Lachnospiraceae ND3007 **group**	0.82%	0.97%	0.69%	0.72%	0.77%	1.18	0.84	0.87	0.93	0.975	0.545	0.841	0.969	0.975	0.775	0.922	0.969
Lachnospiraceae NK4A136 **group**	0.98%	0.61%	0.47%	0.51%	0.40%	0.63	0.47	0.52	0.41	0.088	**0.020**	0.091	**0.032**	0.547	**0.108**	0.454	**0.292**
Lachnospiraceae **unidentified Genus**	7.69%	7.81%	6.47%	5.70%	6.99%	1.02	0.84	0.74	0.91	0.775	0.143	0.092	0.225	0.975	0.537	0.454	0.898
Monoglobus	0.27%	0.25%	0.25%	0.24%	0.24%	0.91	0.91	0.90	0.89	0.875	0.878	0.865	0.861	0.975	0.916	0.922	0.957

taxon	mean relative abundance (%)					foldΔ over baseline				p-value				q-value			
	Wk 0	Wk 2	Wk 6	Wk 9	Wk 12	W0-2	Wk0-6	Wk0-9	Wk0-12	W0-2	Wk0-6	Wk0-9	Wk0-12	Wk0-2	Wk0-6	Wk0-9	Wk0-12
Ruminococcaceae NK4A214 group	0.30%	0.15%	0.27%	0.25%	0.32%	0.49	0.89	0.82	1.05	0.381	0.661	0.732	0.910	0.849	0.816	0.922	0.957
Oscillospiraceae unidentified Genus	0.19%	0.15%	0.12%	0.16%	0.17%	0.78	0.59	0.82	0.88	0.749	0.494	0.796	0.863	0.975	0.762	0.922	0.957
Parabacteroides	0.58%	0.53%	0.32%	0.19%	0.38%	0.91	0.54	0.32	0.65	0.666	0.255	0.098	0.365	0.975	0.553	0.454	0.898
Phascolarctobacterium	1.38%	1.02%	1.16%	1.00%	1.02%	0.74	0.84	0.72	0.74	0.335	0.284	0.056	**0.026**	0.849	0.553	0.413	**0.292**
Romboutsia	0.51%	0.32%	0.46%	0.48%	0.41%	0.63	0.91	0.94	0.81	0.291	0.753	0.435	0.205	0.849	0.844	0.805	0.898
Roseburia	1.54%	0.73%	0.65%	0.67%	0.84%	0.47	0.42	0.44	0.54	0.203	0.145	0.161	0.302	0.849	0.537	0.543	0.898
Ruminococcus	4.68%	3.12%	2.51%	2.53%	2.26%	0.67	0.54	0.54	0.48	0.290	0.188	0.552	0.115	0.849	0.553	0.922	0.854
Ruminococcaceae UCG-002	1.27%	0.87%	0.77%	1.09%	1.23%	0.68	0.61	0.86	0.97	0.199	0.233	0.432	0.705	0.849	0.553	0.805	0.932
Ruminococcus gauvreauii group	0.69%	0.45%	0.42%	0.44%	0.50%	0.65	0.60	0.64	0.72	0.499	0.428	0.482	0.605	0.857	0.720	0.849	0.898
Ruminococcus torques group	0.95%	0.87%	0.66%	0.65%	0.71%	0.91	0.69	0.68	0.74	0.874	0.072	0.309	0.312	0.975	0.333	0.761	0.898
Senegalimassilia	0.45%	0.29%	0.50%	0.41%	0.47%	0.64	1.12	0.92	1.05	0.405	0.891	0.824	0.669	0.849	0.916	0.922	0.932
Streptococcus	0.66%	0.67%	0.45%	0.31%	0.78%	1.02	0.69	0.47	1.20	0.945	0.655	0.380	0.801	0.975	0.816	0.805	0.956
Subdoligranulum	3.14%	2.80%	3.24%	3.04%	3.16%	0.89	1.03	0.97	1.00	0.459	0.566	0.791	0.787	0.849	0.775	0.922	0.956

Wk 0: baseline, week 0; Wk 2: two weeks of intervention; Wk 6: six weeks of intervention; Wk 9: nine weeks of intervention, Wk 12: end of the study after 12 weeks of intervention; foldΔ: fold-change in mean relative abundance over baseline.

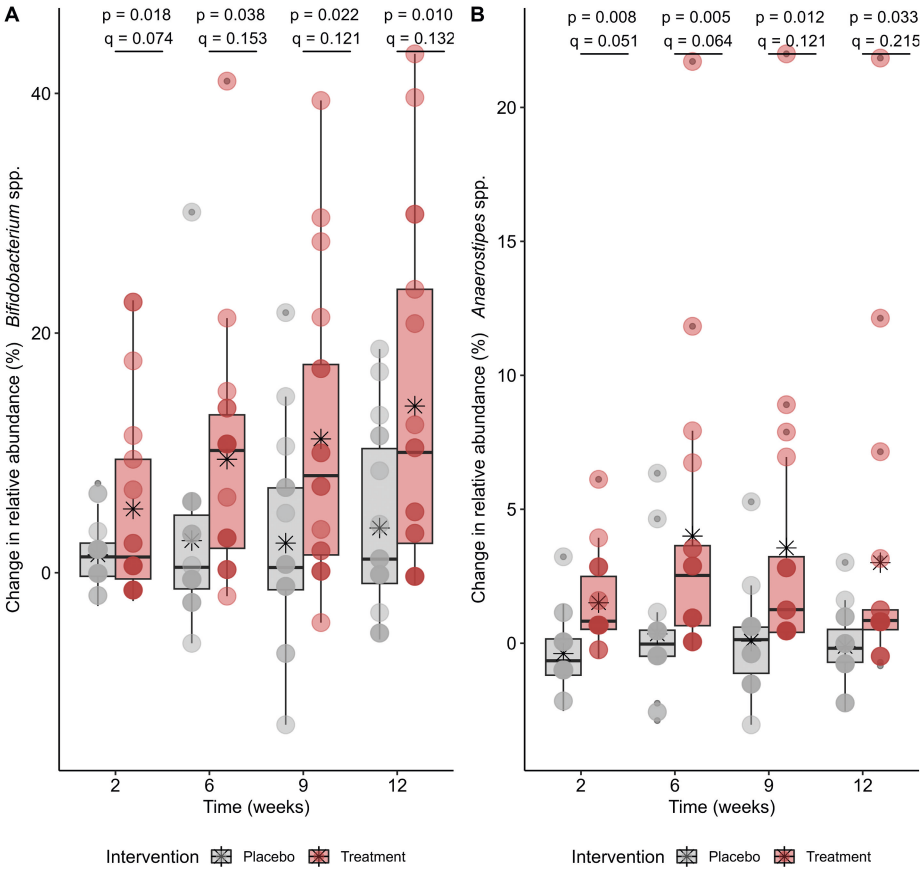
Supplementary Table 5. Changes in mean relative abundances of common taxa (mean relative abundance of at least 1% and mean prevalence in at least 50% of the samples) in the placebo group from baseline (wk0) over time and their respective fold changes, p-values and fdr-corrected q-values.

taxon	mean relative abundance (%)					foldΔ over baseline				p-value				q-value			
	Wk 0	Wk 2	Wk 6	Wk 9	Wk 12	W0-2	Wk0-6	Wk0-9	Wk0-12	W0-2	Wk0-6	Wk0-9	Wk0-12	Wk0-2	Wk0-6	Wk0-9	Wk0-12
Agathobacter	0.28%	0.29%	0.19%	0.18%	0.24%	1.00	0.66	0.64	0.85	0.471	0.192	0.298	0.517	0.997	0.983	0.926	0.982
Alistipes	0.31%	0.25%	0.27%	0.29%	0.32%	0.78	0.87	0.94	1.02	0.483	0.858	0.540	0.743	0.997	0.983	0.926	0.982
Anaerostipes	2.18%	1.79%	2.33%	2.22%	2.06%	0.82	1.07	1.02	0.94	0.300	0.877	0.743	0.747	0.997	0.983	0.926	0.982
Bacteroides	4.24%	4.48%	5.86%	5.06%	5.03%	1.06	1.38	1.19	1.19	0.883	0.443	0.973	0.881	0.998	0.983	0.994	0.982
Bifidobacterium	5.28%	6.78%	7.84%	7.75%	9.06%	1.28	1.48	1.47	1.72	0.669	0.541	0.181	0.091	0.997	0.983	0.926	0.982
Blautia	10.20%	9.46%	8.60%	8.98%	10.22%	0.93	0.84	0.88	1.00	0.325	0.178	0.177	0.909	0.997	0.983	0.926	0.982
Butyricicoccus	0.42%	0.43%	0.38%	0.35%	0.39%	1.03	0.92	0.83	0.92	0.950	0.845	0.662	0.848	0.998	0.983	0.926	0.982

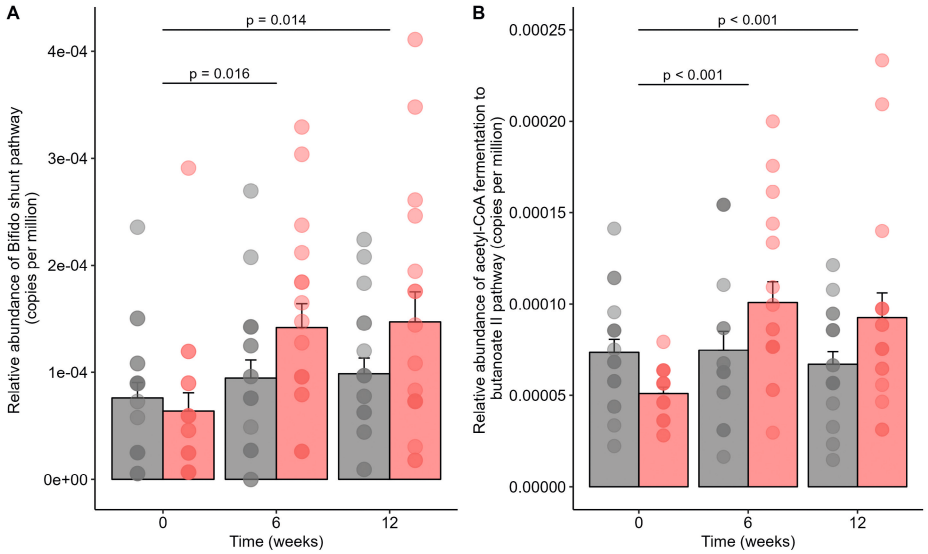
taxon	mean relative abundance (%)					fold Δ over baseline				p-value				q-value			
	Wk 0	Wk 2	Wk 6	Wk 9	Wk 12	W0-2	Wk0-6	Wk0-9	Wk0-12	W0-2	Wk0-6	Wk0-9	Wk0-12	Wk0-2	Wk0-6	Wk0-9	Wk0-12
CAG-56	0.35%	0.33%	0.33%	0.30%	0.33%	0.95	0.95	0.87	0.95	0.603	0.533	0.811	0.594	0.997	0.983	0.926	0.982
Christensenellaceae R7 group	1.23%	1.28%	1.02%	1.14%	0.83%	1.04	0.83	0.93	0.68	0.720	0.968	0.649	0.214	0.997	0.983	0.926	0.982
Clostridia UCG-014 unidentified Genus	0.50%	0.78%	0.50%	0.58%	0.64%	1.56	0.99	1.16	1.27	0.991	0.723	0.866	0.911	0.998	0.983	0.926	0.982
Clostridium sensu stricto 1	0.99%	0.87%	0.58%	0.56%	0.77%	0.88	0.59	0.57	0.78	0.955	0.494	0.555	0.724	0.998	0.983	0.926	0.982
Collinsella	1.82%	2.45%	1.58%	1.61%	1.66%	1.35	0.87	0.88	0.91	0.741	0.874	0.890	0.918	0.997	0.983	0.930	0.982
Coprococcus	1.78%	1.58%	1.33%	1.41%	1.93%	0.89	0.75	0.79	1.08	0.731	0.278	0.187	0.917	0.997	0.983	0.926	0.982
Dorea	1.98%	1.74%	1.66%	1.39%	1.72%	0.88	0.84	0.70	0.87	0.353	0.293	**0.002**	0.336	0.997	0.983	0.086	0.982
Eggerthellaceae unidentified Genus	0.50%	0.58%	0.48%	0.46%	0.51%	1.16	0.96	0.92	1.03	0.798	0.293	0.439	0.314	0.997	0.983	0.926	0.982
Erysipelotrichaceae UCG-003	2.07%	1.82%	1.55%	1.67%	1.50%	0.88	0.75	0.81	0.73	0.200	0.151	0.099	**0.044**	0.997	0.983	0.926	0.982
Eubacterium coprostanoligenes group unidentified Genus	1.03%	1.24%	1.19%	0.72%	1.26%	1.20	1.15	0.70	1.22	0.533	0.785	0.403	0.596	0.997	0.983	0.926	0.982
Eubacterium eligens group	0.46%	0.58%	0.43%	0.37%	0.35%	1.28	0.95	0.81	0.77	0.116	0.944	0.730	0.814	0.997	0.983	0.926	0.982
Eubacterium hallii group	1.59%	1.39%	1.43%	1.45%	1.41%	0.87	0.89	0.91	0.88	0.391	0.389	0.236	0.242	0.997	0.983	0.926	0.982
Eubacterium ventriosum group	0.20%	0.21%	0.20%	0.30%	0.19%	1.08	0.99	1.54	0.97	0.824	0.909	0.581	0.825	0.997	0.983	0.926	0.982
Faecalibacterium	8.39%	9.04%	9.47%	8.66%	7.60%	1.08	1.13	1.03	0.91	0.733	0.966	0.588	0.421	0.997	0.983	0.926	0.982
Fusicatenibacter	2.47%	2.24%	2.26%	2.20%	2.43%	0.91	0.92	0.89	0.98	0.217	0.239	0.261	0.383	0.997	0.983	0.926	0.982
Holdemanella	1.76%	1.39%	1.37%	1.03%	0.92%	0.79	0.78	0.59	0.53	0.937	0.870	0.199	0.212	0.998	0.983	0.926	0.982
Lachnoclostridium	0.34%	0.42%	0.37%	0.32%	0.32%	1.24	1.10	0.94	0.96	0.606	0.793	0.833	0.801	0.997	0.983	0.926	0.982
Lachnospira	0.38%	0.59%	0.39%	0.30%	0.35%	1.57	1.04	0.79	0.94	0.528	0.895	0.625	0.738	0.997	0.983	0.926	0.982
Lachnospiraceae ND3007 group	1.08%	1.20%	0.69%	0.99%	1.07%	1.11	0.64	0.91	0.99	0.686	0.078	0.598	0.814	0.997	0.983	0.926	0.982
Lachnospiraceae NK4A136 group	0.55%	0.71%	0.61%	0.68%	0.64%	1.29	1.10	1.22	1.15	0.918	0.492	0.715	0.895	0.998	0.983	0.926	0.982
Lachnospiraceae UCG-001	0.21%	0.49%	0.33%	0.26%	0.34%	2.28	1.54	1.21	1.59	0.289	0.579	0.802	0.548	0.997	0.983	0.926	0.982
Lachnospiraceae unidentified Genus	6.63%	6.56%	7.84%	6.50%	5.94%	0.99	1.18	0.98	0.90	0.520	0.600	0.386	0.570	0.997	0.983	0.926	0.982

taxon	mean relative abundance (%)					foldΔ over baseline				p-value				q-value			
	Wk 0	Wk 2	Wk 6	Wk 9	Wk 12	W0-2	Wk0-6	Wk0-9	Wk0-12	W0-2	Wk0-6	Wk0-9	Wk0-12	Wk0-2	Wk0-6	Wk0-9	Wk0-12
Monoglobus	0.24%	0.28%	0.22%	0.22%	0.24%	1.15	0.92	0.90	0.99	0.807	0.893	0.856	0.984	0.997	0.983	0.926	0.984
***Muribaculaceae* unidentified Genus**	0.55%	0.68%	0.44%	0.75%	0.71%	1.23	0.79	1.37	1.30	0.530	0.885	0.526	0.624	0.997	0.983	0.926	0.982
***Oscillospiraceae* unidentified Genus**	0.20%	0.16%	0.18%	0.17%	0.19%	0.79	0.93	0.84	0.97	0.610	0.824	0.720	0.954	0.997	0.983	0.926	0.984
Parabacteroides	0.56%	0.41%	0.35%	0.42%	0.44%	0.72	0.62	0.75	0.78	0.363	0.533	0.370	0.476	0.997	0.983	0.926	0.982
Phascolarctobacterium	1.04%	0.85%	0.85%	0.80%	0.72%	0.82	0.81	0.76	0.69	0.552	0.379	0.517	0.061	0.997	0.983	0.926	0.982
Romboutsia	0.57%	0.56%	0.78%	0.78%	0.70%	0.99	1.37	1.37	1.22	0.998	0.983	0.999	0.970	0.998	0.983	0.999	0.984
Roseburia	2.02%	2.33%	2.13%	2.11%	1.75%	1.15	1.05	1.04	0.87	0.669	0.598	0.850	0.617	0.997	0.983	0.926	0.982
***Ruminococcaceae* UCG-002**	1.16%	0.78%	0.78%	0.73%	0.85%	0.68	0.67	0.63	0.74	0.074	0.080	0.079	0.174	0.997	0.983	0.926	0.982
***Ruminococcaceae* UCG-005**	0.27%	0.39%	0.46%	0.33%	0.28%	1.43	1.66	1.19	1.00	0.808	0.884	0.789	0.448	0.997	0.983	0.926	0.982
***Ruminococcaceae* NK4A214 group**	0.45%	0.33%	0.32%	0.35%	0.51%	0.73	0.70	0.78	1.13	0.335	0.552	0.433	0.709	0.997	0.983	0.926	0.982
Ruminococcus	5.82%	4.79%	5.41%	4.27%	3.47%	0.82	0.93	0.73	0.60	0.687	0.806	0.259	0.165	0.997	0.983	0.926	0.982
***Ruminococcus gauvreauii* group**	0.73%	0.49%	0.61%	0.65%	0.53%	0.67	0.84	0.90	0.73	0.501	0.746	0.508	0.861	0.997	0.983	0.926	0.982
***Ruminococcus torques* group**	1.55%	0.86%	1.40%	0.88%	1.18%	0.55	0.90	0.56	0.76	**0.006**	0.328	**0.004**	0.181	0.262	0.983	0.095	0.982
Senegalimassilia	0.38%	0.37%	0.39%	0.38%	0.36%	0.96	1.02	1.01	0.95	0.566	0.303	0.348	0.547	0.997	0.983	0.926	0.982
Streptococcus	0.99%	1.08%	1.32%	2.01%	1.11%	1.10	1.33	2.03	1.12	0.940	0.655	0.273	0.626	0.998	0.983	0.926	0.982
Subdoligranulum	2.54%	3.15%	2.91%	3.32%	3.17%	1.24	1.15	1.31	1.25	0.437	0.882	0.585	0.593	0.997	0.983	0.926	0.982
Sutterella	0.38%	0.26%	0.27%	0.21%	0.27%	0.69	0.72	0.57	0.72	0.266	0.386	0.194	0.353	0.997	0.983	0.926	0.982

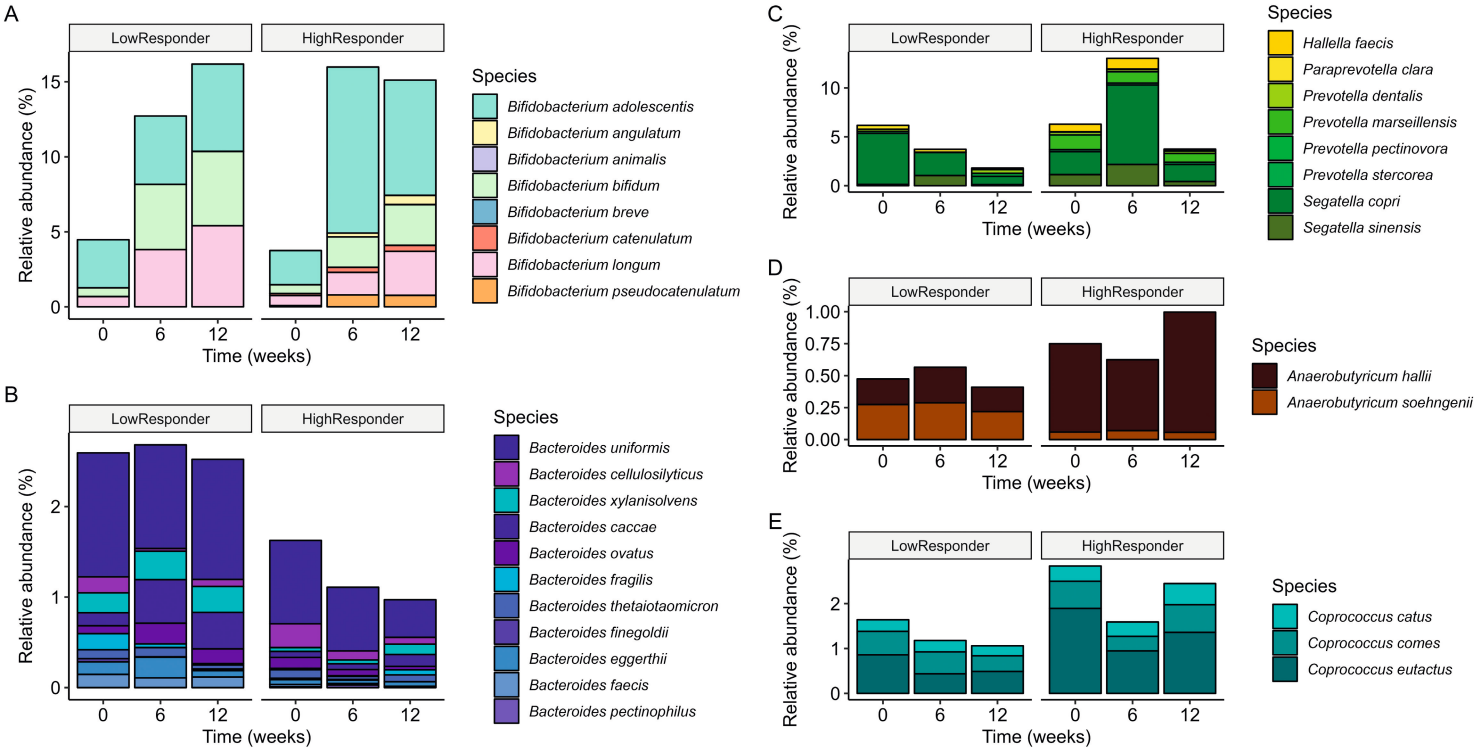
Wk 0: week 0 (baseline); Wk 2: two weeks of intervention; Wk 6: six weeks of intervention; Wk 9: nine weeks of intervention, Wk 12: end of the study after 12 weeks of intervention; foldΔ: fold-change in mean relative abundance over baseline.



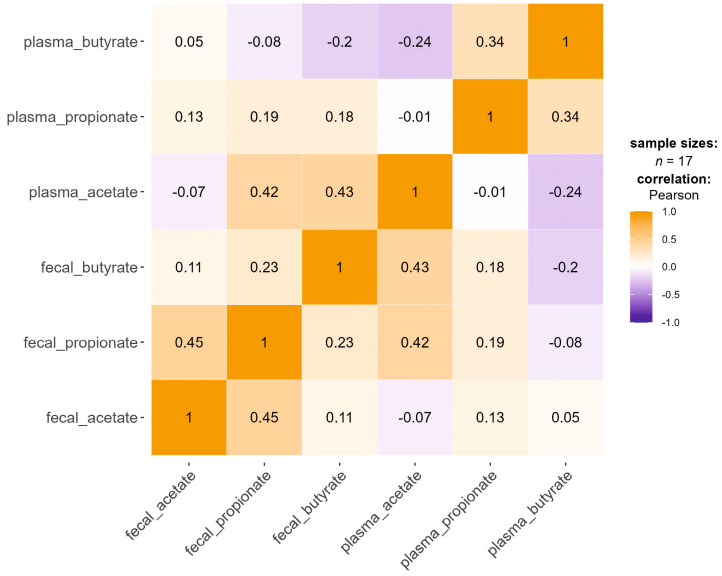
Supplementary Figure 3. Differences in the changes in relative abundances of (A) *Bifidobacterium* spp. and (B) *Anaerostipes* spp. at weeks 2, 6, 9, and 12 compared to baseline between the treatment and placebo groups. The p-values and FDR-corrected q-values are depicted for each comparison of the changes at 2, 6, 9, or 12 weeks.



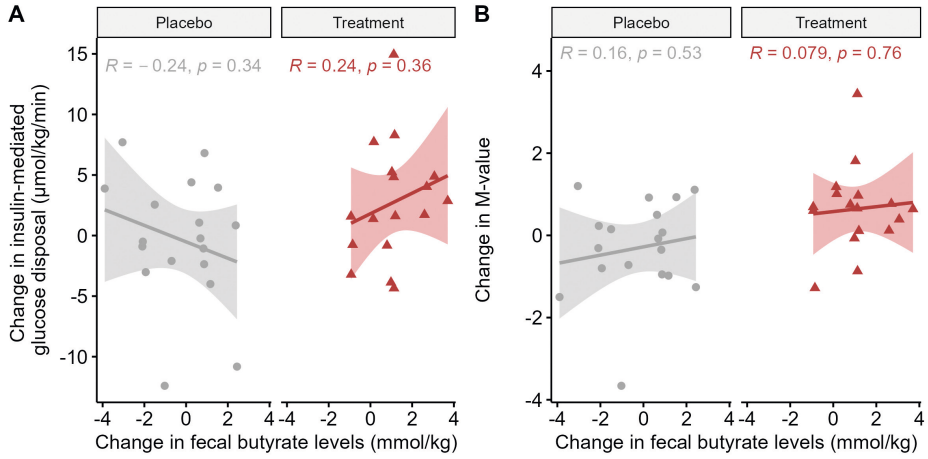
Supplementary Figure 4. Relative levels (copies per million) of the butyrate production pathway (acetyl-CoA fermentation to butanoate II pathway) and the *Bifidobacterium* shunt pathway at baseline (week 0), six and 12 weeks of intervention in the placebo (**black**) and treatment (**red**) groups and the p-values of the statistically significantly differences between the changes from baseline to week six or 12.



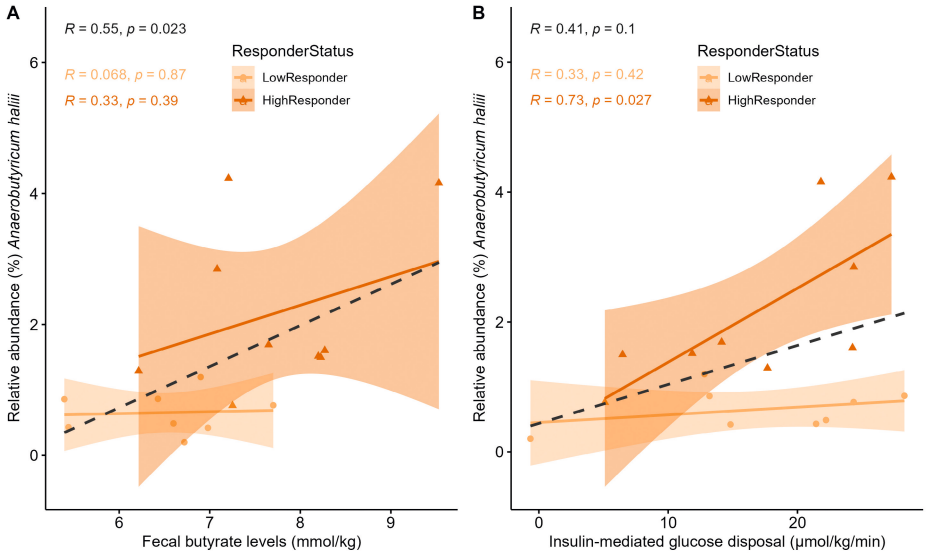
Supplementary Figure 5. Detected species based on taxonomic profiling using metagenomics in low and high responders at baseline (week 0), weeks six, and 12. (**A**) *Bifidobacterium* spp., (**B**) *Bacteroides* spp., (**C**) *Prevotella* spp., (**D**) *Anaerobutyricum* spp. (formerly *Eubacterium hallii group*), (**E**) *Coprococcus* spp..



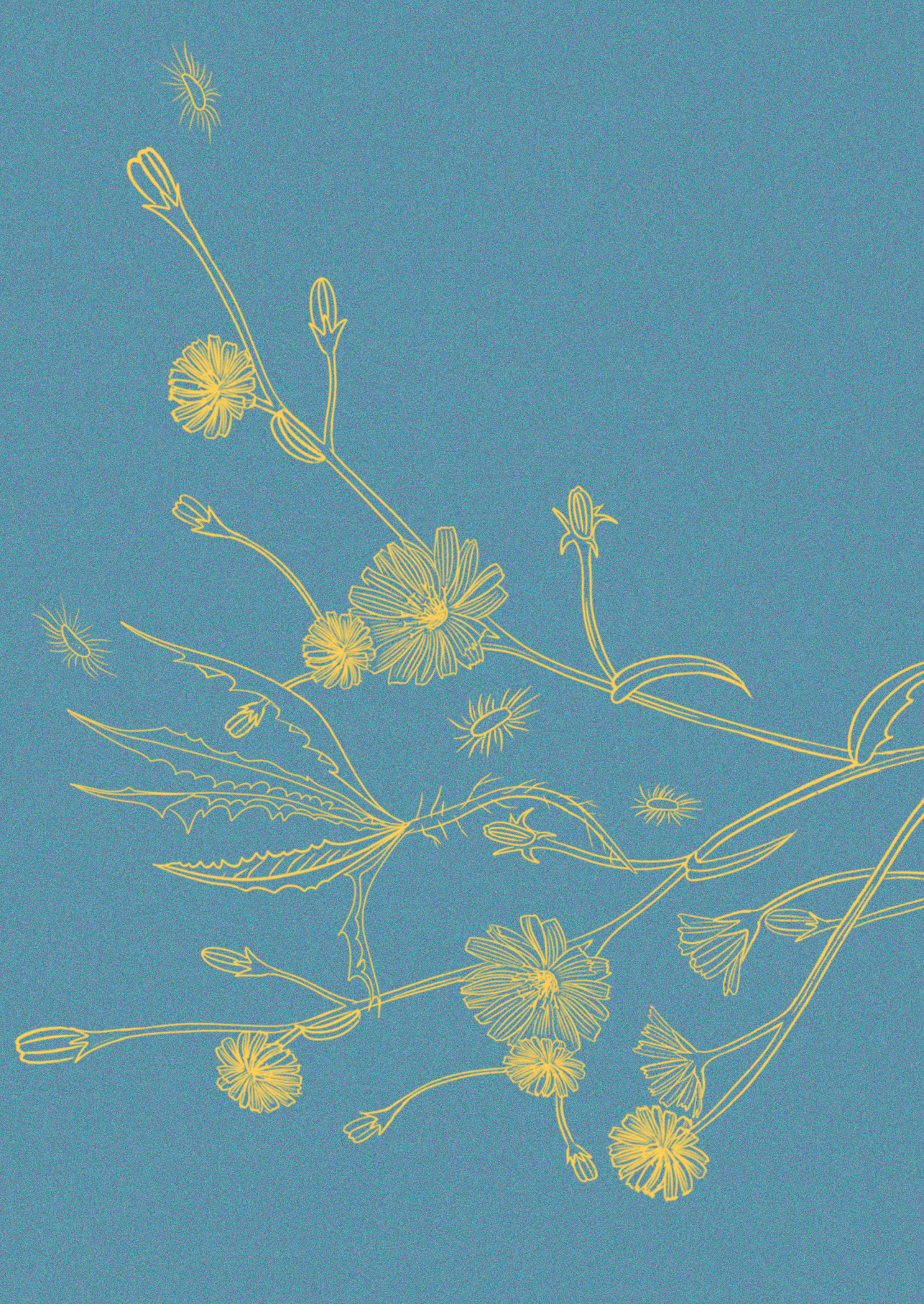
Supplementary Figure 6. Correlations between fecal short-chain fatty acids levels (mmol/kg) and plasma short-chain fatty acids (µmol/L) in the treatment group.



Supplementary Figure 7. Correlations at the intervention level (treatment or placebo group) between changes in (**A**) fecal butyrate levels (mmol/kg) and insulin-mediated glucose disposal as a measure of peripheral insulin sensitivity (µmol/kg/min) (**B**) M-value as a measure of whole-body insulin sensitivity.



Supplementary Figure 8. Correlations in the treatment group between (**A**) fecal butyrate levels (mmol/kg) and relative levels (%) of *Anaerobutyricum hallii* and (**B**) insulin-mediated glucose disposal (µmol/kg/min) as a measure of peripheral insulin sensitivity and relative levels (%) of *Anaerobutyricum hallii*. The dotted black depicts the regression line for the whole treatment group, and the colored lines depict the regression lines for low (light orange) and high responders (dark orange).



CHAPTER 7

Findings of a randomized, double-blind, placebo-controlled study to evaluate the effects of inulin on bowel habit and fecal microbiota in adults with functional constipation

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In preparation for submission

ABSTRACT

Functional constipation is a common disorder of the gut-brain interaction characterized by infrequent bowel movements and hard stools, which substantially affects patients' quality of life. Supplementation with gut microbiome-targeted prebiotics is a promising non-pharmacological alternative to current treatments. In a randomized, double-blind, placebo-controlled, cross-over trial, we investigated the effect of four-week daily 12 g inulin intake in 39 individuals with functional constipation according to Rome III Criteria, mainly females. We assessed stool frequency and consistency, constipation-related quality of life (PAC-QOL) and symptoms (PAC-SYM), and fecal microbiota composition using 16S rRNA gene sequencing. After inulin intake, we observed larger changes in stool frequency, abdominal symptoms, and particularly social and emotional well-being related to quality of life, compared to placebo. Additionally, relative abundances of putative butyrate-producing *Anaerostipes* spp. and *Coprococcus* 1 spp. were higher. Further investigation, however, indicated a possible carry-over. To this end, half of the participants receiving inulin in the first period had a remarkable placebo response in the second period. These participants had the largest improvements in all outcomes during inulin intake and higher baseline and wash-out relative abundances of butyrate-producing *Faecalibacterium* spp. and *Roseburia* spp., and lower but more responsive *Bifidobacterium* spp. relative abundances. To address carry-over induced bias, we analyzed only the first period as a parallel trial and confirmed the observed positive effect of inulin, also affirming its established bifidogenic effect. In conclusion, daily intake of 12 g inulin has the potential to improve functional constipation by modulating the fecal microbiota towards higher relative abundances of butyrate-producing genera.

Key words: non-pharmacological treatment, cross-over effect, prebiotics, gut microbiome, disorders of gut-brain interaction, gut-brain axis

INTRODUCTION

The connection between the gut and the brain is increasingly acknowledged for its substantial impact on human health. These two organs appear to communicate bidirectionally through anatomic, endocrine, and immune pathways, influencing both gut functionality and brain activity. To this end, functional gastrointestinal disorders have recently been reclassified in 2016 as disorders of gut-brain interaction (DGBI) (Drossman & Hasler, 2016; Drossman & Tack, 2022). These disorders differ from organic disorders and motility disorders (Drossman, 2016) as they cannot be diagnosed by pathological changes in organ structure (morphology) or changes in organ function, notably motility dysfunction (altered muscle activity). Instead, their diagnosis is based on the symptoms reported by the patient, as well as the duration, frequency, and interference of these symptoms with the patient's daily life (Drossman, 2016; Drossman & Tack, 2022). Affected patients experience changes in various physiological processes, including central nervous processing, perception of mechanical triggers in the bowel (visceral hypersensitivity), immune and gut barrier function, motility response, digestion of food compounds, and the gut microbiome (Drossman, 2016).

A common DGBI is functional constipation. Functional constipation affects people of all ages and is characterized by infrequent bowel movements, difficult and/or painful passage of (hard) stools. It is estimated that up to eight out of ten adults are affected by constipation-related complaints, although the actual prevalence may vary depending on the definition used for the disorder (Chen et al., 2022; Koppen, Vriesman, et al., 2018; Mugie et al., 2011; Vriesman et al., 2019). The most widely accepted definition is that of the Rome Foundation, which has published a set of diagnostic guidelines, called the Rome criteria (Drossman & Hasler, 2016; Hyams et al., 2016; Schmulson & Drossman, 2017). The most recent version of these guidelines, the Rome IV criteria, differ from the previous version (Rome III) in that criteria 7 and 8 (see Box 1) can be used as part of the two diagnostic criteria for diagnosis instead of needing to be present in addition to two of the other six criteria (Miller et al., 2017). Additionally, the disorder is considered to exist on a continuum with other DGBI rather than treated as separate categories (Schmulson & Drossman, 2017).

Box 1. Rome IV criteria for functional constipation in adults.

FUNCTIONAL CONSTIPATION according to Rome IV criteria
*Diagnostic criteria**
Must include **two or more** of the following:

1. Straining during more than ¼ (25%) of defecations
2. Lumpy or hard stools (Bristol Stool Form Scale 1-2) more than ¼ (25%) of defecations
3. Sensation of incomplete evacuation more than ¼ (25%) of defecations
4. Sensation of anorectal obstruction/blockage more than ¼ (25%) of defecations
5. Manual maneuvers to facilitate more than ¼ (25%) of defecations (e.g., digital evacuation, support of the pelvic floor)
6. Fewer than three stool bowel movements per week
7. Loose stools are rarely present without the use of laxatives
8. Insufficient criteria for irritable bowel syndrome

*Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis

Similar to other DBGI, the etiology of functional constipation is still poorly understood. It is likely caused by a combination of factors, including psychological factors such as stress, lifestyle factors, genetic predisposition, motility disturbances, and the gut microbiome (Vriesman et al., 2019). International guidelines for the treatment of functional constipation recommend lifestyle interventions such as adequate fiber and fluid intake and regular physical activity, along with pharmacological interventions such as osmotic laxatives (Bardisa-Ezcurra et al., 2010; Serra et al., 2020). Despite the availability of these pharmacological treatments, one-third of the patients are not satisfied with their treatment, according to a large survey held in 2013 (Müller-Lissner et al., 2013). Consequently, patients seek alternative or complementary, non-pharmacological treatments. Besides, commonly used polyethylene glycol-based osmotic laxatives have been demonstrated in a humanized mouse model to induce lasting changes in the gut microbiota and the immune response, as well as to impair the mucus layer's barrier function (Tropini et al., 2018). In addition, laxative use has been linked to decreased relative abundances of putative butyrate-producing genera, as reviewed elsewhere (Weersma et al., 2020). As both gut microbiome and altered immune and barrier function are involved in the pathogenesis of functional constipation and other DGBI (Drossman, 2016), such disadvantages of laxatives warrant the need to investigate non-pharmacological treatment alternatives. Ideally, potential treatment alternatives are compounds that address all these aspects by modulating the existing gut microbiota, and simultaneously exerting immunomodulatory and gut barrier-strengthening effects, conferring benefits on the gut-brain interaction. Dietary fiber supplementation may be one such non-pharmacological treatment option, particularly as low fiber consumption has been linked to an increased incidence of constipation (Suares & Ford, 2011). Additionally, alterations in fecal microbiota composition have been reported in individuals with hard stools and low transit times (Asnicar et al.,

2021; Vandeputte et al., 2016), possibly due to intraluminal conditions related to low nutrient (fiber) availability. Therefore, incorporating fiber supplementation in the management of functional constipation could not only offer substantial clinical benefits but also modulate underlying factors associated with the gut microbiota. Notably, a dose-response analysis of the collectively available data conducted in 2013 revealed that increasing dietary fiber intake was linked to significant cost savings in medical expenses for constipation patients, potentially exceeding $12 billion annually among adults in the US (Schmier et al., 2014).

Since dietary fibers are not digested by human endogenous enzymes, they pass through the upper gastrointestinal tract and reach the lower gut, where they affect bowel function in various ways, primarily related to their physicochemical properties (McRorie & McKeown, 2017). Some fibers, such as psyllium, retain water and thereby soften stools, contributing to fecal bulk and softer stools. Others, such as wheat bran, lead to a similar effect not due to water-holding, but through their ability to stimulate the secretion of mucus and fluids (McRorie & McKeown, 2017). Also, fermentable fibers, such as pectin or inulin, do not exert their effect by holding water, but by serving as a substrate for the gut microbiota (McRorie & McKeown, 2017). The microbiota is a collective of primarily bacteria, viruses, fungi, protozoa, and archaea residing in the human gut, which is as unique to individuals as their fingerprints. During fiber fermentation, gut bacteria produce various compounds that have been shown to impact gut motility (Waclawiková et al., 2022). Certain fermentable dietary fibers are of particular interest, as they have the potential to selectively modulate the fecal microbiota and thereby confer health benefits. Such fibers are called prebiotics, as defined by the International Scientific Association of Pro- and Prebiotics (Gibson et al., 2017). One well-established prebiotic is inulin, an intracellular storage carbohydrate found in high amounts in chicory roots (Vandeputte et al., 2017). Inulin is known for its bifidogenic effects, meaning its consumption leads to increased levels of bifidobacteria (Le Bastard et al., 2019; Roberfroid et al., 1998). Moreover, extracted and isolated native chicory root inulin currently holds a European Food Safety Authority (EFSA) approved health claim for the maintenance of normal defecation by increasing stool frequency (EFSA, 2015). In addition to inducing specific changes in the fecal microbiota composition and maintaining stool frequency, several studies have indicated that inulin also has the potential to alleviate constipation symptoms, as reviewed elsewhere (Ahmed & Rashid, 2019). Inulin has also been demonstrated in animal and *in vitro* models to exhibit immune-modulatory functions and improve barrier functionality (Corrêa et al., 2023; Uerlings et al., 2020; Watzl et al., 2005). Most recently, inulin supplementation has also been demonstrated to affect aspects of brain function, such as cognition (Ni Lochlainn et al., 2024) and food decision-making (Medawar et al., 2024), as well as neurological biomarkers in Parkinson's disease (Hall et al., 2023), which suggests a beneficial impact on the gut-brain interaction. Therefore, inulin is a potentially attractive non-pharmacological treatment that may alleviate symptoms of functional constipation, improve quality of life, and promote gut health in such a DGBI.

Here, we aimed to investigate the effect of a four-week daily intake of 12 g inulin versus placebo on stool frequency and stool consistency, constipation-related quality of life and symptoms, resort to laxatives, physical activity, and fecal microbiota composition in adults with functional constipation. The study was designed as a randomized, placebo-controlled, cross-over trial with two arms to assess individual responses to inulin and placebo within each individual, accounting for the unique composition of their gut microbiota, which is expected to influence any potential effects.

MATERIALS AND METHODS

STUDY DESIGN

This study was designed as a randomized, double-blind, placebo-controlled, cross-over trial conducted in adults with functional constipation, in which we compared a four-week period of daily intake of 12 g of inulin against a placebo intake (maltodextrin) with a four-week wash-out period separating the two intervention periods. The primary outcome was the change in stool frequency after treatment compared to baseline. Ethical approval was obtained from the Cork Research Ethics Committee of the Cork Teaching Hospitals (Reference ECM 4 (v) 01/09/15), and the trial design was registered at ClinicalTrials.gov (NCT05447481). The study was conducted from 2015 to 2016 at Atlantia Clinical Trials, Cork, Ireland, and was executed in accordance with the principles of the Declaration of Helsinki and in compliance with the International Council for Harmonization Good Clinical Practice.

PARTICIPANTS

Study participants were recruited through the clinical research organization's database, general practitioners' offices, hospital clinics, and adverts in local newspapers in the surroundings of Cork, Ireland. Written informed consent was obtained from all study participants prior to their participation. To be eligible for the study, participants had to be between 18 and 75 years old and diagnosed with functional constipation according to the Rome III criteria (Drossman, 2006). Individuals were excluded if they had hypersensitivity to any of the test product components or an acute or chronic, unstable, and untreated disease or any condition that contra-indicated entry to the study. Additionally, a history of laxative use, drug and/or alcohol abuse, and intake of any probiotic or prebiotic product or supplement within two weeks of the screening visit resulted in exclusion from the study. Lastly, women who were pregnant, lactating, or wished to become pregnant during the study were not included. Participants were evaluated to be in good general health, as determined by the investigator, and asked to continue their normal diet while avoiding pro- or prebiotic products/supplements or dietary fiber supplements for the duration of the study.

STUDY PROCEDURE

Potential participants were screened for eligibility, and eligible individuals entered a two-week run-in period, after which they were randomized into either the treatment-placebo sequence or the placebo-treatment sequence. Participants received as treatment 12 g inulin (Frutafit® HD native chicory inulin, Sensus B.V., Roosendaal, the Netherlands) and as placebo maltodextrin (MD20, Avebe, Foxhol, the Netherlands) for four weeks. The first intervention period (either treatment or placebo) was followed by a four-week wash-out period before participants crossed over into the second intervention period (Figure 1). The intervention products were consumed in two doses of 6 g per day, except for the first three days of each intervention period when participants only took one dose of 6 g per day. Digestible maltodextrin was chosen as the placebo as it is easily broken down in the upper gastrointestinal tract by human endogenous enzymes (Hofman et al., 2016), making it suitable for interventions expected to be mediated by the lower gut (colonic) microbiota. The study products were similar in flavor, appearance, and packaging, and both participants and research personnel involved in this study were blinded to ensure true allocation concealment. The study products were provided in dark plastic 120 ml bottles. Participants were instructed to add 60 ml of water to the bottle and shake until the study product had dissolved. As a marker of compliance, participants were asked to return all used and unused bottles, and compliance was calculated by counting the actual number of empty bottles returned and dividing it by the expected number of bottles used. Each participant received 60 bottles, with 53 as the total expected number to be used to be 100% compliant. Participants received all bottles of the respective intervention product (inulin or placebo) on day 0 for each four-week intervention period (period 1 or 2). During each intervention period, participants filled in a daily bowel diary in which all outcomes were assessed, including stool frequency, stool consistency, and constipation complaints according to Rome III criteria. At the end of each intervention period, participants also completed three questionnaires covering aspects of constipation-related quality of life and symptoms as well as physical activity. Finally, participants were asked to provide a fecal sample during each of the intervention periods (Figure 1), as a proxy of changes in the gut microbiota composition. Other measurements were blood pressure, heart rate, and temperatures and anthropometric measures such as weight, height, and body mass index (BMI), as well as medication use, which were all taken before the start of the run-in and at the end of each study period (run-in, intervention period 1, wash-out, intervention period 2).

OUTCOME ASSESSMENTS

The primary outcome measure was defined as the change in the number of bowel movements per week from a two-week baseline to the final two weeks of inulin intake, compared to the change from a two-week baseline to the final two weeks of placebo intake. Secondary outcomes included differences between changes after inulin compared to placebo intake in the following parameters: (1) stool consistency, measured using the Bristol Stool Form Scale (BSFS); (2) quality of life related to

constipation, assessed by Patient Assessment of Constipation – Quality of Life (PAC-QOL) scores; (3) constipation-related symptoms, evaluated by Patient Assessment of Constipation – Symptoms (PAC-SYM) scores; (4) use of laxatives; (5) physical activity, determined using the International Physical Activity Questionnaire (IPAQ); and (6) fecal microbiota composition.

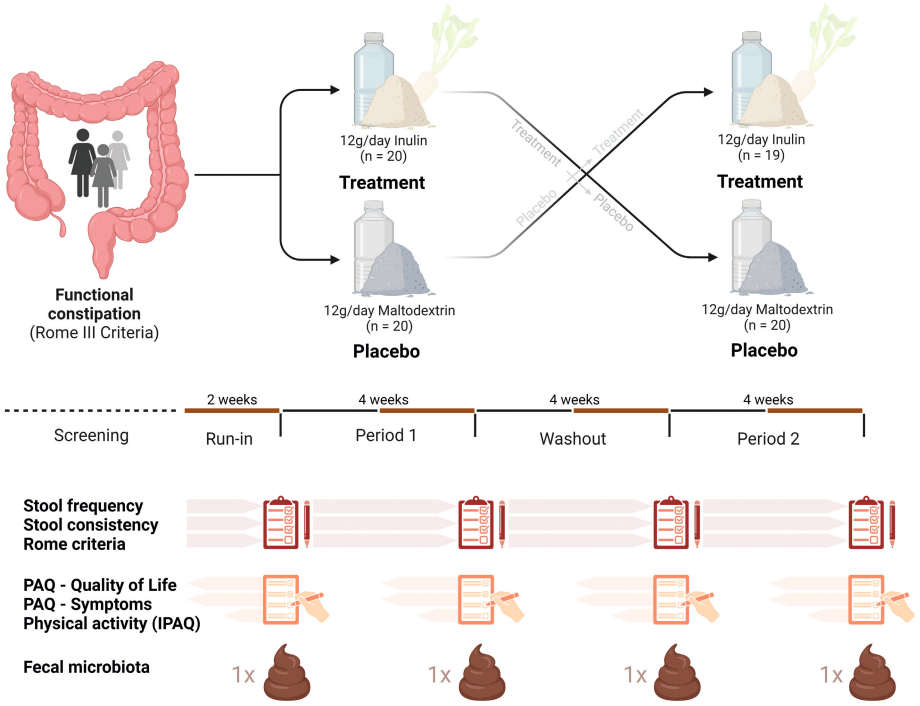


Figure 1. Study design. Overview of the randomized, placebo-controlled cross-over study design and study measurements.

Stool frequency, stool consistency, and resort to laxatives

Stool frequency, stool consistency, Rome criteria, and resort to laxatives were measured by using daily bowel habit diaries. Participants completed daily bowel habit diaries, reporting the number of bowel movements they had and the consistency of the stool using the BSFS.

Patient assessment of constipation – quality of life and symptoms

The PAC-QOL is a retrospective questionnaire with a recall period of two weeks, comprising 28 items scored on a 5-point scale from 0-4, and designed to assess the patient-reported quality of life and the impact of constipation symptoms (Marquis et al., 2005). It generates an overall score and four subscores, including worries/concerns (11 items), physical discomfort (four items), psychosocial discomfort (eight items), and satisfaction (five items). Similarly, the PAC-SYM is a retrospective questionnaire with

a two-week recall period, consisting of 12 items (scored on a 5-point scale from 0-4) assessing the severity of patient-reported symptoms (Frank et al., 1999). This tool also produces an overall score and three subscores, focusing on abdominal symptoms (four items), rectal symptoms (three items), and stool symptoms (five items). In both the PAC-SYM and PAC-QOL, lower scores on the overall score or subscore indicate less severe symptoms and a higher quality of life, respectively.

Physical activity

The IPAQ is a retrospective questionnaire with a recall over the past seven days, comprising 27 questions to evaluate health-related physical activity (Craig et al., 2003). The questions address types of vigorous and moderate physical activity and time spent thereon and are grouped into five parts (job-related, transportation, housework/ house maintenance/ recreation/sport/leisure-time physical activity and time spent sitting). Outcomes are summarized either in three categories of physical activity, as implemented here, which are "high" (one hour of moderate-intensity physical activity or more per day), "moderate" (half an hour of moderate-intensity physical activity on most days) and "low" (not meeting the other categories' criteria) or can be further converted taking energy requirements into account.

SAMPLE SIZE CALCULATION AND RANDOMIZATION

To detect a minimal difference of 1 bowel movement per week between inulin treatment and placebo with a standard deviation (SD) of 2, a total sample size of 39 participants was required. The sample size was calculated using a power of $\beta = 80\%$ and a significance level of $\alpha = 5\%$ based on a two-sided Wilcoxon non-parametric test and takes a drop-out rate of 10% into account. The participants were randomized equally into the treatment-placebo or placebo-treatment arm by an independent statistician, using the uniform random number function in SPSS (IBM Corp. IBM SPSS Statistics for Windows. Armonk, NY: IBM Corp). After an initial blinded data analysis for internal use, the data was unblinded, and the data analysis was performed unblinded.

FECAL MICROBIOTA ANALYSIS

During the study, participants were asked to provide a fecal sample during the run-in period and at the end of each intervention period and the wash-out (Figure 1). Samples were collected at home, stored in home freezers, and then transferred to the study center in cooler bags with a frozen ice pack. The samples were then stored at -20 °C before being processed. DNA was extracted from fecal samples using a repeated bead-beating step and the Maxwell® 16 instrument (Promega, Leiden, The Netherlands). First, 0.25 g of fecal material was added to a bead-beating tube with 700 µl Stool Transport and Recovery (STAR) buffer, 0.5 g of sterilized zirconia beads (0.1 mm), and five glass beads (2.5 mm). These tubes, containing the fecal sample, were bead-beaten three times (60 s × 5.5 ms) and incubated for 15 min at 95 °C at 300 rpm. Samples were then centrifuged for 5 min at 4 °C, and 14,000 g. The resulting supernatants were transferred

to sterile tubes, and the procedure was repeated on the remaining pellets using 300 µl STAR buffer. Both supernatants were pooled, and DNA purification was performed with a customized kit (AS1220; Promega) using 250 µl of the final supernatant pool. DNA was eluted in 50 µl of DNAse- and RNAse-free water, and its concentration was measured using a DS-11 FX+ Spectrophotometer/Fluorometer (DeNovix Inc., Wilmington, USA). The V4 region of the 16S ribosomal RNA (rRNA) gene was amplified in duplicate PCR reactions for each sample in a total reaction volume of 50 µl. The primers used were 515F (5'-GTGTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and 806R (5'-CCGGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015). The master mix contained 1 µl of a unique barcoded primer, 515F-n and 806R-n (10 µM stock concentration), 1 µl dNTPs mixture (200 µM), 0.5 µl Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/µl; Thermo Scientific, Landsmeer, The Netherlands), 10 µl 5× Phusion Green HF Buffer, and 36.5 µl DNAse- and RNAse-free water. The amplification program included 30 s of an initial denaturation step at 98 °C, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 10 s, elongation at 72 °C for 10 s, and a final extension step at 72 °C for 7 min. The PCR product was visualized in 1% agarose gel (~290 bp) and purified with CleanPCR kit (CleanNA, Alphen aan den Rijn, The Netherlands). The concentration of the purified PCR product was measured with Qubit dsDNA BR Assay Kit (Invitrogen, California, USA), and 200 ng of microbial DNA from each sample was pooled for the creation of the final amplicon library, which was sequenced (150 bp, paired-end) on the Illumina HiSeq 2000 platform (GATC Biotech, Constance, Germany). For quality control purposes, two in-house assembled mock communities were included in the library and compared to their theoretical composition (MC3 and MC4; (Ramiro-Garcia et al., 2018)). Additionally, a negative control of the DNA extraction and purification procedure and a water blank were included. The 16S rRNA gene amplicon sequences have been submitted to the European Nucleotide Archive under PRJEB75434.

DATA ANALYSIS

Data was analyzed according to the assigned sequence allocation at baseline, and available data from participants who dropped out or provided incomplete data were included in the analysis. Clinical outcomes were analyzed using R (version 4.0 or higher) (R Core Team, 2023), and the packages tidyverse (Wickham et al., 2019), ggstatsplot (Patil, 2021), ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2023a), lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), emmeans (Lenth, 2023), rstatix (Kassambara, 2023b) and tableone (Yoshida & Bartel, 2022). Outcomes recorded in the bowel habit diaries, being stool frequency, stool consistency, and resort to laxatives, were summarized over the last two weeks of each period (run-in, period 1, wash-out, and period 2) and averaged to reflect measurements per week (e.g., stool frequency as bowel movements per week). Total and subscores of the PAC-QOL and PAC-SYM were calculated as previously described (Frank et al., 1999; Marquis et al., 2005) and covered the same two-week periods as the bowel habit outcomes. Changes in all these outcomes were then assessed by calculating the difference between the average of each

intervention period (period 1 or period 2; post-intake levels) and that of each respective baseline (run-in for period 1 and wash-out for period 2; pre-intake levels). Normality was checked using QQ-plot, and outcomes were summarized using parametric descriptive statistics, except stool frequency, which was also summarized using nonparametric statistics for comparability with other studies. Physical activity was calculated and expressed as categories "low", "moderate," and "high", as previously described (Craig et al., 2003) and analyzed as categorical data. All other outcomes (blood pressure, heart rate, anthropometry) were summarized at baseline using descriptive statistics. Inference of statistical significance was made at $\alpha = 0.05$.

The main analysis consisted of assessing whether the change during treatment (inulin post-pre intake) differed from that during placebo intake (maltodextrin post-pre intake), for which we used linear mixed modeling. Time (pre vs. post) and intervention (inulin vs. placebo) were included as fixed effects, and individual was included as random effect. The change was modeled as a fixed effect by the interaction term *intervention*time*. To model potential carry-over, we also included the interaction term *intervention*period* (period 1 vs. period 2), indicating whether the effect of the intervention is impacted by the period, thus reflecting differences between sequences. Including the *intervention*period* interaction term in the linear mixed models improved the models according to the Akaike information criterion (AIC) and was found to be statistically significant for some outcome assessments, although not all. Based on this finding, we summarized and visualized outcome measures for each study period (run-in, period 1, wash-out, period 2) according to the intervention sequences (treatment-placebo and placebo-treatment). Building on these observations, we identified differences in response to the placebo, particularly notable for the primary outcome (stool frequency) within the treatment-placebo sequence. Further sub-group analysis was then conducted based on the differentiation between high (stool frequency of >3x/week) and low (stool frequency of <=3x/week) responses to placebo, which allowed us to gain additional insights into the observed differences.

The presence of a significant *intervention*period* term indicates a potential bias due to carry-over, which might weaken the intervention effect. In addressing data analytical challenges related to potential carry-over in cross-over designs, various approaches have been proposed and equally fueled criticism (Jones & Kenward, 1989; Senn, 2002). One common approach is the analysis of the first period as a parallel trial, although recognized for its tendency to increase the likelihood of type 1 errors (false-positives) (Senn, 2002). To mitigate this risk, applying a stricter α-level, such as 0.005, has been proposed (Senn, 2002) However, all these approaches have been acknowledged not to provide a comprehensive solution (Jones & Kenward, 1989; Senn, 2002). In our study, acknowledging the limitations of existing approaches, we incorporate the first-period analysis to function as a sensitivity analysis alongside the cross-over analysis. This decision was informed by the presence of the significant *intervention*period* interaction for some outcomes, though not all, and by the smaller number of samples available in period 2 for the assessment of the mediating role of the fecal microbiota. The first

period was, hence, analyzed by assessing the difference in change reflected in the *intervention*time* interaction between inulin and placebo in the first period using the same linear mixed model approach as for the cross-over analysis.

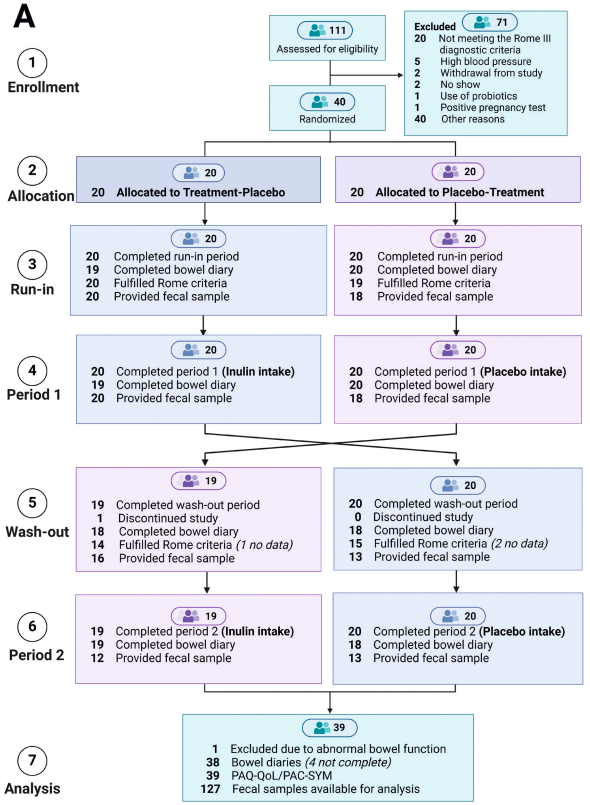
For fecal microbiota composition data analysis, data filtering and taxonomy assignment of the 16S rRNA gene amplicon sequences were performed using the NG-Tax pipeline with default settings (Poncheewin et al., 2020). A table based on Amplicon Sequence Variants (ASVs) was created for each sample and taxonomically annotated using the SILVA 138 database (Quast et al., 2013). Low abundance ASVs were discarded, using a minimum relative abundance threshold of 0.1% (Poncheewin et al., 2020; Ramiro-Garcia et al., 2018). Fecal microbiota composition and changes in individual taxa were analyzed using the microViz (Barnett et al., 2021) and the microbiome (Lahti & Shetty, 2019) packages, which use the phyloseq (McMurdie & Holmes, 2013) and the vegan (Oksanen et al., 2022) packages. Moreover, the mare package (Korpela, 2016) was used, which relies on the vegan package, the MASS (Venables & Ripley, 2002) and glmmADMB (Fournier et al., 2012; Skaug et al., 2016) packages. Microbiota composition was compared by calculating β-diversity based on Bray-Curtis dissimilarity, a measure of between-sample variation. Differences in β-diversity were assessed using permutational analysis of variance (PERMANOVA). Differences in relative abundance of individual taxa at pre- and post-intake and changes for both the cross-over as well as the responder analysis and analysis of the first period only were calculated as implemented in mare, and details of such analyses have been previously described elsewhere (Puhlmann et al., 2022). In short, the mare package includes the individual as random factor in the repeated measure analysis and offers the possibility of analyzing data using zero-inflated negative binominal models as well as models excluding samples where respective taxa are not observed (non-zero models).

RESULTS

PARTICIPANT CHARACTERISTICS AND ADVERSE EVENTS

Forty participants were randomly assigned to the study, with 20 allocated to the treatment-placebo sequence and 20 to the placebo-treatment sequence (Figure 2A). One participant in the placebo-treatment sequence dropped out after the wash-out period. A second participant in the placebo-treatment sequence was excluded due to inexplicable spontaneous improvements in symptoms. Additionally, five participants provided incomplete bowel habit data. However, data from the PAC-QOL, PAC-SYM, and IPAQ questionnaires were available from all participants who completed the study, and all available data was included in the data analysis. Thirty-eight of the 40 participants provided a fecal sample at baseline and 38 after period 1, but only 29 provided a fecal sample after wash-out and 15 after period 2. No differences at baseline were detected between participants in the treatment-placebo sequence and those in the placebo-treatment sequence (Figure 2B). Participants were primarily females, and all fulfilled the Rome III criteria at run-in.

A



B

Baseline characteristics	Total (n = 39)	Treatment-Placebo sequence (n = 20)	Placebo-Treatment sequence (n = 20)	p-value
Sex female, n (%)	36 (92.3%)	18 (90.0%)	18 (94.7%)	
male, n (%)	3 (7.7%)	2 (10.0%)	1 (5.3%)	1.00
Age, years	37.00 [18.00, 66.00]	36.50 [18.00, 59.00]	37.00 [19.00, 66.00]	0.855
BMI, kg/m^2	24.71 [20.29, 43.43]	24.22 [21.86, 27.87]	25.58 [20.29, 43.43]	0.227
Fulfilling Rome III criteria, n(%)	39 (100.0%)	20 (100.0%)	19 (100.0%)	n.a.
Systolic blood pressure, mm Hg	113.56 (13.22)	112.11 (12.72)	115.18 (13.97)	0.495
Diastolic blood pressure, mm Hg	74.19 (8.35)	74.63 (9.08)	73.71 (7.70)	0.745
Pulse, bpm	74.92 (10.70)	73.00 (11.77)	77.06 (9.23)	0.262
Temperature, °C	36.27 (0.31)	36.28 (0.32)	36.26 (0.30)	0.852
Height, cm	165.90 (8.32)	165.25 (8.46)	166.58 (8.35)	0.625
Weight, kg	69.72 (11.62)	66.89 (9.25)	72.69 (13.29)	0.12
IPAQ score, High, n(%)	6 (15.4%)	3 (15.0%)	3 (15.8%)	
IPAQ score Low n(%)	9 (23.1%)	5 (25.0%)	4 (21.1%)	0.958
IPAQ score Moderate n(%)	24 (61.5%)	12 (60.0%)	12 (63.2%)	
PAQ-QOL, total score	1.97 (0.60)	2.04 (0.62)	1.89 (0.60)	0.429
PAQ-SYM, total score	1.94 (0.56)	1.98 (0.53)	1.90 (0.60)	0.661
Stool frequency, x/week	2.00 [1.00, 3.00]	2.00 [1.50, 3.00]	2.00 [1.00, 2.50]	0.963
Stool consistency, BSFS	1.87 (0.50)	1.92 (0.53)	1.82 (0.47)	0.561

Figure 2. Participants in the cross-over study. (A) Consort statement flow diagram (B) Baseline characteristics.

BMI, Body Mass Index; BSFS, Bristol Stool Form Scale; IPAQ, International Physical Activity Questionnaire; PAQ-QOL, Patient Assessment of Constipation-Quality of Life; PAQ-SYM, Patient Assessment of Constipation-Symptoms

Data are presented as mean ± SD or median [range] if not specified otherwise; p-values represent outcomes of corresponding parametric or nonparametric statistical testing.

Compliance was excellent, with all participants exceeding the minimum product intake of 80% (range 92.5% - 113.2%). None of the participants from either group used laxatives during the intervention periods. During the intervention, 16 out of the 40 participants reported 21 possibly intervention-related adverse events. Overall, these adverse events were limited, and their nature was in line with expectations (gastrointestinal symptoms upon increased dietary fiber intake). The most frequently reported adverse event was flatulence (inulin: n = 8), followed by bloating (inulin: n = 5, placebo: n = 1) and cramps (inulin: n = 4, placebo: n = 1).

CROSS-OVER ANALYSIS OF BOWEL HABIT CHANGES

First, we compared changes in bowel habits between inulin and placebo intake. Stool frequency increased by 1.43 (0.19) defecations per week with inulin and by 0.90 (0.19) with placebo. The difference between these changes was 0.53 (0.26) defecations per week ($p = 0.046$, Table 1). On average, participants defecated 3.44 (0.15) times per week with inulin and 3.15 (0.15) times with placebo (Table 2). Stool consistency increased similarly after both inulin and placebo by about half a BSFS unit, resulting in comparable final post-intake BSFS scores (Table 1). However, we found a significant *intervention*period* interaction for stool frequency ($p = 0.014$), which also improved the model for stool consistency ($p = 0.093$), suggesting that carry-over effects could not be excluded for either outcome.

CROSS-OVER ANALYSIS OF PATIENT ASSESSMENT OF CONSTIPATION – QUALITY OF LIFE AND SYMPTOMS AND PHYSICAL ACTIVITY

Next, we analyzed the outcomes of constipation-related quality of life and symptoms questionnaires. Improvements in these scores are indicated by a negative change, with -0.5 for PAC-QOL and -0.6 for PAC-SYM being the minimum important differences (Marquis et al., 2005; Yiannakou et al., 2017). The total PAC-QOL score decreased by -0.61 (0.10) after inulin intake, which was larger than the -0.21 (0.10) decrease after placebo, resulting in a difference between inulin and placebo of -0.41 (0.15) ($p = 0.007$, Table 1). Three of the four PAC-QOL subscores (physical discomfort, psychological discomfort, and worries discomfort) improved evidently more with inulin than with placebo (Table 1). The most notable changes were in psychosocial discomfort and worries discomfort, which virtually remained unchanged after placebo intake (Table 1). Changes in the satisfaction subscore did not differ between inulin and placebo, but and we found a significant *intervention*period* interaction ($p = 0.002$) for this outcome. No significant interaction was detected for the total PAC-QOL score or other subscores.

Table 1. Changes in stool frequency, stool consistency, PAC-QOL, PAC-SYM and physical activity observed in the cross-over trial. Pre, and post-intake levels and changes are reported as estimated marginal means (standard error) if not otherwise indicated. In addition, for differences between inulin and placebo also the 95% CI and related p-values are given.

	Inulin			**Placebo**			**Difference change Inulin vs. placebo**		
	Pre-intake	*Post-intake*	*Change*	*Pre-intake*	*Post-intake*	*Change*	*Difference*	*95%CI*	*p-value*
Stool frequency (x/week)[1]	2.01 (0.15)	3.44 (0.15)	1.43 (0.19)	2.25 (0.15)	3.15 (0.15)	0.90 (0.19)	0.53 (0.26)	[0.02, 1.04]	0.046*
Stool consistency (BSFS)	1.95 (0.12)	2.57 (0.12)	0.62 (0.12)	2.03 (0.12)	2.55 (0.12)	0.52 (0.12)	0.09 (0.17)	[-0.25, 0.43]	0.600*
PAC-QOL									
Total score	2.01 (0.11)	1.39 (0.11)	-0.61 (0.10)	1.81 (0.11)	1.60 (0.11)	-0.21 (0.10)	-0.41 (0.15)	[-0.69, -0.12]	0.007
Physical discomfort	2.29 (0.14)	1.49 (0.14)	-0.80 (0.13)	2.03 (0.14)	1.66 (0.14)	-0.37 (0.13)	-0.43 (0.19)	[-0.08, -0.07]	0.023
Psychosocial discomfort	1.44 (0.14)	0.94 (0.14)	-0.50 (0.12)	1.23 (0.14)	1.21 (0.14)	-0.02 (0.12)	-0.48 (0.17)	[-0.81, -0.14]	0.007
Worries and concerns	1.85 (0.13)	1.28 (0.13)	-0.56 (0.11)	1.67 (0.13)	1.51 (0.13)	-0.16 (0.10)	-0.40 (0.15)	[-0.69, -0.11]	0.009
Satisfaction	3.17 (0.13)	2.28 (0.13)	-0.89 (0.16)	3.05 (0.13)	2.44 (0.13)	-0.62 (0.16)	-0.28 (0.22)	[-0.71, 0.16]	0.218*
PAC-SYM									
Total score	1.96 (0.11)	1.34 (0.11)	-0.61 (0.11)	1.72 (0.11)	1.45 (0.11)	-0.27 (0.11)	-0.35 (0.15)	[-0.64 -0.06]	0.022
Abdominal symptoms	2.08 (0.13)	1.46 (0.14)	-0.62 (0.14)	1.73 (0.13)	1.58 (0.13)	-0.15 (0.14)	-0.47 (0.19)	[-0.84, -0.09]	0.017
Rectal symptoms	1.25 (0.12)	0.73 (0.12)	-0.52 (0.12)	1.22 (0.12)	0.97 (0.12)	-0.25 (0.12)	-0.27 (0.17)	[-0.60, 0.05]	0.104*
Stool related symptoms	2.26 (0.12)	1.33 (0.13)	-0.94 (0.13)	2.04 (0.12)	1.41 (0.12)	-0.64 (0.13)	-0.30 (0.19)	[-0.66, 0.06]	0.110*
IPAQ[2]									
"low"	9 (23.7%)	11 (28.9%)	-	8 (20.5%)	8 (21.1%)	-			
"moderate"	21 (55.3%)	18 (47.4%)	-	22 (56.4%)	16 (42.1%)	-	-		0.432**
"high"	8 (21.1%)	9 (23.7%)	-	9 (23.1%)	14 (36.8%)	-			

BSFS, Bristol Stool Form Scale; IPAQ, international physical activity questionnaire; PAC-QOL, patient assessment of constipation – quality of life; PAC-SYM, patient assessment of constipation – symptoms

[1]*Stool frequency values expressed as median(IQR) were for inulin: pre-intake = 2.0 (1.5, 2.5), post-intake = 3.5 (3.0, 4.0) and change = 1.5 (0.5, 2.5), and for placebo: pre-intake = 2.0 (2.0, 2.5), post-intake = 3.0 (2.5, 4.0) and change = 1.0 (0.5, 1.5). The median [95%CI] difference between changes after inulin versus placebo intake was 0.75 [0.00, 1.50] and p = 0.042*

[2] *Values are expressed as count (%)*

**p-value biased due to contribution of intervention*period interaction term that improved the model*

*** represents the p-value for the difference in post-intake counts*

Similar to the PAC-QOL outcomes, the total PAC-SYM score decreased more after inulin intake (0.61 (0.11)) compared to placebo (-0.27 (0.11)), with a difference between inulin and placebo of -0.35 (0.15) ($p = 0.022$, Table 1). This was mainly due to a significant decrease in abdominal symptoms (-0.62 (0.14) with inulin vs. -0.15 (0.14) with placebo, $p = 0.017$, Table 1), despite adverse events of flatulence and bloating after inulin intake. Rectal and stool-related symptoms also decreased after inulin intake but did not differ significantly from placebo intake (Table 2). None of the outcomes had a significant *intervention*period* interaction, although the interaction term improved the model for stool-related symptoms.

Physical activity levels, as assessed by IPAQ scores, remained consistent during inulin intake (pre- versus post-intake). However, during placebo intake, a slightly higher proportion of participants engaged in high-intensity physical activities, but these differences were not statistically significant (Table 2). Additionally, none of the participants resorted to laxatives during any of the study periods.

CROSS-OVER ANALYSIS OF FECAL MICROBIOTA OUTCOMES AND RELATION WITH CLINICAL OUTCOMES

To investigate how changes in clinical outcomes may be mediated by the gut microbiota, we analyzed fecal microbiota composition using 16S rRNA gene amplicon sequencing. We had 65 samples to assess inulin-induced changes and 62 to assess placebo-induced changes due to fewer samples from the second period (Figure 2). We observed no differences in β-diversity after inulin compared to placebo intake, assessed by Principal Coordinate analysis (PCoA) based on β-diversity using Bray-Curtis dissimilarity at genus level (1% of variation explained, PERMANOVA $p = 0.88$; Figure 3A). However, similar to the clinical changes, we observed a significant *intervention*period* interaction in the PCoA, which explained 9% of the variation (PERMANOVA $p = 0.001$; Figure 3A). This result indicated a potential carry-over effect in the fecal microbiota as well.

Assessing individual genera and changes in their relative abundances, *Bifidobacterium* spp. was observed to be the most abundant genus across all samples (Figure 3B). *Bifidobacterium* spp. relative abundance increased from 16.4% to 18.4% during inulin intake, compared to a minimal increase from 14.4% to 15.1% during placebo intake. This resulted in a post-intake difference between inulin and placebo of 1.2-fold ($p = 0.565$). Most notably, post-intake relative abundances of *Anaerostipes* spp. and *Coprococcus* spp. were 1.75-fold and 1.59-fold higher after inulin intake than placebo, respectively, based on samples where these taxa were present (*Coprococcus* 1 spp.: 78% and *Anaerostipes* spp.: 87% of all samples; Figure 3C).

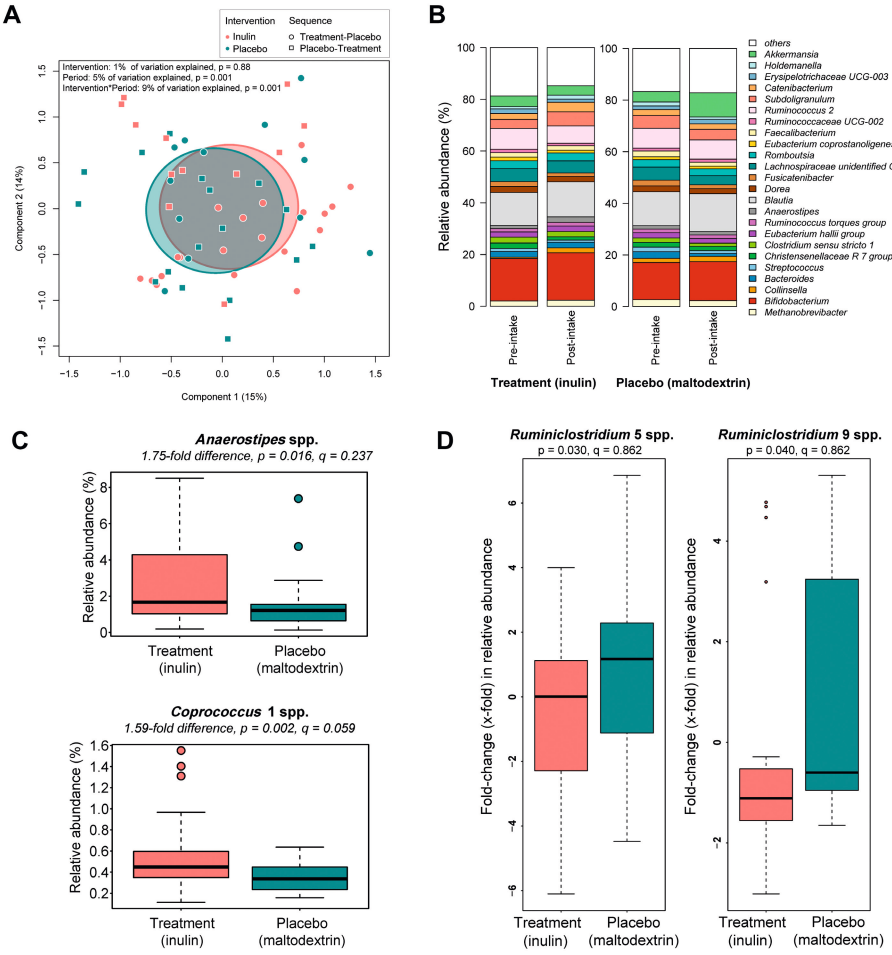


Figure 3. Fecal microbiota composition and changes in individual taxa observed in the cross-over trial. (A) Fecal microbiota composition at the genus level was visualized and assessed by principal coordinate analysis using β-diversity based on Bray-Curtis dissimilarity after inulin intake compared to after placebo intake. The difference between interventions, periods, and the contribution of the *intervention*period* interaction was tested using PERMANOVA, and corresponding p-values are depicted in the upper left of the figure. (B) Common genera (>1.0% abundance in at least 50% of the samples) pre-intake and post-intake (inulin or placebo) relative levels. (C) Genera identified to significantly differ in their post-intake levels between inulin and placebo. (D) Genera identified to significantly differ in their pre-post-intake changes between inulin and placebo intake.

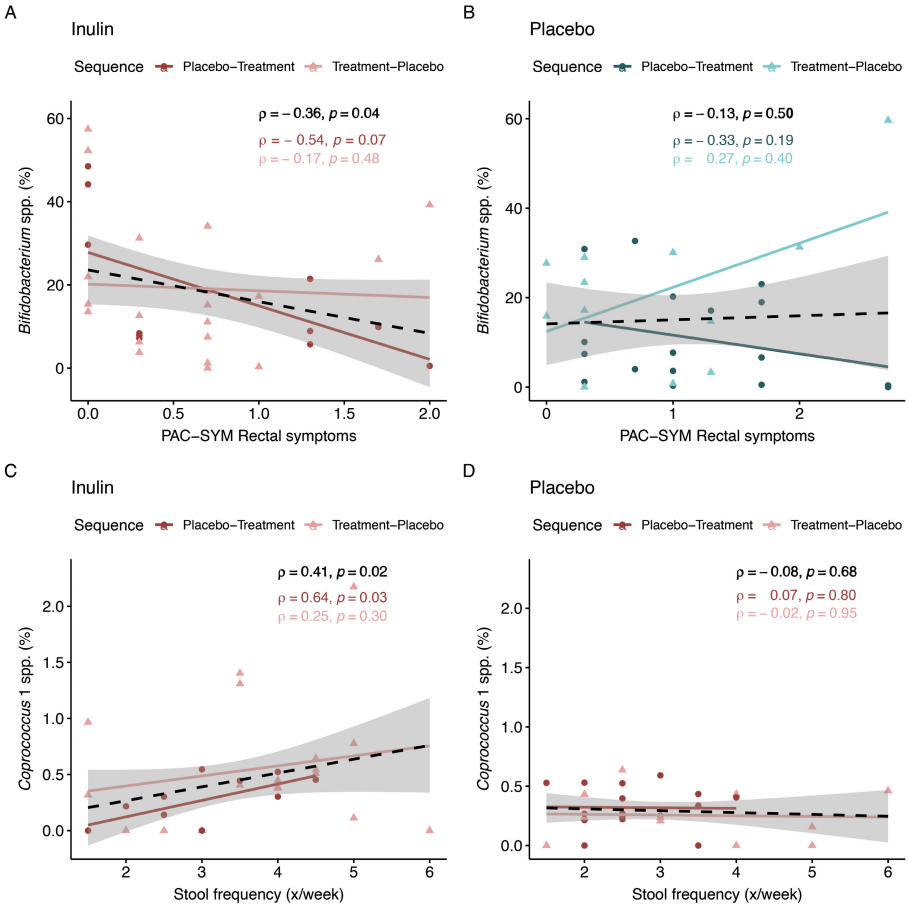


Figure 4. Correlation between fecal microbial taxa and clinical outcomes observed in the cross-over trial. *Bifidobacterium* spp. relative levels and PAC-SYM rectal symptoms after (A) inulin and (B) placebo intake, as well as the correlation between *Coprococcus* spp. relative levels and stool frequency after (C) inulin and (D) placebo intake. The black dotted lines and Spearman correlation coefficients depict the overall correlation outcomes (both sequences combined). The colored lines and correlation coefficients depict the correlation outcomes per respective sequence in each intervention intake.

Additionally, two less abundant genera (<1% relative abundance) differently changed after inulin compared to placebo. These were *Ruminiclostridium* 5 spp. (inulin: 0.75-fold from 0.34% to 0.25%; placebo: 1.74-fold from 0.28% to 0.48%) and *Ruminiclostridium* 9 spp. (inulin: 0.33-fold from 0.05% to 0.02%; placebo: 1.15-fold from 0.04% to 0.04%; Figure 3D). Several other genera of the *Ruminococcaceae* family also decreased after inulin, but not placebo. However, these differences were not significantly different between interventions.

Building on these observations, we assessed potential relationships between the relative abundance of bacterial taxa and clinical outcomes. Post-inulin intake levels of

Bifidobacterium spp. correlated moderately with PAC-SYM rectal symptoms (ρ = -0.36, p = 0.04; Figure 4A), indicating higher relative abundance of bifidobacteria was associated with lower rectal symptom scores, while this relationship was very weak after placebo intake (ρ = -0.13, p = 0.50; Figure 4B). Also, post-inulin intake *Coprococcus* 1 spp. levels correlated moderately with stool frequency (ρ = 0.40, p = 0.02; Figure 4C), indicating higher levels of *Coprococcus* 1 spp. were associated with better stool frequency outcomes, but this relationship was absent after placebo intake (ρ = 0.08, p = 0.68; Figure 4D).

INTERVENTION SEQUENCE DIFFERENCES IN THE CROSS-OVER TRIAL

Given the significant *intervention*period interaction* observed in stool frequency, stool consistency, PAC-QOL satisfaction, PAC-SYM stool-related symptoms, and fecal microbiota composition, we further investigated potential differences related to the intervention sequence (Table 2). Both sequences initially had similar run-in starting values, but differed during wash-out. This was primarily caused by participants in the treatment-placebo sequence, who maintained improved levels of stool frequency, consistency, PAC-QOL, PAQ-SYM total and subscores even after the four-week wash-out period (Table 2). Considering physical activity, no differences were found between the run-in and wash-out periods in the treatment-placebo sequence, indicating that improved outcomes during wash-out were not due to changes in physical activity. In the placebo-treatment sequence, slightly more participants engaged in high-intensity activities and fewer in low- and moderate-intensity activities (Table 2). Investigating differences in fecal microbiota composition per intervention sequence, we observed that β-diversity during wash-out in the treatment-placebo sequence differed significantly from that observed during run-in, whereas for the placebo-treatment sequence, compositions remained comparable (Supplementary Figure 1).

Collectively, these differences between intervention sequences suggested a lasting effect of inulin in the treatment-placebo sequence despite a four-week washout, and that potential carry-over in clinical outcomes was linked to changes in fecal microbiota.

INVESTIGATING DIFFERENCES IN THE TREATMENT-PLACEBO SEQUENCE RELATING TO POTENTIAL CARRY-OVER

To understand potential carry-over, we first assessed individual responses (Figure 5A-H and Supplementary Figure 2). The key observation was a dichotomous response in the primary outcome, stool frequency, within the treatment-placebo sequence during placebo intake (Figure 5E and Supplementary Figure 2A). Approximately half of the participants in the treatment-placebo sequence reported stool frequencies exceeding three defecations per week during placebo intake, while the other half had lower frequencies. Considering this and the clinical relevance of three bowel movements per week according to the Rome criteria, participants were categorized into high-placebo-responders (more than three defecations/week, n = 9) and low-placebo-responders (three or fewer defecations/week, n = 9; Figure 5E-H). One participant was not categorized due to missing data.

Table 2. Stool frequency, consistency and PAC-QOL, and SYM, and physical activity per sequence. Descriptive statistics of each outcome are reported as mean (SEM) if not indicated otherwise.

	Treatment-Placebo				Placebo-Treatment			
	Run-in	*Period 1*	*Washout*	*Period 2*	*Run-in*	*Period 1*	*Washout*	*Period 2*
Stool frequency (x/week)[1]	2.00 [2.00, 2.12]	4.00 [2.75, 4.50]	2.25 [2.00, 3.00]	3.50 [2.50, 4.50]	2.00 [2.00, 2.25]	2.50 [2.25, 3.25]	2.00 [1.50, 2.50]	3.25 [3.00, 3.50]
Stool consistency (BSFS)	1.92 (0.12)	2.75 (0.18)	2.19 (0.22)	2.88 (0.27)	1.82 (0.11)	2.23 (0.14)	1.97 (0.16)	2.33 (0.16)
PAC-QOL								
Total score	2.05 (0.14)	1.26 (0.14)	1.74 (0.17)	1.50 (0.21)	1.89 (0.14)	1.71 (0.15)	1.97 (0.15)	1.57 (0.15)
Physical discomfort	2.37 (0.12)	1.36 (0.16)	1.81 (0.19)	1.55 (0.22)	2.24 (0.19)	1.76 (0.22)	2.21 (0.23)	1.69 (0.21)
Psychosocial discomfort	1.58 (0.19)	0.93 (0.13)	1.25 (0.21)	1.28 (0.25)	1.21 (0.17)	1.14 (0.19)	1.29 (0.21)	1.00 (0.16)
Worries and concerns	1.84 (0.17)	1.16 (0.17)	1.62 (0.2)	1.46 (0.24)	1.72 (0.16)	1.55 (0.17)	1.85 (0.18)	1.46 (0.16)
Satisfaction	3.18 (0.13)	1.95 (0.22)	2.75 (0.17)	1.94 (0.24)	3.34 (0.11)	2.94 (0.14)	3.15 (0.13)	2.6 (0.21)
PAC-SYM								
Total score	1.98 (0.12)	1.25 (0.13)	1.54 (0.17)	1.35 (0.19)	1.90 (0.14)	1.56 (0.17)	1.93 (0.16)	1.47 (0.17)
Abdominal symptoms	2.13 (0.12)	1.34 (0.15)	1.52 (0.20)	1.51 (0.21)	1.94 (0.18)	1.64 (0.2)	2.02 (0.22)	1.63 (0.22)
Rectal symptoms	1.25 (0.16)	0.59 (0.12)	0.93 (0.2)	0.82 (0.19)	1.52 (0.15)	1.11 (0.17)	1.26 (0.17)	0.88 (0.16)
Stool related symptoms	2.29 (0.18)	1.58 (0.17)	1.96 (0.2)	1.07 (0.13)	2.12 (0.17)	1.75 (0.2)	2.24 (0.19)	1.09 (0.14)
IPAQ[2]								
"low"	5 (25%)	7 (36.8%)	4 (20%)	6 (30%)	4 (21.1%)	2 (11.1%)	4 (22.2%)	4 (21.1%)
"moderate"	12 (60%)	7 (36.8%)	10 (50%)	7 (35%)	12 (63.2%)	9 (50%)	9 (50%)	11 (57.9%)
"high"	3 (15%)	5 (26.3%)	6 (30%)	7 (35%)	3 (15.8%)	7 (38.9%)	5 (27.8%)	4 (21.1%)

BSFS, Bristol Stool Form Scale; IPAQ, international physical activity questionnaire; PAC-QOL, patient assessment of constipation – quality of life; PAC-SYM, patient assessment of constipation – symptoms

[1] *Values are expressed as median [IQR]*

[2] *Values are expressed as count (%)*

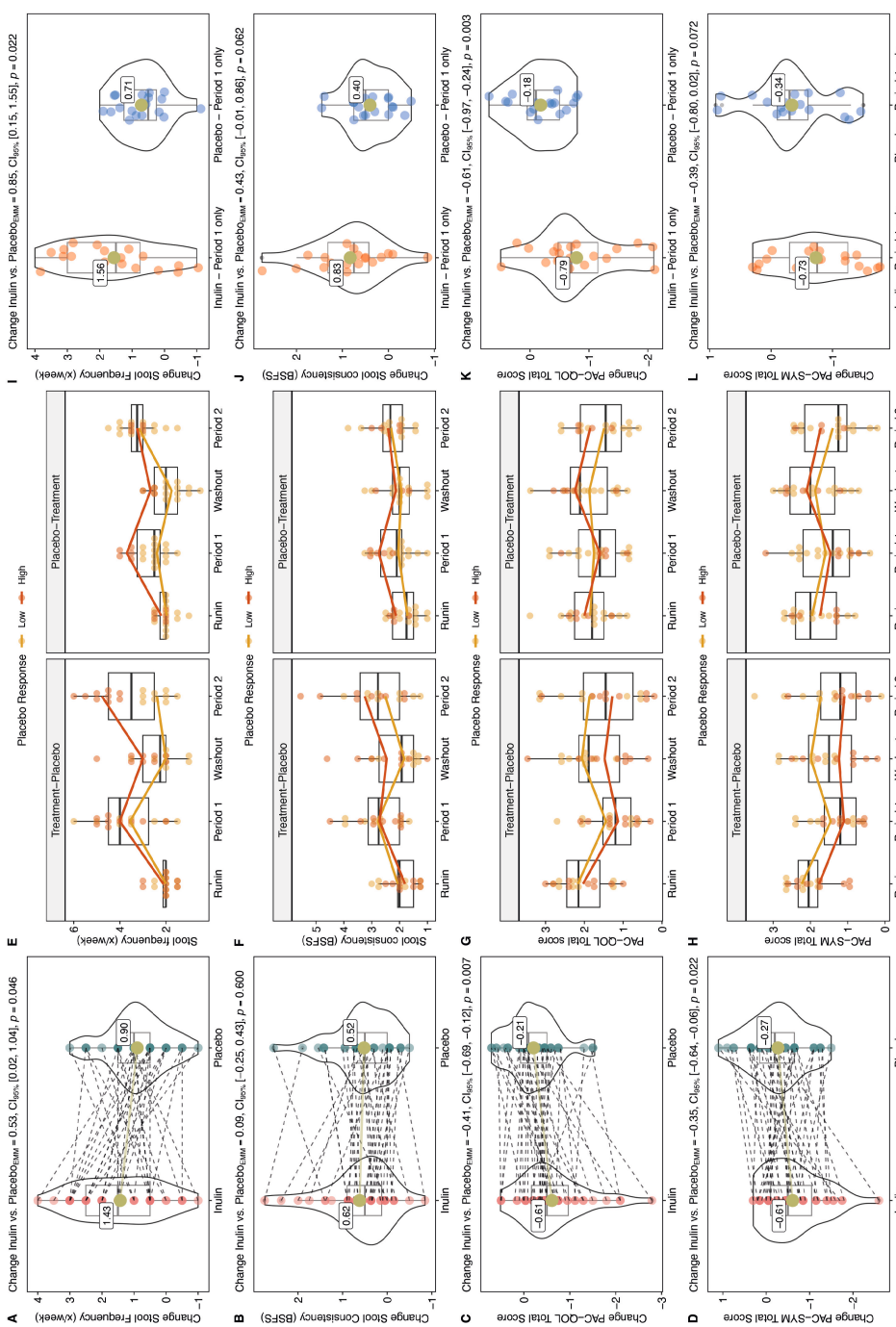

A
Change Inulin vs. Placebo$_{ΔAM}$ = 0.53, CI$_{95\%}$ [0.02, 1.04], p = 0.046
Change Stool Frequency (x/week)
1.43
0.90
Inulin
Placebo
B
Change Inulin vs. Placebo$_{ΔAM}$ = 0.09, CI$_{95\%}$ [−0.25, 0.43], p = 0.600
Change Stool Consistency (BSFS)
0.62
0.52
Inulin
Placebo
C
Change Inulin vs. Placebo$_{ΔAM}$ = −0.41, CI$_{95\%}$ [−0.69, −0.12], p = 0.007
Change PAC−QOL Total Score
−0.61
−0.21
Inulin
Placebo
D
Change Inulin vs. Placebo$_{ΔAM}$ = −0.35, CI$_{95\%}$ [−0.64, −0.06], p = 0.022
Change PAC−SYM Total Score
−0.61
−0.27
Inulin
Placebo
E
Placebo Response
Low
High
Stool Frequency (x/week)
Treatment−Placebo
Placebo−Treatment
Runin
Period 1
Washout
Period 2
F
Placebo Response
Low
High
Stool Consistency (BSFS)
Treatment−Placebo
Placebo−Treatment
Runin
Period 1
Washout
Period 2
G
Placebo Response
Low
High
PAC−QOL Total Score
Treatment−Placebo
Placebo−Treatment
Runin
Period 1
Washout
Period 2
H
Placebo Response
Low
High
PAC−SYM Total Score
Treatment−Placebo
Placebo−Treatment
Runin
Period 1
Washout
Period 2
I
Change Inulin vs. Placebo$_{ΔAM}$ = 0.85, CI$_{95\%}$ [0.15, 1.55], p = 0.022
Change Stool Frequency (x/week)
1.56
0.71
Inulin − Period 1 only
Placebo − Period 1 only
J
Change Inulin vs. Placebo$_{ΔAM}$ = 0.43, CI$_{95\%}$ [−0.01, 0.86], p = 0.062
Change Stool Consistency (BSFS)
0.83
0.40
Inulin − Period 1 only
Placebo − Period 1 only
K
Change Inulin vs. Placebo$_{ΔAM}$ = −0.61, CI$_{95\%}$ [−0.97, −0.24], p = 0.003
Change PAC−QOL Total Score
−0.79
−0.18
Inulin − Period 1 only
Placebo − Period 1 only
L
Change Inulin vs. Placebo$_{ΔAM}$ = −0.39, CI$_{95\%}$ [−0.80, 0.02], p = 0.072
Change PAC−SYM Total Score
−0.73
−0.34
Inulin − Period 1 only
Placebo − Period 1 only

Figure 5. Individual changes in stool frequency, stool consistency, PAC-QOL and PAQ-SYM score. (A-D) Cross-over analysis. Individual changes for (A) stool frequency, (B) stool consistency, (C) patient assessment of constipation quality of life (PAC-QOL) total score, and (D) symptoms (PAC-SYM) total score. (E-H) Intervention sequence differences. Changes in treatment-placebo and placebo-treatment for (E) stool frequency, (F) stool consistency), (G) PAC-QOL total score and (H) PAC-SYM total score. Each point represents participants' individual values. Note the dichotomy in the response in stool frequency in the treatment-placebo sequence during placebo intake (period 2). Lines represent the average of participants categorized into high- (dark orange) or low- (light orange) placebo-responders based on the individual responses in stool frequency observed during placebo intake. (I-L) First period analysis. Changes based on the analysis of the run-in and period 1 as a parallel trial for (I) stool frequency, (J) stool consistency), (K) PAC-QOL total score and (L) PAC-SYM total score. For the cross-over analysis and the first period analysis, inside the figures the estimated marginal means for the change after inulin or placebo are reported. Additionally, the estimated marginal means of the difference between inulin and placebo intake are reported on top of the figures together with the 95% confidence interval, and related p-values. Note that for the cross-over analysis p-values of stool frequency and stool consistency are biased due to the detection of a significant *intervention*period* interaction.

By categorizing placebo responses into high and low, our aim was to understand whether differences in stool frequency and other outcomes emerged during previous study periods [run-in, period 1 (inulin intake), wash-out]. High- and low-placebo-responders had similar stool frequencies and consistencies during run-in and inulin intake (Figure 5E-F). Additionally, high-placebo-responders had lower PAC-QOL and PAC-SYM scores during inulin intake and seemed to start with lower PAC-SYM scores compared to low-placebo-responders (Figure 5G-H).

Notable differences began to emerge during the wash-out, where it became clear that high-placebo-responders also drove the lasting improvements in stool consistency, PAC-QOL and PAC-SYM for the whole sequence. For comparison, we categorized the placebo-treatment sequence, similarly, though resulting in a less even distribution (high response: n = 5, low response: n = 14). Minor differences were observed during inulin intake but were less pronounced compared to the treatment-placebo sequence (Figure 5E-H).Using the available fecal microbiota data, we investigated potential differences in the fecal microbiota composition in the treatment-placebo sequence between high- and low-placebo-responders (Figure 6 and Supplementary Figure 3A-C). Neither during run-in nor inulin intake PCoA of β-diversity suggested differences in fecal microbiota composition (Figure 6A-D) between high-and low-placebo-responders, but notable differences existed in the wash-out samples (Figure 6C). When we investigated individual taxa, several differences became apparent at run-in, wash-out and during placebo intake (Figure 6A-E).

At run-in high-placebo-responders had lower relative abundances of *Bifidobacterium* spp. (0.6-fold difference) and *Methanobrevibacter* spp. (0.8-fold difference) compared to low-placebo-responders (Figure 6F). Moreover, at run-in, high-placebo-responders had substantially higher relative abundances of the butyrate-producers *Faecalibacterium* spp. and *Roseburia* spp. (Figure 6F), as well as higher relative abundances of *Fusicantenibacter* spp., *Intestinibacter* spp., and an unidentified genus within the *Lachnospiraceae* family, and lower relative abundances of bacteria from

the *Ruminococcus gauvreauii* group (Supplementary Figure 3B). *Anaerostipes* spp. relative abundances did not evidently differ between high-placebo-responders (1.4%) and low-placebo-responders (1.3%) based on samples where this taxon was present.

During inulin intake (period 1), the fecal microbiota composition of high and low-placebo-responders became more similar (Figure 6B). Notably, *Bifidobacterium* spp. relative abundances increased in high-placebo-responders 1.8-fold (from 11.7% to 21.7%), and not only reached but exceeded those in low-placebo-responders (periods 1: 18.7%). Additionally, *Anaerostipes* spp. relative abundances increased 2.4-fold in high-placebo-responders (from 1.4% to 3.4%) but only 1.7-fold in the low-placebo responders (from 1.3% to 2.1%).

After the four-week wash-out, the run-in differences re-emerged. *Bifidobacterium* spp. relative abundances returned to baseline levels, differing nearly two-fold between high-placebo-responders (11.8%) and low-placebo-responders (22.9%). Also, relative abundances of the butyrate-producers *Faecalibacterium* spp. and *Roseburia* spp. were again higher in high-placebo-responders compared to low-placebo-responders. Notably, the relative abundances of putative butyrate-producer *Subdoligranulum* spp. was also higher in high-placebo-responders compared to low-placebo-responders (Figure 6G). During wash-out, *Subdoligranulum* spp. constituted 10.4% of the fecal microbiota in high-placebo-responders, representing a 2.4-fold increase over run-in and a 1.7-fold increase compared to inulin intake. In contrast, in low-placebo-responders, *Subdoligranulum* spp. relative abundances decreased after inulin intake (run-in: 3.2%; inulin: 5.2%) to 2.5% during wash-out. Additionally, *Anaerostipes* spp. relative abundances remained elevated in high-placebo-responders during wash-out (2.0%) but returned to run-in levels in low-placebo-responders (1.4%), based on samples where this taxon was present.

After placebo intake (period 2), wash-out differences were no longer present. High-placebo-responders once again had lower relative abundances of bifidobacteria (0.6-fold difference) and *Methanobrevibacter* spp. (0.1-fold difference) compared to low-placebo-responders (Figure 6H). Collectively, this observation in the fecal microbiota composition suggested that potential carry-over effects related to differences in baseline fecal microbiota composition, including *Bifidobacterium* spp. and their responsiveness to inulin, as well as butyrate-producing bacteria that notably remained elevated during wash-out.

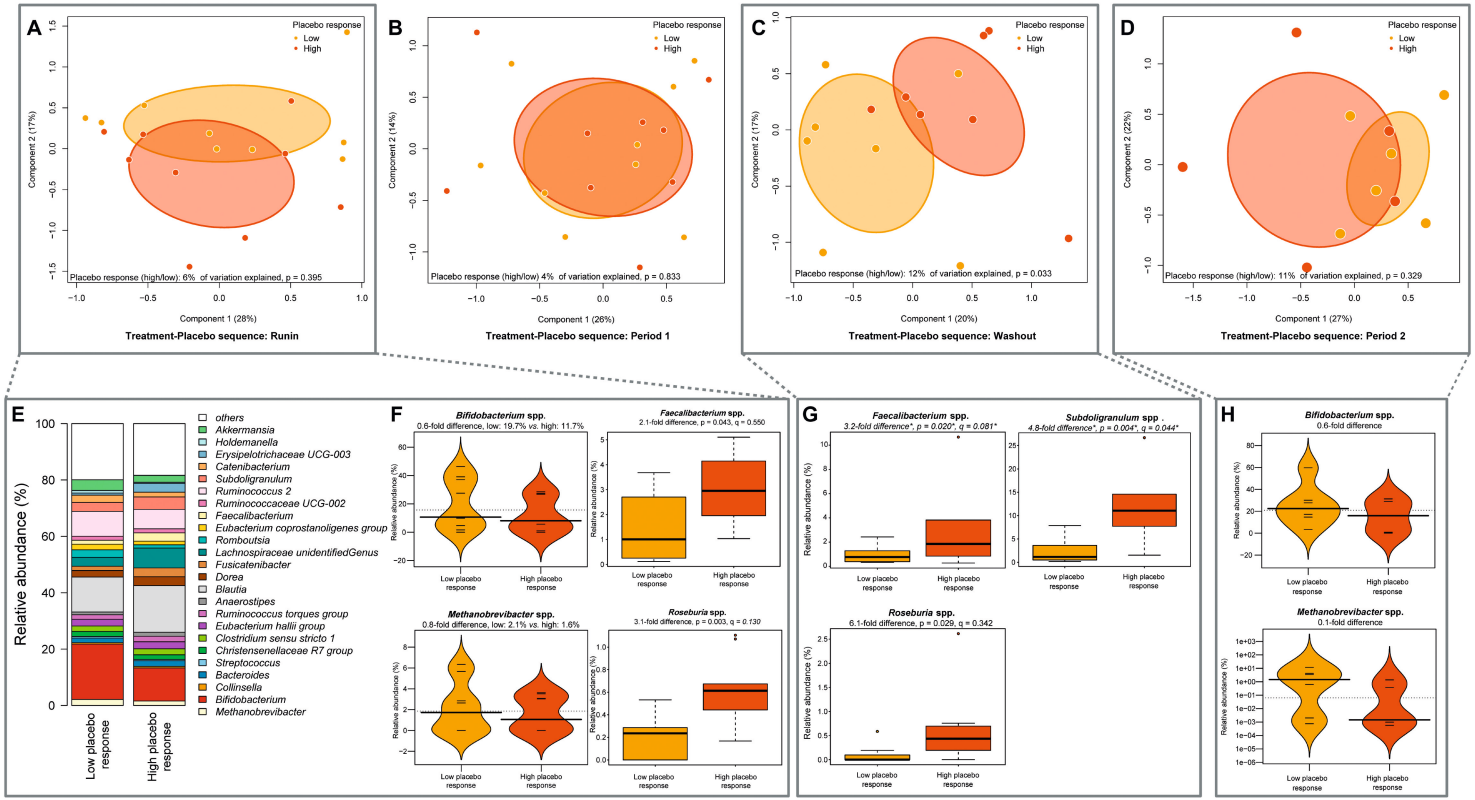


Figure 6. Fecal microbiota differences in the treatment-placebo sequence split out based on placebo response in period 2. (A-D) Overall gut microbiota composition at genus level visualized and assessed by principal coordinate analysis using β-diversity based on Bray-Curtis dissimilarity at (A) run-in, (B) period 1 inulin intake), (C) washout and (D) period 2 (placebo intake) for participants in the treatment-placebo sequence responding either strongly to the placebo in period 2 (high placebo response) or not (low placebo response). The difference between each group was tested using PERMANOVA and corresponding p-values are depicted in the lower left of the figures. Note the decreasing number of samples over time, attributed to the decreasing availability of fecal samples from all participants. (E) Common genera (>1% abundance in 50% of the samples) at run-in. Genera differing in relative levels between participants with low and high placebo responses a run-in (F), after washout (G), and after period 2 (placebo intake) (H). old differences, p-values, and FDR-corrected q-values denoted with an asterisk * are based only on samples with the taxon present. For differences in *Bifidobacterium* spp. and *Methanobrevibacter* spp. relative abundances no p-values were estimated due to model violations.

ANALYSIS OF THE RUN-IN AND FIRST PERIOD AS PARALLEL TRIAL

Considering the indications of carry-over effects on some outcomes and the constrained explanatory power of the observed fecal microbiota composition changes posed by the limited fecal microbiota samples at the end of period 2, we conducted an analysis of the changes after the first period only (first period analysis) as a sensitivity analysis. Changes in stool frequency and PAC-QOL were consistent with those observed in the cross-over analysis (Supplementary Table 1). However, for stool consistency and PAC-SYM outcomes, certain results differed.

After inulin intake, stool consistency increased by 0.83 (0.16) BSFS units, which was twice as high compared to placebo (placebo: 0.40 (0.16) units) and resulted into a notable difference between inulin and placebo of 0.43 (0.22) (p = 0.062). Consequently, final BSFS scores were substantially higher after inulin intake (2.75 (0.14)) compared to placebo (2.23 (0.14); p = 0.011). Additionally, the difference in the decrease in PAC-QOL score after inulin compared to placebo intake was 0.61 (0.16) (p = 0.003) and thereby exceeded the minimum important difference of 0.5 for PAC-QOL (Marquis et al., 2005)Also, the PAC-QOL satisfaction subscore improved, which was not observed in the cross-over analysis, where potential carry-over was detected for this outcome (Supplementary Table 1). Again, the subscores for psychosocial discomfort and worries and concerns notably improved after inulin intake but changed minimally after placebo intake (Supplementary Table 1). Finally, the PAC-SYM final rectal symptom subscore differed after inulin (1.58 (0.18)) compared to placebo intake (1.75 (0.18); p = 0.018), which was not detected in the cross-over analysis.

Changes in fecal microbiota composition assessed by PCoA on β-diversity using Bray-Curtis indicated that fecal microbiota composition in participants consuming inulin became more similar but did not significantly differ from that in participants consuming placebo (p = 0.163; Supplementary Figure 4A-B). Considering individual taxa, we confirmed the observed increase in the relative abundance of *Bifidobacterium* spp. and *Anaerostipes* spp., and decreases in genera of the *Ruminococcaceae* family seen in the cross-over analysis. Changes in bifidobacteria relative abundances differed significantly (p = 0.013, q = 0.192; Supplementary Figure 5A) after inulin intake (run-in: 14.8%, period 1: 19.2%; fold-change: 1.3-fold) compared to placebo (run-in: 12.8%, period 1: 10.9%; fold-difference: 0.8-fold). Post-intake relative abundances of *Anaerostipes* spp. were 2.5-fold higher after inulin intake compared to placebo (p = 0.001, q = 0.025; Supplementary Figure 5B). Additionally, *Akkermansia* spp. relative abundances were higher after placebo intake compared to inulin intake, however, this difference seemed to be skewed by one individual (Supplementary Figure 5B).

Collectively, the analysis of the first period alone as a parallel trial confirmed the inulin-induced improvements in notably stool frequency and PAC-QOL total and subscores as well as the increased relative abundances of butyrate-producer *Anaerostipes* spp..

DISCUSSION

Here, we assessed the impact of four-week daily 12 g inulin intake on stool frequency, stool consistency, constipation quality of life, symptoms, and physical activity, as well as fecal microbiota composition in 39 individuals with functional constipation according to Rome III criteria. In the cross-over analysis of the predominantly female study population, we observed that inulin had a positive impact on stool frequency, which improved from once every three to four days to once every second day despite no observed changes in stool consistency. This was concurrent with improvements in constipation-related quality of life and symptoms, notable for the decrease in worries, concerns, psychosocial discomfort, and abdominal symptoms alongside an increase in the relative abundance of genera of known butyrate-producing bacteria. After detecting potential carry-over in several outcomes, we analyzed the first period only, confirming the positive effect of inulin on bowel habits, quality of life, symptoms, and fecal microbiota.

Considering that the gut microbiome is a recognized factor in the poorly understood etiology of DGBI (5), we aimed to investigate how inulin-induced improvements were reflected in the gut microbiota composition using fecal samples. We observed that improvements in stool frequency, constipation-related quality of life, and symptoms were accompanied by an increase in the relative abundances of *Anaerostipes* spp. and *Coprococcus* 1 spp., two well-known putative butyrate producers (Louis & Flint, 2017; Sheridan et al., 2022). Butyrate is a short-chain fatty acid (SCFA) produced by the gut microbiota during fiber fermentation and is well-known to benefit gut health directly by serving as an energy source for colonocytes, improving gut barrier function (Hamer et al., 2008), and potentially promoting gut motility, although the latter has been mainly shown in animal models (Hodgkinson et al., 2023). Some reports have suggested a less favorable effect of elevated levels of (specific) butyrate-producing bacteria in constipation based on studies with a limited number of individuals (Parthasarathy et al., 2016; Zhu et al., 2014). This has led to anecdotal interpretations that butyrate might induce constipation (Zhang et al., 2021; Zhao & Yu, 2016), possibly due to its known role in counteracting diarrhea (Canani et al., 2011) and conflicting effects on gut motility (Hodgkinson et al., 2023). However, larger studies present opposing, notably beneficial findings. Generally, butyrate-producing taxa appear to be reduced in relative abundance compared to healthy controls, as observed from fecal samples of individuals with functional constipation diagnosed according to Rome III criteria (Mancabelli et al., 2017) or colonic tissue samples of individuals with severe constipation requiring surgery (Yarullina et al., 2020). Similar findings have been made in individuals with irritable bowel syndrome (IBS) (Chassard et al., 2012; Pozuelo et al., 2015), also a DGBI. Another notable finding from studies in individuals with constipation is the positive relationship between butyrate-producing bacteria such as *Faecalibacterium* spp., *Coprococcus*, and *Roseburia* spp., and faster transit (Parthasarathy et al., 2016) or higher fecal water content (with and without fiber treatment) (Jalanka et al., 2019). These

observations suggest that improvements in bowel function and the relative abundance of butyrate-producing taxa might be linked. Here, we did not investigate the relationship between butyrate and bowel function through the measurement of fecal SCFAs, which reflects the net levels of production and uptake; a limitation of this study. However, we found that higher *Coprococcus* 1 spp. relative abundances correlated with higher stool frequencies, pointing towards a role of butyrate production in the inulin-induced bowel habit improvements. Furthermore, higher relative abundances of *Bifidobacterium* spp. during inulin intake correlated with lower scores for PAC-SYM rectal symptoms, underscoring the known positive impact of bifidobacteria on gut health. Finally, inulin intake decreased relative abundances of several *Ruminococcaeae* members, potentially indicating a beneficial modulation of the gut microbial environment, given the reported higher relative abundance of this bacterial family in constipated individuals versus healthy controls (Mancabelli et al., 2017; Yarullina et al., 2020; Zhu et al., 2014). Nevertheless, for stool frequency, stool consistency, PAC-QOL satisfaction, and PAC-SYM rectal and stool-related symptom subscores, we observed a considerable contribution of the *intervention*period* interaction term, which indicated we could not rule out bias in these outcomes due to carry-over.

Carry-over effects are a primary source of bias in cross-over designs and may attenuate treatment effects. Despite this issue, cross-over trials are the preferred study design when the outcome is hypothesized to depend strongly on an individual's characteristics (Jones & Kenward, 1989; Senn, 2002). This is especially true when the gut microbiota is expected to affect the response, as its composition is highly individual and has been shown to influence how individuals respond to dietary stimuli (Korpela et al., 2014; Salonen et al., 2014). Therefore, here, a cross-over design was chosen to compare individual responses to the treatment and placebo intake, mediated by the gut microbiota. To avoid carry-over, a sufficiently long wash-out period is essential (Dwan et al., 2019; Jones & Kenward, 1989; Senn, 2002). Currently, however, no guidelines exist on appropriate wash-out length for human gut microbiota studies (Leeming et al., 2019). Here, a four-week wash-out was estimated to be sufficient, as the summarized evidence of several studies has suggested that the effect of inulin-type fructans on the fecal microbiota progressively disappears after one to two weeks (Roberfroid, 2005). In addition, the adoption of the cross-over design was underpinned by previous cross-over trials that used inulin. These trials used a similar dosage (12 g/day), albeit in healthy individuals without impaired bowel function (Micka et al., 2017), or lower dosages (3 g/day or 7 g/day) in healthy individuals with low fiber intake (Reimer et al., 2020).

There is ongoing debate on how to address potential carry-over, given the lack of a robust test for it and its possible concealment within other effects (Dwan et al., 2019; Jones & Kenward, 1989; Senn, 2002). In an AB/BA design like ours, including a parameter for carry-over in the model has been proposed. Since carry-over cannot be distinguished from a treatment by period effect (Dwan et al., 2019; Jones & Kenward, 1989), we used the *intervention*period* interaction term to model carry-over. While carry-over bias could be seen as a design failure, leading researchers to report no or

limited outcomes, we chose to investigate and report potential reasons for carry-over, as recommended (Dwan et al., 2019). We aimed to learn from this trial and understand part of the observed effect while being aware of the limitations. Furthermore, we analyzed the first period alone as a parallel trial to assess the robustness of the cross-over analysis findings. This strategy to deal with carry-over has been extensively debated (Dwan et al., 2019; Jones & Kenward, 1989; Senn, 2002). Here, we decided to include such an analysis as we did not observe a significant contribution of the *intervention*period* interaction term for all outcomes and because of the limited fecal microbiota data for the second period.

Following the observation of the significant *intervention*period* interaction for stool frequency, we first set out to understand the notable high placebo response. Water intake and physical activity are known modulators of bowel function (Dukas, 2003; Tantawy et al., 2017; Verkuijl et al., 2020). It is unlikely that the daily 60 mL of water, in which the intervention product was consumed, substantially affected bowel habits. Also, participants who received inulin first (the treatment-placebo sequence) did not engage in higher-intensity physical activity during the placebo intake. However, during placebo intake in the treatment-placebo sequence, we observed a notable difference in stool frequency, primarily driven by nine participants with distinctly high stool frequencies (>3 times per week). Consequently, we categorized participants based on their placebo response (>3 or ≤ 3 defecations per week on placebo) into high-placebo-responders and low-placebo-responders and assessed how they reacted differently during inulin intake and wash-out. High-placebo-responders also had a stronger response to inulin across all other outcomes. Additionally, these nine participants demonstrated lasting improvements in stool consistency, PAC-QOL, and PAC-SYM scores during the wash-out period, explaining why wash-out levels did not return to run-in levels for the entire treatment-placebo sequence. Thus, we aimed to explore the lasting effect of inulin by investigating differences among participants, particularly their baseline characteristics such as fecal microbiota composition, and its potential role in influencing the observed effects related to carry-over.

First, we noticed that participants in the treatment-placebo sequence who responded strongly to the placebo had lower run-in PAC-SYM scores, mainly due to fewer rectal symptoms. This suggests that their baseline experience of physical discomfort was comparatively milder. In line with this, they also had a lower PAC-QOL physical discomfort subscore, even though their total PAC-QOL score was similar to that of the other participants. Intriguingly, these high-placebo-responders also had higher relative abundances of known butyrate-producing genera *Faecalibacterium* spp. and *Roseburia* spp. at run-in. During inulin intake, high-placebo-responders had a higher increase in relative abundances of *Anaerostipes* spp., the butyrate-producing genus, which differed significantly between inulin and placebo intake in both the cross-over and first-period analyses. In the wash-out period, higher relative abundances of butyrate producers re-emerged, accompanied by impressively increased relative abundances of the putative butyrate-producing genus *Subdoligranulum* spp. compared to run-in.

It is therefore possible that these higher placebo responders may have had a more favorable gut microbial environment characterized by higher relative abundances of butyrate-producing bacteria. This predisposition could have contributed to their better response during the intervention, despite their run-in stool frequency, consistency, and PAC-QOL scores being similar.

Another notable difference at run-in between high- and low-placebo-responders in the treatment-placebo sequence was the differences in relative abundances of *Bifidobacterium* spp.. High-placebo-responders started with half the relative abundances of bifidobacteria compared to low-placebo-responders. However, during inulin intake, their response to inulin was nearly twice as strong, surpassing the post-intake relative abundances of those with low placebo responses. Inulin is recognized for its bifidogenic effect, meaning it specifically promotes the growth of bifidobacteria. *Bifidobacterium* spp. metabolize inulin into acetate and lactate, which can then serve as substrates for butyrate production by other microorganisms through cross-feeding. Intriguingly, participants with the constipation subtype of IBS have been reported to have lower levels of lactate-utilizing and butyrate-producing bacteria (Chassard et al., 2012). *Anaerostipes* spp. is such a taxon that can utilize lactate for butyrate production (Louis et al., 2022; Shetty et al., 2020) and showed a stronger response among high-placebo-responders in our study. This suggests that baseline *Bifidobacterium* spp. relative abundances and their change may have influenced cross-feeding to *Anaerostipes* spp., contributing to stronger clinical responses to inulin. Previous research with inulin-type fructans has shown similar findings, indicating greater metabolic responsiveness in individuals with lower baseline bifidobacteria levels (Bouhnik et al., 2004; De Preter et al., 2008). These findings point towards a potential value of *Bifidobacterium* spp. as predictors in responder analysis.

The differences in baseline relative abundances of *Bifidobacterium* spp. and *Anaerostipes* spp., their response to inulin, and variations in other butyrate-producing taxa, alongside the sustained clinical improvement during wash-out in this subgroup, are intriguing. It should be noted that only 70% of participants provided fecal samples during wash-out, limiting our ability to conclusively link lasting gut microbiota changes to carry-over effects. Nevertheless, the differences in relative abundances and changes of butyrate-producing bacteria, along with previous research linking such taxa beneficially to amelioration of constipation, suggest that variations in gut microbiota could have contributed to carry-over effects. Additionally, besides impacting microbiota composition directly, inulin might have influenced other aspects of the gut-brain interactions, such as gut barrier function and neuro-immunological aspects, which were not specifically investigated in this study.

One notable outcome following inulin intake, seemingly unaffected by carry-over effects, was the improvement in PAC-QOL scores. Specifically, the psychosocial discomfort and worries and concerns subscores showed improvement during inulin intake, contrasting with minimal change during placebo intake. Generally, baseline PAC-QOL scores aligned with reference values but were slightly higher for these subscores,

indicating a greater impact on participants' overall quality of life (Marquis et al., 2005). The psychosocial discomfort subscore encompasses aspects of the condition's interference with daily life, such as embarrassment, worries related to toilet visits, and considerations regarding routine, appetite, and food intake (Marquis et al., 2005). The worries and discomfort subscore encompasses aspects regarding emotional well-being, including irritability, upset, obsession with the condition, stress, impaired self-confidence, and feelings of control over one's body and condition (Marquis et al., 2005). It is important to note that habitual dietary intake throughout the study was not assessed, which is a limitation as it could have provided additional insights into changes related to these subscores. Nonetheless, the improvement in these subscores of social and emotional well-being was consistent across both the cross-over and first-period analyses and absent during placebo for both sequences, indicating a genuine effect of active inulin intake. While improved stool frequency certainly contributed to social and emotional well-being, changes in frequency alone are unlikely to be the sole driver, given that stool frequency also improved significantly during placebo intake. Instead, the improvement in these subscores may be partly attributed to inulin-induced reductions in abdominal symptoms, including discomfort, bloating, and cramps, as measured with the PAC-SYM. The reduction in abdominal symptoms was particularly noteworthy, considering that adverse events indicated that inulin increased bloating and flatulence, yet these effects did not worsen PAC-SYM scores. Collectively, the observed improvements suggest that inulin-mediated modulation of the intestinal environment benefits specific aspects of the gut-brain interaction, potentially leading to perceived changes in social and emotional well-being.

Although the exact mechanism of such an effect remains unclear due to the lack of additional biomarkers, initial applications of inulin in animal models of constipation-related depression and anxiety have demonstrated beneficial effects by affecting neuroimmunology and gut barrier function aspects of the gut-brain interaction (Zou et al., 2024). Inulin-derived butyrate may play a role here, as seen in animal models where butyrate induced neuroplastic changes in the enteric nervous system and affects vagus nerve activity (Mörkl et al., 2023; Suply et al., 2012). Additionally, other microbial-derived metabolites and direct activation of the vagus nerve by bifidobacteria have been suggested (Mörkl et al., 2023). Future studies on DGBI should consider integrating assessments of common bowel habit markers like stool frequency and consistency with selected gut microbial, neuroimmunological, and barrier function biomarkers (e.g., zonulin (Jian et al., 2022)) to comprehensively understand their impact on quality of life, particularly emotional and social well-being.

The analysis of the first period, serving as an assessment of the robustness of the cross-over analysis despite the observed carry-over effects, largely confirmed the observed inulin-induced changes in stool frequency and PAC-QOL. However, it also highlighted some differences. In the cross-over analysis, we did not observe a significant increase in the relative abundance of bifidobacteria or a decrease in rectal symptoms, but *Bifidobacterium* spp. relative levels were inversely associated with

rectal symptoms. When analyzing the first period only, we noticed an evidently larger fold-change in bifidobacteria, higher final BSFS scores, better satisfaction with bowel habits, and lower final rectal symptom scores. It seems plausible that softer stools correspond to reduced rectal symptoms, which include rectal burning, bleeding, and the feeling of incomplete evacuation (Frank et al., 1999), thereby resulting in better bowel habit satisfaction. Conversely, the lack of changes in stool consistency in the cross-over analysis might explain why we did not observe marked effects on stool-related symptoms, including hard, small stools and straining (Frank et al., 1999). In both the cross-over and first-period analyses, the inulin-induced decrease in PAC-QOL score exceeded the minimum important difference of -0.50 in either analysis, but the difference between inulin- and placebo-induced changes only exceeded this threshold in the first-period analysis (Marquis et al., 2005). Similarly, the inulin-induced decrease in total PAC-SYM symptoms exceeded the minimum important difference of -0.60, but the difference between inulin- and placebo-induced changes did not meet the -0.75 minimum important difference proposed for placebo-controlled studies (Yiannakou et al., 2017). While the PAC-QOL and PAC-SYM scores showed clinically meaningful changes, it might seem surprising that overall well-being (PAC-QOL) and treatment satisfaction (from the first-period analysis) improved significantly while symptoms (PAC-SYM) did not. However, the PAC-QOL score is influenced by improvements in stool frequency and abdominal pain, both of which were enhanced by inulin intake. Traditional dietary fiber interventions typically focus heavily on assessing stool frequency, consistency, and symptoms. Recent guidelines underscore that perceived symptoms and their impact on patients' lives, including their "bothersomeness", are crucial in diagnosing DGBI (Drossman & Tack, 2022). Therefore, relying solely on fecal/stool-related aspects (symptoms and stool consistency) provides an incomplete picture when assessing the outcomes of non-pharmacological treatments for DGBI. Instead, broader, more patient-centric measures are necessary.

The findings from our study highlight the importance of carefully considering the use of cross-over designs and the duration of wash-out periods in future studies assessing the effects of dietary fiber on the fecal microbiota. While cross-over trials can reveal differences between individuals, their suitability for DGBI should be debated. Previous recommendations for studying pharmacological treatments in such disorders have preferred parallel designs due to the variability of symptoms over time in both adults and children (Irvine et al., 2006; Koppen, Saps, et al., 2018; Veldhuyzen Van Zanten et al., 1999). We argue that similar considerations should apply to non-pharmacological treatments aimed at altering the gut microbiota.

In addition to the challenge posed by the suspected carry-over effect of inulin on the statistical evaluation of the treatment effect, its lasting impact may also be advantageous. Inulin could induce sustained changes in the gut environment, benefiting gut-brain interaction, as evidenced by reductions in PAC-QOL worries, concerns, and psychosocial discomfort subscores. Unlike pharmacological interventions such as laxatives, inulin, as a dietary component, likely influences multiple aspects of the

gut environment simultaneously, including gut microbiota composition, metabolite production, epithelial proliferation, and immune function. These effects may develop gradually over time without the drawbacks associated with laxative use. Future research should explore how inulin's lasting effects relate to specific fecal microbiota signatures, given the limited fecal samples in our study. These studies should also assess other biomarkers relevant to DGBI, such as gut barrier and immune function markers.

In conclusion, our cross-over study confirmed that inulin is a promising non-pharmacological treatment alternative. It improved constipation-related quality of life, social-emotional well-being as well as abdominal symptoms, and promoted gut health by increasing stool frequency and modifying the fecal microbiota in functional constipation.

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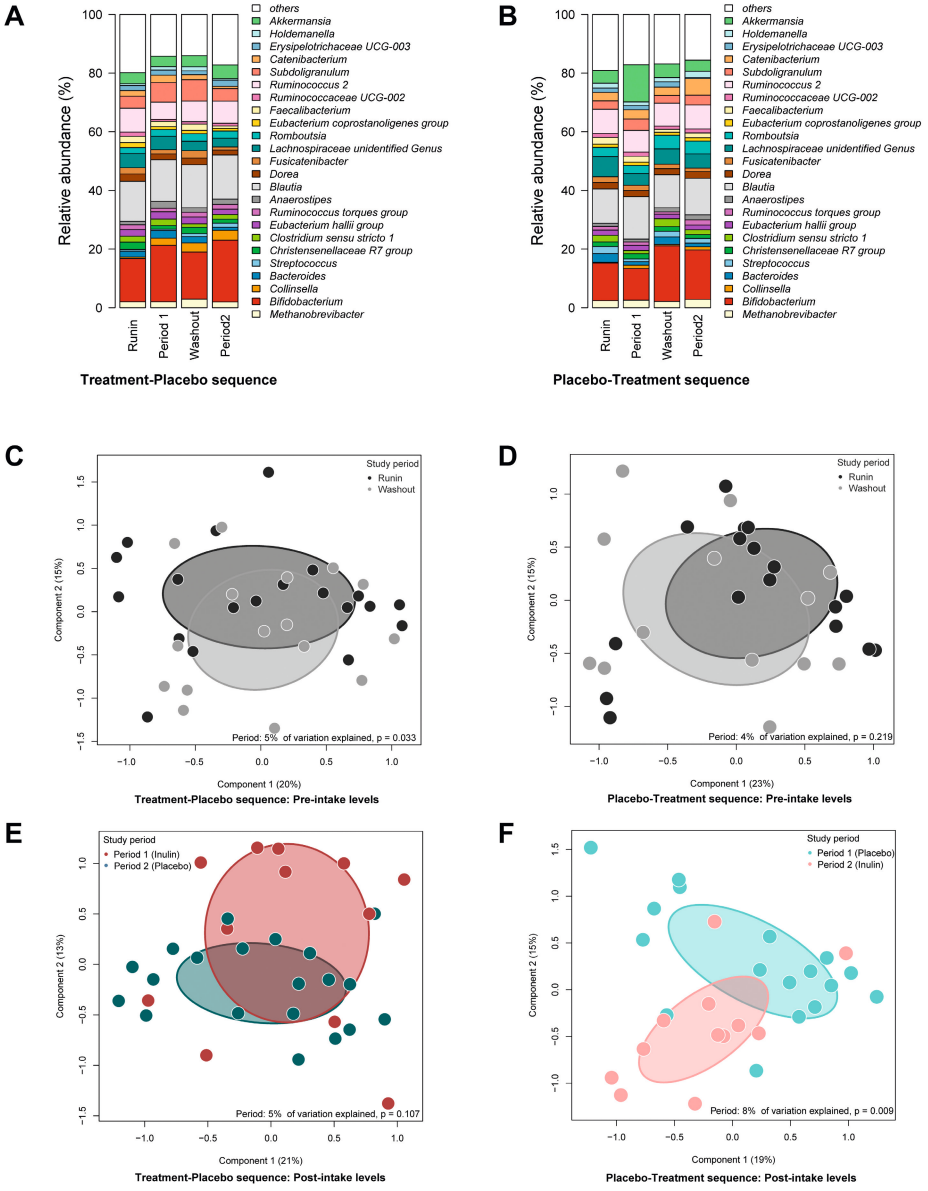
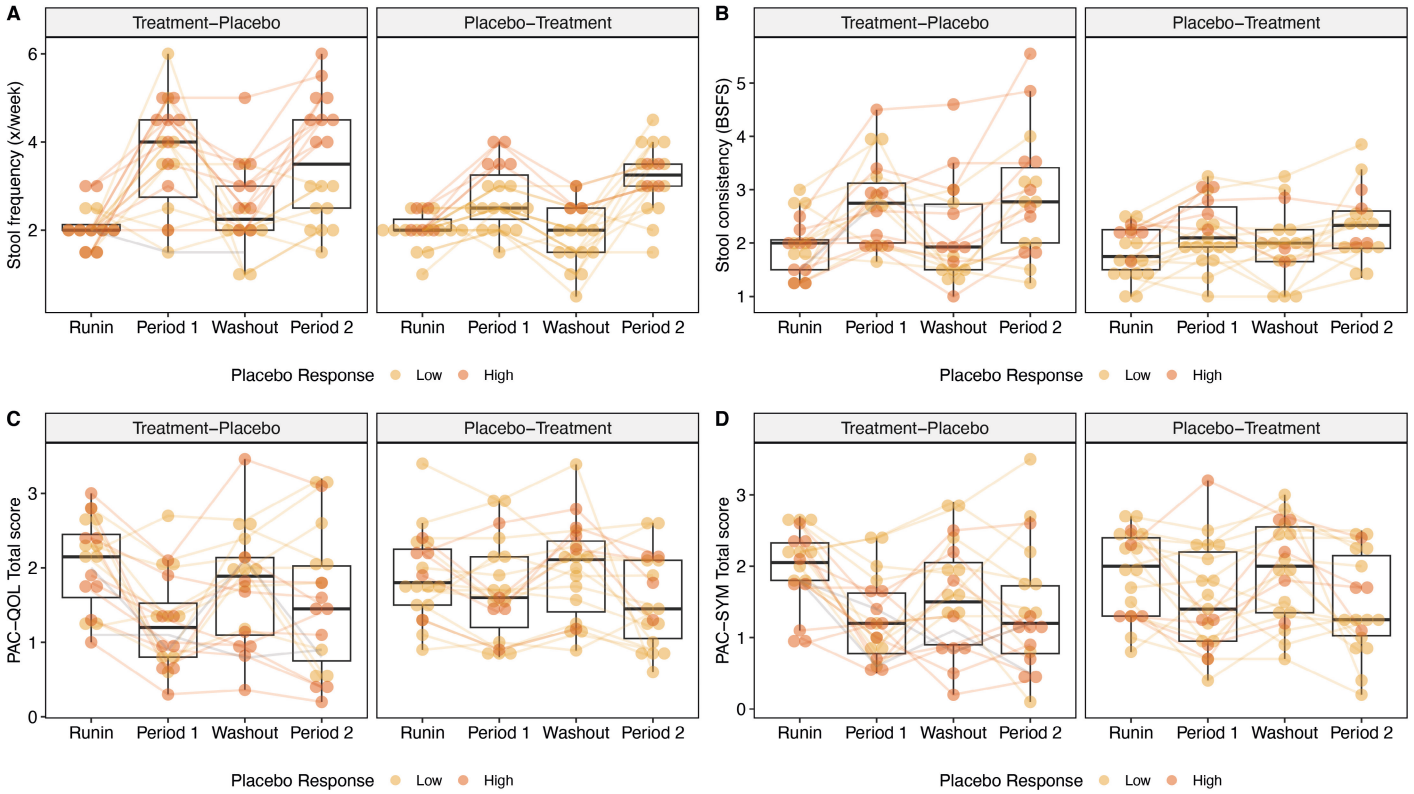
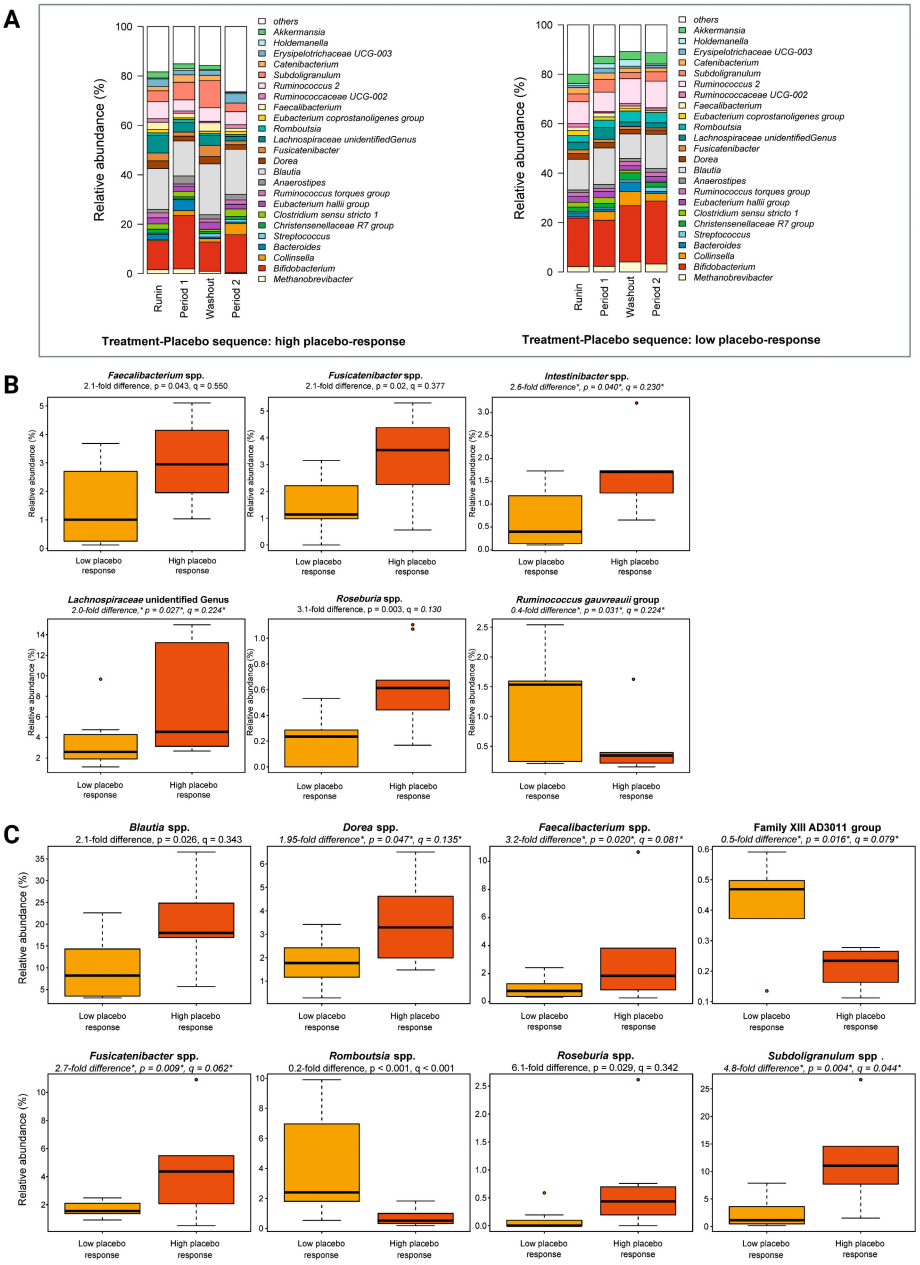
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Fecal microbiota composition in each intervention sequence. (A-B) Common genera (>1% abundance in 50% of the samples) at each study period in (A) the treatment-placebo sequence and (B) the placebo-treatment sequence. (D-F) fecal microbiota composition at genus level visualized and assessed by principal coordinate analysis using β-diversity based on Bray-Curtis dissimilarity for pre-intake levels in (C) the treatment-placebo sequence and (D) the placebo-treatment sequence and for post-intake levels in (E) the placebo-treatment sequence and (F) the treatment-placebo sequence. The difference between each group was tested using PERMANOVA and corresponding p-values are depicted in the lower right of the figures.



Supplementary Figure 2. Individual responses in each intervention sequence. Differences in the treatment-placebo or placebo-treatment sequence comparing participants with a high placebo response (stool frequency > 3x/week) and those with a low placebo response (stool frequency <= 3x/week) for (A) stool frequency (defecations per week), (B) stool consistency (Bristol Stool Form Scale), (C) patient-assessment of constipation - Quality of life (PAC-QOL) total score, patient-assessment of constipation - Symptoms (PAC-SYM) total score.



Supplementary Figure 3. Differences in fecal microbiota between high- and low-placebo-responders in the treatment-placebo sequence. (A) Common genera (>1% abundance in 50% of the samples) at each study period in subjects of the treatment-placebo sequencing with either a high or low response to placebo intake. Note that group averages are based on decreasing the number of fecal samples during wash-out and period 2. (B) Run-in differences in genera identified to differ statistically significantly in relative abundances between subjects with low and high placebo responses. (C) Wash-out differences in genera identified to differ statistically significantly in relative abundances between subjects with low and high placebo response. Fold differences, p-values, and FDR-corrected q-values denoted with an asterisk * are based only on samples with the taxon present.

Supplementary Table 1. First period analysis of changes in stool frequency, stool consistency, PAC-QOL, PAC-SYM and physical activity. Pre, post-intake levels and changes are reported as mean (SEM) if not indicated otherwise, and differences in changes are reported as estimated differences (95% CI) between treatments with the number of observations included (n) and p-values.

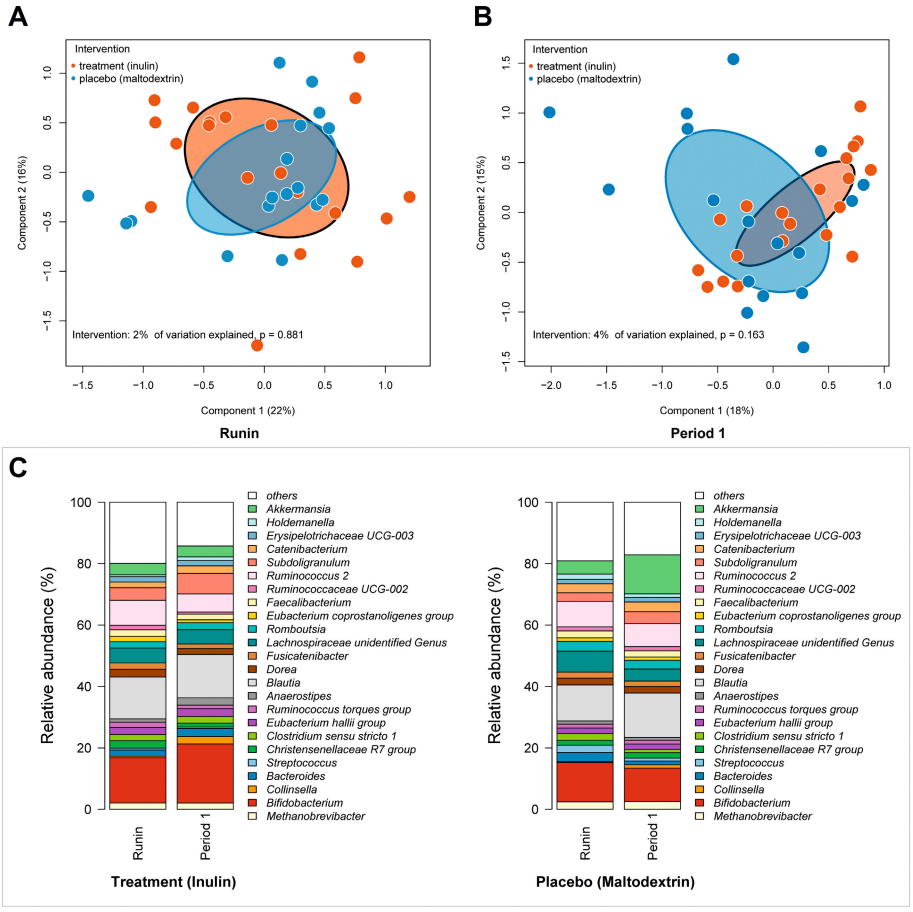
	Inulin			**Placebo**			**Difference change Inulin vs. Placebo**		
	Run-in	*Period 1*	*Change*	*Run-in*	*Period 1*	*Change*	*Difference*	*95%CI*	*p-value*
Stool frequency (x/week)[1]	2.10 (0.18)	3.66 (0.18)	1.56 (0.26)	2.03 (0.18)	2.74 (0.18)	0.71 (0.26)	0.85 (0.36)	[0.15, 1.55]	0.022
Stool consistency (BSFS)	1.92 (0.14)	2.75 (0.14)	0.83 (0.16)	1.82 (0.14)	2.23 (0.14)	0.40 (0.16)	0.43 (0.22)	[-0.01, 0.86]	0.062
PAC-QOL									
Total score	2.05 (0.14)	1.26 (0.14)	-0.79 (0.13)	1.89 (0.16)	1.71 (0.16)	-0.18 (0.13)	-0.61 (0.16)	[-0.97, -0.24]	0.003
Physical discomfort	2.37 (0.17)	1.37 (0.17)	-1.00 (0.17)	2.24 (0.17)	1.76 (0.17)	-0.48 (0.18)	-0.52 (0.25)	[-1.01, -0.04]	0.042
Psychosocial discomfort	1.58 (0.17)	0.93 (0.17)	-0.65 (0.15)	1.21 (0.17)	1.14 (0.17)	-0.07 (0.16)	-0.58 (0.22)	[-1.01, -0.15]	0.011
Worries and concerns	1.84 (0.17)	1.16 (0.17)	-0.68 (0.14)	1.72 (0.17)	1.55 (0.17)	-0.16 (0.15)	-0.52 (0.20)	[-0.92, -0.12]	0.016
Satisfaction	3.18 (0.16)	1.95 (0.16)	-1.23 (0.22)	3.34 (0.17)	2.94 (0.16)	-0.40 (0.23)	-0.82 (0.32)	[-1.44, -0.21]	0.013
PAC-SYM									
Total score	1.98 (0.14)	1.25 (0.14)	-0.73 (0.15)	1.90 (0.14)	1.56 (0.14)	-0.34 (0.15)	-0.39 (0.21)	[-0.80, 0.02]	0.072
Abdominal symptoms	2.13 (0.16)	1.34 (0.16)	-0.79 (0.19)	1.94 (0.17)	1.64 (0.17)	-0.30 (0.19)	-0.50 (0.27)	[-1.03, 0.03]	0.075
Rectal symptoms	1.25 (0.15)	0.59 (0.15)	-0.66 (0.18)	1.52 (0.15)	1.11 (0.15)	-0.41 (0.18)	-0.25 (0.26)	[-0.75, 0.25]	0.338
Stool related symptoms	2.29 (0.18)	1.58 (0.18)	-0.71 (0.17)	2.12 (0.18)	1.75 (0.18)	-0.37 (0.17)	-0.34 (0.24)	[-0.81, 0.12]	0.158
IPAQ[2]									
"low"	5 (25%)	7 (36.8%)	-	4 (21.1%)	2 (11.1%)	-			
"moderate"	12 (60%)	7 (36.8%)	-	12 (63.2%)	9 (50%)	-	-		0.189*
"high"	3 (15%)	5 (26.3%)	-	3 (15.8%)	7 (38.9%)	-			

BSFS, Bristol Stool Form Scale; IPAQ, international physical activity questionnaire; PAC-QOL, patient assessment of constipation – quality of life; PAC-SYM, patient assessment of constipation – symptoms

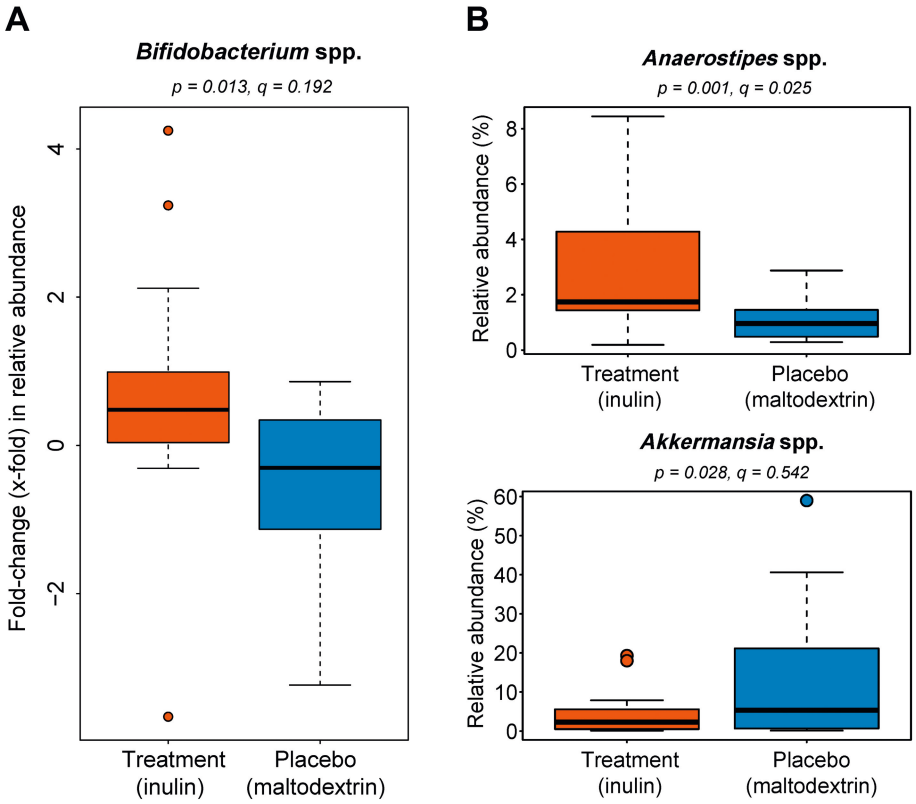
[1]Stool frequency values expressed as median (IQR) were for inulin: pre-intake = 2.00 (2.00, 2.12), post-intake = 4.00 (2.75, 4.50), and change = 1.50 (0.75, 3.00), and for placebo: pre-intake = 2.00 (2.00, 2.25), post-intake = 2.50 (2.25, 3.25) and change = 0.50 (0.25, 1.25). The median [95%CI] difference between changes after inulin versus placebo intake was 1.00 [>0.00, 1.50] and p = 0.030

[2] Values are expressed as count (%)

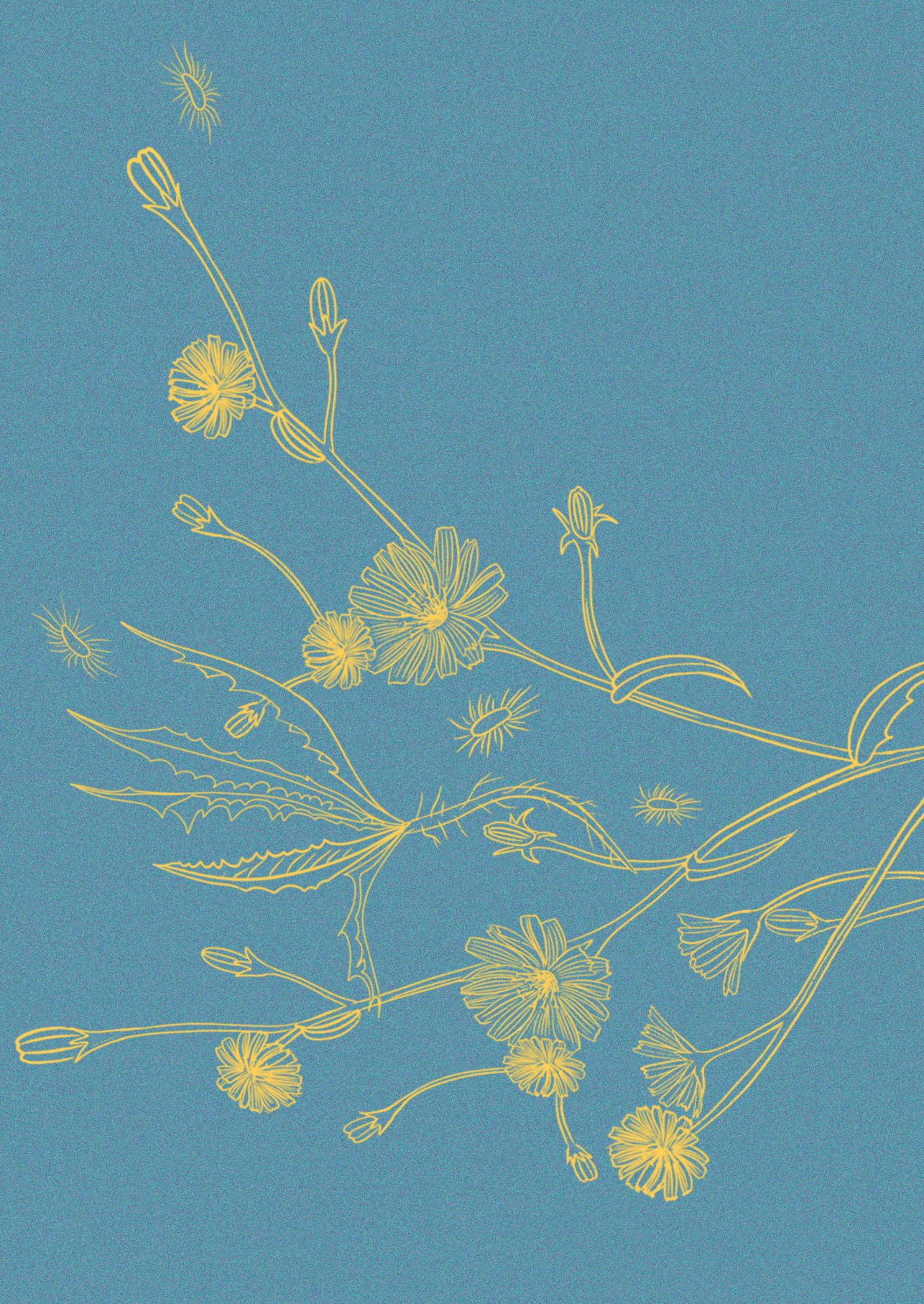
** represents the p-value for the difference in post-intake counts*



Supplementary Figure 4. First period analysis of fecal microbiota composition. (A-B) Fecal microbiota composition at genus level was visualized and assessed by principal coordinate analysis using β-diversity based on Bray-Curtis dissimilarity at (A) run-in and (B) after period 1 of the intervention (either inulin or placebo intake). (C) Common genera (>1% abundance in 50% of the samples) at run-in and after period 1 in participants consuming either the treatment (inulin) or the placebo (maltodextrin).



Supplementary Figure 5. First period analysis of differences in individual genera. (A) Differences in fold-changes after period 1 between interventions (inulin versus placebo) in *Bifidobacterium* spp. relative abundances. (D) Differences in period 1 relative abundances of *Anaerostipes* spp. and *Akkermansia* spp. between interventions (inulin versus placebo). Note that the difference in *Akkermansia* spp. relative abundances were mainly caused by one individual consuming the placebo, where *Akkermansia* spp. comprised 80% of the fecal microbiota.



CHAPTER 8

The impact of dried chicory root on bowel function and the gut microbiota in adults with bowel function issues in the Netherlands: a study protocol for a double-blinded randomized controlled trial (HappYFiber study).

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In preparation for submission

ABSTRACT

Background: Bowel function issues, characterized by a low defecation frequency and hard stools without warranting medical intervention, are widespread and fundamentally affect the overall quality of life. A commonly hypothesized cause is inadequate fiber consumption. Therefore, increasing dietary fiber is often recommended to alleviate bowel function issues. The gut microbiota contributes to bowel function by mediating the colonic effects of fiber, making it a promising target for improving bowel function satisfaction. The aim of this study is to assess the impact of dried chicory root on bowel function and the gut microbiota in healthy individuals with self-reported bowel function issues.

Methods: This study is a double-blinded, randomized, placebo-controlled, parallel trial with four arms in which subjects are exposed to a four-week intake of three different dosages of dried chicory root particles or placebo. A total of 160 healthy adults aged 20 to 80 years with bowel function issues living in the Netherlands will be recruited. The primary objective of the study is to evaluate whether the intake of dried chicory root improves bowel function, which will be assessed through subjective measures, including defecation ease, feeling of complete bowel emptying, and satisfaction, as well as objective measures such as stool frequency and consistency. Secondary objectives are to assess whether these effects are dose-dependent and associated with the modulation of the gut microbiota and activity. Furthermore, the time-dependent adaption of bowel function and the gut microbiota and its activity will be investigated.

Discussion: Bowel function is a complex interplay of factors related to the experience surrounding the process of defecation. The formation, movement, and elimination of feces are partly influenced by the residing gut microbiota, which may be modulated by dietary fiber intake. This study will illuminate the role of gut microbiota modulation through increased dietary fiber intake in alleviating bowel function issues. Our findings will increase the understanding of the role of the gut microbiota in bowel function issues, highlighting possible avenues for therapeutic interventions for nonmedical bowel function issues.

Trial registration: The trial is registered prospectively at ClinicalTrials.gov under NCT05473793 on 22/07/2022.

Keywords: bowel function satisfaction, intrinsic dietary fiber, prebiotics, dried chicory root, gut microbiota, stool consistency, stool frequency, quality of life, functional constipation

BACKGROUND

A healthy bowel that is perceived as well-functioning by individuals is essential to human health and substantially impacts the overall quality of life. Bowel function is a combination of several simultaneous processes involved in the formation, movement, and elimination of stools. These processes encompass the frequency of bowel movements, the softness of stools, and their subjective evaluation, including perceived ease of defecation, completeness of bowel emptying, and general satisfaction with bowel functions (Grønlund et al., 2018; Mysonhimer & Holscher, 2022; Ueberall et al., 2011). Studies indicate that only approximately half of the general population has normal bowel function (Heaton et al., 1992). In the Netherlands, approximately one-sixth of the population is estimated to fulfill the diagnostic definition (Rome IV criteria) for functional constipation, warranting professional attention (Verkuijl et al., 2020).

The exact cause of bowel function issues is not clear. Although factors such as low physical activity and water intake have been proposed as potential causes (Arnaud, 2003; Boilesen et al., 2017; Tantawy et al., 2017; Wilson, 2020), bowel function issues are most commonly attributed to insufficient dietary fiber intake (Lemay et al., 2021; Yang et al., 2012). Fiber intake affects bowel function in several ways (McRorie & McKeown, 2017). As fibers are by definition not digested in the upper gastrointestinal tract, they reach the lower gut largely unchanged. There, fibers interact with the gut microbiota, a complex community of gut bacteria and other microorganisms. Fibers that cannot be broken down by the gut microbiota, called non-fermentable fibers, may hold water or mechanically stimulate the colon, resulting in softer, bulkier stools and faster transit time (McRorie & McKeown, 2017). Thereby, non-fermentable fibers may impact the flux of nutrients to the gut microbiota (Daniel, 2022). In contrast, fermentable fibers can be broken down and used by gut bacteria as a source of carbon and energy for growth. This may increase bacterial mass and lead to the production of bacterial fiber fermentation metabolites, notably short-chain fatty acids (SCFAs). SCFAs play a crucial role in human (gut) health, extending the beneficial effect of fibers beyond their physicochemical properties (Blaak et al., 2020). For instance, butyrate, which serves as fuel for colonocytes, is also one of the most important SCFAs involved in various G protein-coupled receptor-dependent signaling pathways and strengthens the gut lining and barrier function (Canfora et al., 2015; Hamer et al., 2008; Wells et al., 2017). Taken together, resident gut bacteria and their metabolites are hypothesized to influence bowel function and indeed, the gut microbiota composition has been associated with stool consistency, frequency, and transit (Asnicar et al., 2021; Vandeputte et al., 2016). Nevertheless, the interplay between gut microbiota and bowel function is still poorly understood as it is mediated by the abundance of the resident bacteria, the flux of nutrients, including dietary fiber, and the stool water content, all of which affect the gut microbiota's activity toward fibers (Daniel, 2022). Interindividual differences in gut microbiota composition most likely play a role in this context, determining the amount of fiber needed to modulate the gut microbiota toward beneficial impacts on bowel function.

The ability of fiber to modulate aspects of bowel function, particularly fecal output, has long been of interest to the research field (Cummings, 2001), and in a number of studies, additional fiber intake has been demonstrated to improve bowel function (Eswaran et al., 2013; So et al., 2021). For instance, the fermentable fiber native inulin has been recognized for maintaining gut regularity and for selectively stimulating gut bacteria, such as *Bifidobacterium* spp. (EFSA, 2015; Le Bastard et al., 2019). The poorly fermentable fiber wheat bran is known for its effect on transit time when used as coarse particles, while its finely milled form can contribute to constipation despite the increase in fermentability of smaller particles (Brodribb & Groves, 1978; Deroover et al., 2017; Kirwan et al., 1972; Stewart & Slavin, 2009). Another poorly fermentable fiber, polydextrose, has been administered as well, but has not always shown an effect on bowel function (Duncan et al., 2018; Ibarra et al., 2019; Röytiö & Ouwehand, 2014). Additionally, in the clinical setting, psyllium is often recommended due to its water-holding capacity, but its intake has reportedly had only minor effects on the gut microbiota (Jalanka et al., 2019).

Most of the intervention studies with dietary fiber assessed a specific dosage of one type of fiber that is either synthetic or extracted and purified from their plant food source. These approaches, however, have two problems. First, administering only a single dose of fiber fails to consider possible interindividual differences in response to the fiber product, mediated by the unique composition of an individual's gut microbiota and its fiber fermentation activity (Korpela et al., 2014; Anne Salonen et al., 2014). Second, the structural organization of extracted and purified fibers differs considerably from how they are intrinsically present in their plant food source. In the plant tissue, fibers are part of the complex structures of plant cell walls (cellulose, hemicellulose, and pectins), or enclosed inside plant cells as nonstructural storage carbohydrates (such as starch and inulin) (Puhlmann & de Vos, 2022). To distinguish these complex structures from their extracted counterparts, these fibers have been coined 'intrinsic fibers' (Figure 1) (Augustin et al., 2020; Grundy et al., 2016; Hansen & Sams, 2018). The natural combination of different fiber types in intrinsic fibers likely leads to a synergistic effect on bowel function through the associated modulatory effects of water-holding capacity, mechanical irritation, and fermentation, predisposing intrinsic fibers to behave physiologically differently compared to extracted and purified fibers.

Differences in the physiological behavior between extracted and intrinsic fibers are primarily attributed to the kinetics of their breakdown in the human gut. *In vitro*, the plant cell matrices of intrinsic fiber slow bacterial fermentation, which, *in vivo,* likely results in fiber fermentation persisting throughout the entire colon instead of ceasing beyond the proximal colon, as observed for isolated fibers (Figure 1) (Dagbasi et al., 2020; Hamaker & Tuncil, 2014; Lu et al., 2020; So et al., 2021). Such a gradual release of fiber components and slow fermentation likely have beneficial physiological outcomes since they are hypothesized to reduce gas production (So et al., 2021) and generate SCFAs that can be taken up in the distal colon, benefiting metabolic health (Neis et al., 2019; van der Beek et al., 2016). Hence, intrinsic fibers are notable, minimally processed food products that promote gut health through gut microbiota modulation, emphasizing the need to explore their application in treating bowel function issues (Eswaran et al., 2013; So et al., 2021).

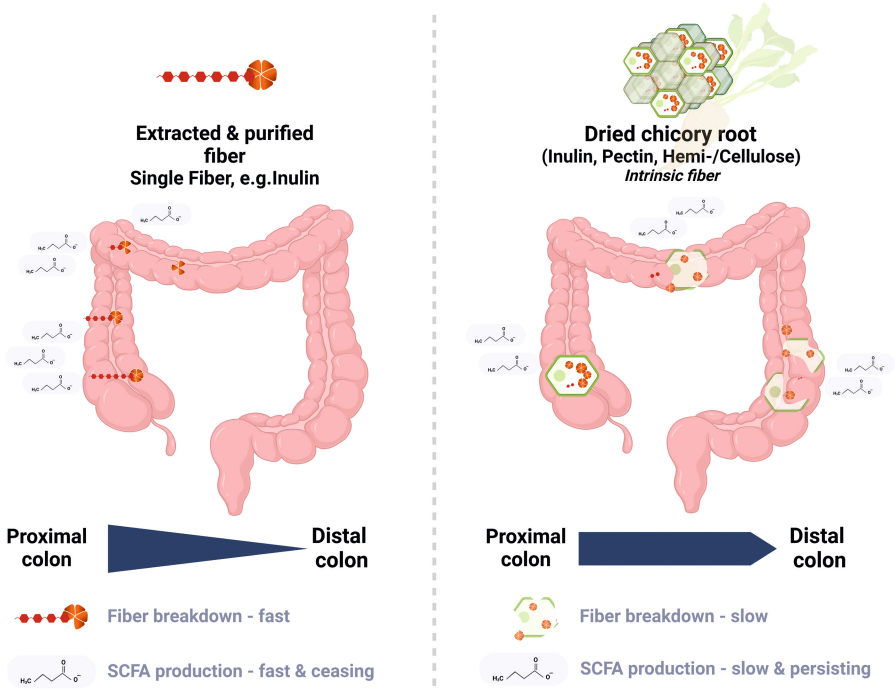

Figure 1. Proposed mechanism through which extracted, purified versus intrinsic fibers benefit bowel function. Extracted and purified fibers, such as inulin, are hypothesized to undergo proximal colon fermentation, resulting in a rapid increase in SCFAs due to bacterial fiber breakdown that ceases beyond the proximal colon. In contrast, intrinsic fibers, which are naturally present in plant foods and form plant tissues, are believed to undergo slow fermentation. Slower fermentation leads to persistent SCFA production from the proximal to the distal colon and thereby benefits gut health throughout the entire lower gastrointestinal tract.

Dried chicory roots are an intrinsic fiber product that may contribute to alleviating self-reported bowel function issues and improving bowel function satisfaction. Chicory roots have been consumed for centuries as both a culinary and medicinal foodstuff, and are naturally remarkably high in fiber due to their intracellular inulin content enclosed in the plant matrix, which consists of pectin, cellulose, and hemicellulose (Puhlmann & de Vos, 2020). We previously assessed the modulatory effect of dried chicory root as particles on the gut microbiota as well as on gut and systemic health in subjects at risk for type 2 diabetes (Puhlmann et al., 2022). We observed that a dose of 15 g/day could already modulate bowel function in predominantly elderly subjects who had no self-reported bowel function issues. Notably, dried chicory root intake increased fecal SCFA levels and modulated gut microbiota composition (Puhlmann et al., 2022). In particular, the relative abundance of bacteria belonging to *Bifidobacterium* spp. and *Anaerostipes* spp. increased, and members of these bacterial taxa were demonstrated to form a trophic chain that produced butyrate. For these reasons, we hypothesize that the additional intake of 15 g/day of intrinsic fibers in the form of dried chicory root

particles could modulate bowel function, thereby benefiting gut health through colonic bacterial fiber breakdown and overcoming reportedly insufficient fiber intake (Mertens et al., 2019; Stephen et al., 2017) to reach the levels recommended by the Dutch Health Council (Health Council of the Netherlands, 2006).

Here, we outline a study to assess the impact of dried chicory root, administered at different daily dosages (5 g, 10 g, and 15 g), compared with a control (placebo) on bowel function and gut microbiota composition and activity in subjects with self-reported bowel function dissatisfaction in combination with low stool frequency and/or hard stools. As bowel function consists of more than one aspect, we assess subjective outcomes (perceived ease of defecation, feeling of incomplete emptying, bowel function satisfaction) alongside commonly measured objective outcomes (stool frequency and consistency). This study is expected to contribute to our understanding of the impact of intrinsic fibers and the mechanisms of action by which these fibers modulate bowel function mediated by the gut microbiota.

MATERIALS/DESIGN

The primary objective is to study the effect of dried chicory root particles (WholeFiber™) on bowel function assessed by stool frequency, stool consistency, ease of defecation, feeling of incomplete bowel emptying, and bowel function satisfaction as well as on the gut microbiota and its activity in subjects with self-reported bowel function issues. The secondary objectives are to assess whether these effects are dose-dependent and associated with the modulation of the gut microbiota and its activity. Furthermore, the adaptation of bowel function and the gut microbiota and its activity over time will be analyzed.

STUDY DESIGN

The study is designed as a parallel, randomized, double-blind, placebo-controlled trial with four intervention arms consisting of three different dosages of dried chicory root particles versus a placebo for four weeks (Figure 2). The study period will be preceded by a two-week screening period during which a subject's bowel function is monitored to assess whether the subject meets the inclusion criteria for stool frequency and consistency. Thereafter, eligible subjects are randomly allocated to one of the four study arms and consume one of the intervention products (dried chicory root or placebo) for four weeks. The outcomes of the screening period will serve as baseline bowel function assessment (stool frequency and consistency, ease of defecation, feeling of incomplete bowel emptying, bowel function satisfaction, and gastrointestinal symptom occurrence) for subjects eligible to participate in the study. Baseline measurements regarding the quality of life and gastrointestinal symptom intensity, dietary intake, and physical activity are taken at the start of the study period (after the screening period) and will be retaken at the end of the last study week. During the intervention period,

subjects will record their bowel function daily, and collect a fecal sample weekly. We aim to recruit 160 subjects aged 20 to 80 years from the general population living in the Netherlands (speaking Dutch or English) with self-reported bowel function issues.

PARTICIPANT RECRUITMENT

Subjects will be recruited from within the Netherlands using the database of volunteers of the Division of Human Nutrition & Health and the Laboratory of Microbiology, Wageningen University & Research. Moreover, the following channels will be used to recruit subjects: social media channels of Wageningen University & Research, local newspapers in Wageningen and Ede, posters, and leaflets. We aim to include subjects in the age range of 20 to 80 years, reflecting the demographic age distribution within the Netherlands (Centraal Bureau voor de Statistiek, 2022). The research team will be responsible for recruitment, informing the subjects about the study and obtaining informed consent. Subjects interested in the research will receive the patient information letter and an invitation for an information session, which can be either online or in person at the Health Research Unit of Wageningen University & Research. After the information session, subjects who are willing to participate in the study and have no further questions are asked to sign an (electronic) informed consent form and a (digital) copy will be provided to them.

INCLUSION CRITERIA

To be eligible to participate in this study, a subject must meet the following criteria:

- 20–80 years old
- Unsatisfied with bowel function (self-reported), rated on a visual analog scale (VAS, 0-10) as <6
- Four or fewer bowel movements per week (low stool frequency) and/or
- Hard, lumpy, or solid stools (Bristol stool form scale 1-4) during 90% of bowel movements (hard to solid consistency)
- Able to read and understand Dutch or English

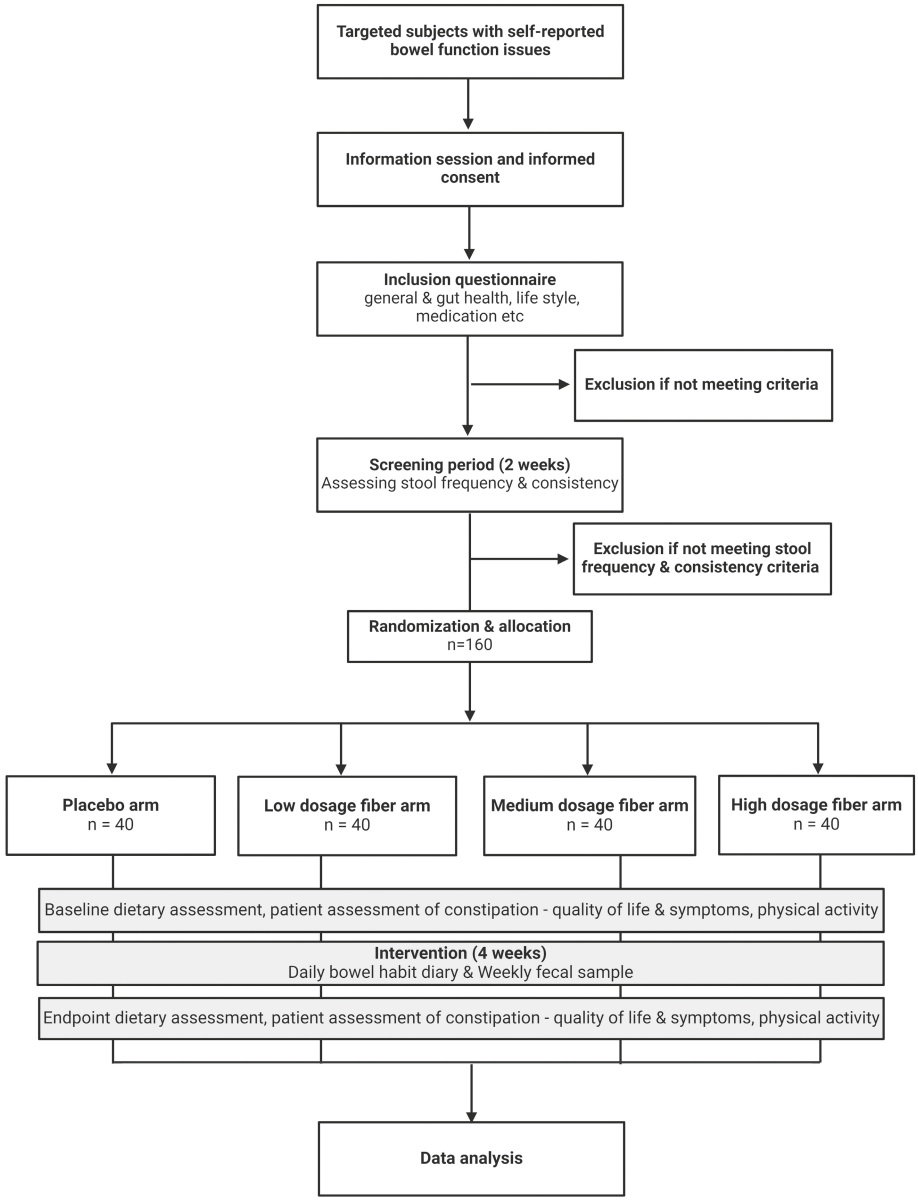

Figure 2. Flow chart of participants through the study.

EXCLUSION CRITERIA

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Having a history of medical or surgical events that may significantly affect the study outcome, e.g. irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD) patients and subjects with medically diagnosed constipation (i.e., constipation related to anatomic, medication, or readily identifiable physiological causes)
- Less than one bowel movement per week during the screening
- Medical drug use:
    - Antibiotic use within three months of the study screening day
    - Chronic use of antacids and proton pump inhibitors (PPIs)
    - Use of laxatives during the screening
- Chronic use of blood glucose-lowering medication
- Consumption of supplements containing fiber (other than laxatives), pro-, post-, or synbiotics one month before the screening
- Not willing to provide fecal samples
- Unable to comply with proper study procedures
- Current or planned pregnancy, lactation for subjects of reproductive age
- Known allergic reactions to plants from the *Asteraceae* (*Compositae*) family (e.g., lettuce, daisies, sunflowers, artichokes, sage, tarragon, chamomile, chicory, etc.)
- Reported unexplained weight loss or weight gain of > 5 kg in the month before pre-study screening
- Reported slimming or medically prescribed diet
- Reported macrobiotic lifestyle
- Personnel of the Division of Human Nutrition & Health or the Laboratory of Microbiology of Wageningen University & Research, The Netherlands
- Current participation in other medical scientific research
- Not having a general practitioner
- Not willing to be informed about accidental discoveries concerning the subject's health.

INTERVENTION

The study comprises four arms, consisting of three treatment arms that each receive a different dosage of the intervention product and a control arm that receives the placebo. The investigational product consists of dried chicory root particles (WholeFiber™ obtained from WholeFiber BV, Emmeloord, The Netherlands), a fiber product made from whole chicory roots. The dried chicory root particles contain approximately 85% dietary fiber, 70% of which is inulin enclosed within the plant cell matrix composed of pectin and hemi-/cellulose (15%). The dosages for the three intervention arms will be 5 g, 10 g, or 15 g of product per day. The average intake of ten grams of product per day delivers approximately 8.5 g of fiber, 1.0 g of other nutrients (proteins, sugars, organic acids), and 0.5 g of water per day. The energy content of each dosage is approximately 10.5 kcal for

5 g, 21.3 kcal for 10 g, and 32 kcal for 15 g per day, based on an estimated energy content of 2 kcal/g for fiber and 4 kcal/g for the other food compounds. As this additional energy intake is negligible compared to the total daily energy requirements, no additional recommendations regarding energy intake are needed for the subjects. The placebo arm will receive 5.5 g of rice puffs per day. This is a compromise between the energy contents of the highest and lowest doses of the intervention product (corresponding to the 10 g/day dried chicory root dosage). With an energy content of 3.86 kcal/g, 5.5 g of rice puff particles provide ~21.2 kcal per day, which is also considered negligible. Moreover, the fiber intake from rice puff crisps in this quantity is negligible as well, with 1.6 g fiber/100 g rice puff crisps amounting to 0.9 g fiber per day. Intervention products will be consumed daily in two portions throughout the day. The subjects will receive the intervention products packed per portion in individual sachets and will be instructed to consume two sachets per day.

The subjects are instructed to incorporate the intervention products into their regular diet, allowing them to choose the foods and meals of their choice. Compliance will be assessed by counting the unused and opened empty sachets, which subjects will be asked to return. Furthermore, subjects will be instructed to maintain their habitual diet and lifestyle (e.g., level of physical activity). Medications other than those leading to exclusion are not expected to affect the study outcomes and are thus allowed during the study. If subjects experience physical complaints related to ongoing low defecation frequency (more than three days without bowel movements), they will be allowed to use the over-the-counter enema Microlax. The enema will be administered and dosed according to the manufacturer's instructions. Subjects are monitored weekly and instructed to contact the researcher at any time in case of complaints to discuss potential follow-up measures and, if needed, to consult the research physician.

RANDOMIZATION, TREATMENT ALLOCATION, AND BLINDING

Subjects are allocated to one of the four arms at a randomization ratio of 1:1:1:1 (n = 40 per arm). The allocation sequence will be generated in R using the package mergedblocks with the assistance of an independent statistician (van der Pas, 2021). Randomization within the four arms will be stratified by age group (20–40 years or >40–80 years), sex, and baseline bowel function inclusion criteria (only low frequency, only hard-to-solid stools, or their combination). The age group distribution will represent the current demographics of the Netherlands (Centraal Bureau voor de Statistiek, 2022), with approximately one-third of the subjects aged 20–40 years and two-thirds of the subjects aged >40–80 years. Moreover, a balanced distribution of sexes in all study arms is required to account for bias in bowel function patterns in subjects of female sex and reproductive age due to hormonal fluctuations throughout the menstrual cycle (Heaton et al., 1992; Judkins et al., 2020; Verkuijl et al., 2020). Finally, based on preliminary screening results, we anticipate recruiting a study population of which approximately 40% of the subjects only has a low stool frequency, approximately 40% of the participants only has hard-to-solid stools, and approximately

20% of the participants has a combination of both. To mitigate potential bias, we will randomize subjects across strata of age and sex. Subjects are enrolled and assigned to the intervention by a member of the research team and both the researchers and the participating subjects are blinded to the study product allocation. Study products are packaged in nontransparent sealed sachets inside boxes and a randomization code will be assigned to each of the study products. The labelling of the products is handled by an independent researcher. The randomization key will not be broken until after the primary data analysis. Only in case of an emergency, the treatment can be unblinded after consultation with the research physician and the principal investigator at Wageningen University & Research.

OUTCOME MEASUREMENTS

Bowel habit diary

Bowel function assessed by stool frequency, stool consistency, ease of defecation, feeling of incomplete bowel emptying, and bowel function satisfaction will be recorded using a daily (online) questionnaire. Subjects will be asked daily whether they had any bowel movement(s) on that day. For each bowel movement, subjects are asked to score their stool consistency using the Bristol Stool Form Scale (BSS). For subjects without a shelf-style toilet, a flushable, paper feces catcher will be provided to be able to score the stool consistency. Subjects will also indicate for each bowel movement the ease of defecation, the feeling of incomplete bowel emptying, and bowel satisfaction, using a five-point scale. This five-point scale is identical to the subscales of the Patient Assessment of Constipation (PAC) questionnaires (Marquis et al., 2005). The bowel habit diary will be completed daily during both weeks of the screening period and the four weeks of the study period. Additionally, during the two screening weeks and the last two weeks of the intervention period, subjects will record daily the occurrence of twelve gastrointestinal symptoms, which are identical to the subscales of the PAC-SYM questionnaire (Frank et al., 1999).

Patient assessment of constipation – quality of life and symptoms

Bowel function-associated quality of life (QoL) and symptoms (SYM) are assessed using the PAC-Quality of Life (QoL) (Marquis et al., 2005) and PAC-Symptoms (SYM) (Frank et al., 1999) questionnaires. Both questionnaires retrospectively assess over the past two weeks different aspects of QoL (28 items) and the intensity of gastrointestinal symptoms (12 items) using five-point scales. In addition to symptom intensity, subjects will be asked to monitor the occurrence of the respective symptoms in their bowel habit diaries (see above). The PAC-QoL and PAC-SYM questionnaires will be administered at the beginning and end of the study over the last two weeks.

Transit time assessment
Transit time will be assessed using the blue dye method, which is a newly established method relating transit time to stool frequency, consistency, and gut microbiota composition (Asnicar et al., 2021). The blue dye method involves the consumption of muffins colored with a food-grade blue dye, which is water soluble and stains the luminal contents of the gastrointestinal tract. Due to its poor absorbability, the dye is excreted in the feces. Recording the time between ingestion and excretion of the blue dye, based on the first appearance in the feces, makes it possible to calculate the transit time. The transit time assessment is optional for subjects. Those willing to participate in this assessment will be instructed to consume the two muffins during the morning and to record the date and time of ingestion and the date and time of the blue dye appearing in the feces (Asnicar et al., 2021). Transit time will be assessed during the screening phase before the start of the study and during the last week of the study.

Stool sampling and subsequent analysis of the gut microbiota and SCFAs
Subjects will receive a home sampling kit with instructions for collecting fecal samples. Each fecal collection kit contains flushable paper feces catchers, gloves, a cold transport container (Sarstedt, Nümbrecht, Germany), and sterile feces sample tubes with a screw cap and attached spoon. The subjects will be instructed to freeze the cold transport container in their home freezers at −20°C. Feces will be collected using a paper feces catcher to prevent contamination of feces with urine or toilet water. Fecal tubes will then be filled for one-third with a fecal sample taken from the middle part of the feces. After collection of the sample, the tubes will be stored immediately in pre-frozen cold transport containers in the home freezer until they are returned frozen to the research facility, where they are stored at −80°C until further processing. DNA extraction, 16S rRNA gene amplicon sequencing, and fecal SCFA assessment will be performed according to previously described methods (Puhlmann et al., 2022). The baseline fecal sample will be taken before starting the intake of the intervention products, and subsequent fecal samples will be collected once per week at the end of each study week.

Physical activity assessment
Physical activity will be measured using the Baecke questionnaire (Baecke et al., 1982). The Baecke questionnaire is a short validated questionnaire (16 questions) developed in the Netherlands to assess current habitual physical activity using five-point scales. Similar to the PAC-QoL/SYM, the Baecke questionnaire will be administered at the beginning and end of the study.

Dietary assessment
Three-day food intake will be measured at the beginning and the end of the study using a smartphone-based application (TRAQQ (Lucassen et al., 2021)) on two weekdays and one weekend day to assess background intake of dietary fiber and other nutrients as

well as fluid intake. Additionally, fiber intake will be specifically assessed at the start of the study (screening period) using the short questionnaire FiberScreen (Rijnaarts et al., 2021).

Table 1. Study outcomes and their respective measurement tools and time points.

Outcome	Measurement tool	Screening		Study				
		Wk -2	Wk -1	Wk 1		Wk 2	Wk 3	Wk 4
Bowel function	Bowel habit diary	Daily	Daily		Daily	Daily	Daily	Daily
Gastrointestinal symptom occurrence	Bowel habit diary	Daily	Daily				Daily	Daily
Transit time	Blue dye method		1x					1x
Fiber intake	FiberScreen		1x					
Dietary intake	Traqq smartphone application for dietary assessment			1x				1x
Quality of life & constipation symptoms	PAC – QoL & SYM			1x				1x
Physical activity	Baecke questionnaire			1x				1x
Gut microbiota	Home stool sampling kit			1x	1x	1x	1x	1x

PAC-QoL, Patient Assessment of Constipation – Quality of Life; PAQ-SYM, Patient Assessment of Constipation – Symptoms

SAMPLE SIZE CALCULATION, DATA HANDLING, AND DATA ANALYSIS

Sample size calculation

Sample size calculations were performed in R version 4.0.3 (R Core Team, 2023) using the power.t.test() function from base R and the pwr package (Champely et al., 2020). A t-test was used with Bonferroni correction accounting for the four arms (alpha/number of arms). The sample size in this study was based on available information regarding stool consistency from a previous study using dried chicory root (Puhlmann et al., 2022) and was supported by the sample sizes applied in a previous trial using isolated inulin to assess changes in stool frequency (Micka et al., 2017). These studies were considered relevant for all aspects of bowel function, as an increase in stool frequency and consistency is likely interrelated with an increase in perceived ease of defecation, feeling of incomplete bowel emptying, and overall bowel function satisfaction. In a previous randomized controlled trial that used dried chicory root in prediabetic subjects without bowel function issues, also stool frequency (x/week) and consistency (using the Bristol Stool Form Scale [BSS]) (Puhlmann et al., 2022) were assessed. There, none of the subjects in the treatment group had a stool frequency of four times per week or less, but eight subjects had hard stools at baseline, with an average BSS score of 1–2.5. For these subjects, stool consistency increased after two weeks of 15 g/day dried chicory root intake by 1.5 units, with a standard deviation (SD) of 1.3. We consider

a one-unit increase in stool consistency (BSS score) to be clinically relevant. After a one-unit increase, individuals with a BSS score of 1-2 would have a final score of 2-3, where scores between 3-4 are considered normal (Lewis & Heaton, 1997). In our study, allowing for a 1.0 increase in BSS score with an SD of 1.3, using a t-test with a Bonferroni corrected significance level of $\alpha = 0.0125$ and a power of $\beta = 0.8$, a sample size of 40 per arm and a total of 160 subjects is needed. This sample size is in line with the sample size of a previous crossover study assessing the effect of four weeks of 12 g/day of isolated inulin on stool frequency with sample sizes of a total of 40 subjects (Micka et al., 2017). Subjects who do not complete the study for any reason will be considered drop-outs and will be replaced with subjects from the same randomization stratum.

Data handling and analysis

The data are collected and stored in the cloud-based Electronic Data Capture platform Castor EDC (Castor, 2024) using electronic case report forms (eCRFs). The final dataset will be accessible to only the research team under the supervision of the principal investigator. The data analysis will be performed using R (R Core Team, 2023) or comparable software. Normality will be checked for all continuous outcome variables by inspecting Q–Q plots, and statistical inference will be calculated using a two-sided alpha. For the descriptive analyses, the results will be presented as mean ± SD or median and interquartile range, depending on their distribution or percentage. The data will be analyzed according to the intention-to-treat principle as well as per-protocol. Bowel function outcomes will be primarily based on the data collected during the two-week run-in period and the last two weeks of the study period. Stool frequency will be calculated as the total number of bowel movements averaged per week. Stool consistency will be calculated by averaging the BSS score per bowel movement per week. In the same way, average scores for ease of defecation, feeling of incomplete bowel emptying, and bowel function satisfaction will be calculated. Gut microbiota composition will be calculated using between-sample (β-diversity) and within-sample (α-diversity) measures, as well as relative abundances of individual taxa. Descriptive statistics will be used to summarize values and changes from baseline for each time point.

The main analysis compares the highest treatment arm dose (15 g/day) with the control arm by assessing end-point differences (end of four-week intervention) from baseline in stool consistency, frequency, ease of defecation, feeling of incomplete bowel emptying, and bowel function satisfaction. For this purpose, analysis of covariance (ANCOVA) will be used with endpoint differences in stool consistency, frequency, ease of defecation, and bowel function satisfaction as outcome variables; treatment group as a factor; and baseline values of the outcome variables as covariates. Moreover, the analysis will be stratified according to the randomization stratification factors age group (20-40 vs >40-80), sex, and baseline bowel function inclusion criteria (only low frequency, only hard-to-solid stools, and their combination). Additionally, these factors, together with menstrual stage (for female sex), and physical activity, will be assessed as covariates in the main

analysis. The differences in least squares means between the treatment groups and the corresponding 95% confidence intervals (CIs) and p-values will be presented.

For the secondary study outcomes, end-point differences between all intervention arms (5 g, 10 g, and 15 g) and the control arm will be assessed regarding stool consistency, frequency, ease of defecation, feeling of incomplete bowel emptying, and bowel function satisfaction, as well as PAC-QoL, PAC-symptoms, fecal SCFA levels, and gastrointestinal symptom occurrence. For this purpose, methods similar to those used for the primary study parameters will be used.

The analysis of other study outcomes assesses the time-dependent adaption of stool consistency, frequency, ease of defecation, feeling of incomplete bowel emptying, bowel function satisfaction, and fecal SCFA levels. For this purpose, repeated-measures mixed effect modeling will be used with group, time and their interaction as fixed factors and subject as random factor. Similarly, the time-dependent adaption of the gut microbiota composition and differences of individual taxa will be assessed according to established methods including repeated-measures analysis with mixed effect modeling (Korpela et al., 2014; Korpela, 2016; Puhlmann et al., 2022). Additionally, associations will be assessed between transit time and primary outcomes and the gut microbiota as well as transit time for subjects who participate in the blue dye method. Finally, body mass index, fiber intake, physical activity, menstrual stage, and transit time will be summarized using descriptive statistics.

Adjusting for multiple testing will be performed for the primary study parameter comparison between the three intervention doses using Bonferroni correction and for gut microbiota analysis using false discovery rate (FDR) correction for multiple testing of individual taxa according to established methods (Korpela, 2016). For all the other tests, no adjustment for multiple testing will be performed; for exploratory purposes, parameter estimates with CIs will be reported together with corresponding calculated p-values as supportive evidence. Additionally, stool frequency and consistency, ease of defecation, feeling of incomplete bowel emptying, and bowel function satisfaction will be reported as categorical variables with the proportion (%) of subjects within each category.

ETHICAL CONSIDERATIONS

Ethical approval was granted by the medical ethical committee METC Oost-Nederland (protocol ID: NL80274.091.22), and the study was prospectively registered at ClinicalTrials.gov under NCT05473793. Protocol amendments will be submitted to the medical ethical committee for review. All protocol modifications will be updated in the trial registry, and relevant changes will be communicated to the participants and the journal. The study will be executed in accordance with the Declaration of Helsinki. No data monitoring committee is assigned as the study involves a low-risk intervention and no vulnerable population. Participation is voluntary, and subjects can withdraw from the study at any timepoint without providing a reason. Subjects may be withdrawn from the study in consultation with the research physician if bowel function issues worsen or if other (serious) adverse events occur. Information on (serious) adverse events is

collected weekly and documented by the research team. All subjects need to sign an informed consent form before the start of the study, and the subjects will be given at least one week to consider participation before being contacted again by the research team. Before the study, the subjects are informed about the study outcomes related to gut microbiota adaptation over time, and after completion of the study, subjects are informed about all study outcomes. All data will be handled confidentially and coded (in agreement with the General Data Protection Act (GDPA; in Dutch "AVG"). Before the start of the screening, subjects will be assigned a pseudonymized study code that is linked with the name, and contact information of the subject. This study code is securely stored in a password-protected file accessible solely to the research team, and only this code will be used for data collection, storage, and analysis.

DISCUSSION

Bowel function issues that warrant no immediate medical attention are widespread and substantially impact the quality of life. While insufficient fiber intake is often hypothesized to cause bowel function issues, the gut microbiota has been recognized as an equally important factor given its ability to metabolize fibers and thereby mediate the effects of fibers in the human colon. Until now, the majority of trials aiming to improve bowel function outcomes through fiber supplementation have focused predominantly on single-dosed, extracted, and purified fibers. However, intrinsic fiber, constituting the complex plant tissue matrix present in whole plant foods, could offer additional (gut) health benefits beyond local effects such as stool softening. Here, we describe the design of a randomized, placebo-controlled, parallel trial in adults living in the Netherlands that aims to elucidate the dose-dependent impact of microbiota-mediated fiber effects on self-reported bowel function issues using an intrinsic fiber product high in prebiotic fiber.

This study is designed as a parallel trial in line with longstanding recommendations on study designs addressing bowel function issues in adults and children in the medical field (Irvine et al., 2006; Koppen et al., 2018; Veldhuyzen Van Zanten et al., 1999). In addition to the potential bias by an order effect, where the experience of the first period influences that of the second—commonly known to impact crossover studies—the parallel design also circumvents challenges arising from the instability of problems over time (Irvine et al., 2006; Veldhuyzen Van Zanten et al., 1999). Moreover, defining a sufficiently long wash-out period for crossover trials targeting the gut microbiome remains challenging and could lengthen the study period, potentially compromising its timely and realistic execution and completion. Finally, the different dosages of the study products, combined with the appearance and sensory properties, may compromise blinding in a nonparallel design.

The choice of the intervention dosage, duration, and preceding screening period is based on a previous study using dried chicory root, the underlying timeframe of the

applied instruments, and other studies in the field. In a previous human randomized controlled trial in which dried chicory root particles were used in subjects at risk of type 2 diabetes with average normal bowel function, already a two-week intake of 15 g of dried chicory root per day modulated bowel function by increasing stool frequency and softness (Puhlmann et al., 2022). Here, we aim to assess whether a similar dosage can produce modulatory effects in a population with self-reported bowel function issues over a period of four weeks. Additionally, we are interested in whether such effects are dose-dependent and potentially mediated by the individual's gut microbiota composition and activity. To achieve this, we selected equal, increasing increments of an additional five grams of fiber product, consistent with findings from other previous fiber studies reporting measurable benefits (Ibarra et al., 2019; Lawton et al., 2013). To allow sufficient time for establishing potential effects based on the adaptation of the colon to increased fiber intake, we chose a four-week intervention with the last two weeks aligning with the timeframe of the retrospective PAC questionnaires (Frank et al., 1999; Marquis et al., 2005). The intervention duration of four weeks is in line with the findings of a previous study in which a substantial modulation of stool frequency was detected using 12 g of extracted, purified inulin—equivalent to the amount present in 15 g of dried chicory root (EFSA, 2015; Micka et al., 2017). Discrepancies between self-reported and measured bowel function have been reported in fiber intervention studies, as reflected in baseline stool frequencies and consistencies exceeding the inclusion criteria once subjects were included in the study (Ibarra et al., 2019; Watson et al., 2019). To avoid such discrepancies between self-reported and measured bowel function issues, a two-week screening period precedes the intervention period, during which subjects monitor their actual bowel function using a daily online diary.

Bowel function issues are not well-defined in nonmedical contexts, posing a challenge for designing a study applicable to the broader population. For this study, we will recruit subjects who report being unsatisfied with their bowel functions related to low stool frequency and/or consistency. Currently, only the Rome IV criteria provide an official definition of constipation in the medical context (Aziz et al., 2020; The Rome Foundation, 2021). Here, we aim to address a broader target population of nonmedical cases, i.e., those not requiring the use of medication to defecate and not currently receiving medical treatment for bowel function issues. Hence, we set the cutoff for stool frequency to four bowel movements per week (low frequency). Similarly, we set the criteria for stool consistency to include stool types of the Bristol Stool Form Scale from types 1 to 4, including hard, lumpy, and solid stools (types 3 and 4), as we deem these relevant for our target population (hard-to-solid consistency). We allow for other BSS types in 10% of the defecations, as subjects may also occasionally experience a softer bowel movement (e.g., one softer defecation out of 8-9 defecations within the 14-day screening period). Furthermore, a low stool frequency does not necessarily coincide with hard-to-solid stools. Therefore, we consider each of these criteria individually and in combination for subjects who are not satisfied with their bowel function. These criteria have been adapted to include a broader target population and were successfully

applied in an earlier study conducted with Dutch adults without constipation-related bowel complaints (Rijnaarts et al., 2022).

Bowel function combines several physiological processes whose perception by an individual determines overall satisfaction with a bowel movement. In this study, we use two commonly applied objective measures of bowel function, namely stool frequency (bowel movements per week) and stool consistency (BSS score). However, these objective measures alone provide no information on individuals' perceptions of successful bowel movements (Grønlund et al., 2018; Mysonhimer & Holscher, 2022; Ueberall et al., 2011). Hence, we incorporate subjective measures by including the individual's perception of ease of defecation, feeling of incomplete bowel emptying, and satisfaction related to bowel function. These outcome measures are complemented by two validated questionnaires, patient assessment of constipation related quality of life (PAC-QoL; (Marquis et al., 2005)) and symptoms (PAQ-SYM; (Frank et al., 1999)). We hypothesize that potential changes in bowel function resulting from intrinsic fiber intake are due to the adaptation of the gut microbiota over time and its activity, which are reflected in the production of SCFAs. Accordingly, in addition to objective and subjective markers of bowel function, we collect weekly stool samples to impart meaning to observed changes in the gut microbiota. By analyzing the microbiota composition and activity over time in combination with bowel function, questionnaire scores, and transit time as a function of dose, we expect to obtain further mechanistic insight into how intrinsic fiber may exert its effect.

Studies assessing therapeutic interventions for bowel function issues are commonly subjected to a largely variable placebo response of up to 80% (Irvine et al., 2006; Veldhuyzen Van Zanten et al., 1999), which appears to be a combination of methodological and psychobiological aspects (Enck & Klosterhalfen, 2005). Minimization of the placebo response by identifying the factors contributing to this response warrants therefore attention, especially in light of the subjective outcome measures (Enck & Klosterhalfen, 2005; Irvine et al., 2006). In study designs assessing the psychobiological aspects of eating behavior, it is common practice not to reveal the study aim until after the end of the study to control possible confounding effects related to revealing the true study aim beforehand. In line with this practice, we aim to inform subjects with a primary focus on the secondary outcome, aligning with our hypothesis that the underlying biology of bowel function issues is linked to gut microbiota composition and activity. After completion of the study, subjects will be informed about the primary aim of the study concerning bowel function. We believe this approach is justified given the study's limited invasiveness, its alignment with the gut microbiota mechanism, and the importance of establishing scientific findings.

Aside from the placebo effect, several factors are known to influence bowel function and thereby potentially bias outcome assessment. As dietary fiber intake has been related to constipation (Lemay et al., 2021; Yang et al., 2012) and the background diet is known to influence the gut microbiota composition at baseline and in response to a dietary intervention (A Salonen & de Vos, 2014; Anne Salonen et al., 2014), fiber intake

will be measured via three-day food intake records (Lucassen et al., 2021) and, more specifically, using the established FiberScreen questionnaire (Rijnaarts et al., 2021). Three-day food records will also record fluid intake, which has been hypothesized to impact bowel function (Boilesen et al., 2017). Similarly, as mixed evidence suggests that physical activity affects bowel function and thereby might bias outcome assessment, habitual physical activity will be recorded using the short Baecke questionnaire (Baecke et al., 1982). Another well-recognized but rarely measured factor that impacts the colonic environment is transit time, as it possibly affects the gut microbiota's activity by determining nutrient and water fluxes throughout the gut (Daniel, 2022; Vandeputte et al., 2016). A simple method to estimate transit time based on a noninvasive blue food dye method has recently been established within the PREDICT 1 study (Berry et al., 2018) and has been demonstrated to relate stool consistency, frequency, and gut microbiota composition (Asnicar et al., 2021). Here, we include this blue dye method as an optional measurement for estimating transit time in subjects interested in participating in the method. Furthermore, the use of certain medication and supplements is not allowed during the study. Medication known to affect the gut microbiota, including antibiotics, antacids, and proton pump inhibitors, as well as glucose-lowering medication due to the potential effect of inulin on glucose metabolism, all lead to exclusion (Fishbein et al., 2023; Imhann et al., 2016; Wang et al., 2019). Supplements that alter bowel function or the gut microbiota composition, such as fiber supplements and pro-, post-, or synbiotics, must be discontinued at least one month before screening. Finally, (planned) pregnancy and lactation lead to exclusion of a subject from the study due to potential bias by the temporal hormonal and mechanical (physiological) changes related to bowel function in this specific population group.

To enhance the generalizability of the study findings to the broader population, we aim to include a wide age range and not restrict participation to a single sex. The age group of 20–80 years represents the current demographic of the Netherlands, with approximately one-third of subjects aged 20–40 years and two-thirds of the subjects aged >40–80 years (Centraal Bureau voor de Statistiek, 2021). While bowel function issues are present at all ages, females tend to experience hard stools more often than males (Heaton et al., 1992; Verkuijl et al., 2020), notably during reproductive age. This difference has been linked to hormonal fluctuations during the menstrual cycle even when oral contraceptives are used (Judkins et al., 2020) and may also be associated with sex-related differences in the gut microbiota (Korpela et al., 2021). Hence, we also inquire about the menstrual cycle stage at the start of the study and aim to achieve balanced sex distribution in all study arms to account for hormonal changes throughout the menstrual cycle affecting bowel function. It should be noted that these fluctuations differ from the temporal bowel function issues experienced during pregnancy and lactation (Cullen & O'Donoghue, 2007).

Here, we present the study design of a randomized, placebo-controlled parallel trial to assess the impact of dried chicory root, administered at different dosages, on bowel function and the gut microbiota in subjects with self-reported bowel function issues.

We expect that the treatment product at any dosage has the potential to affect the gut microbiota but that the magnitude of the effect and its translation into a physiological response—meaning an improvement in objective or subjective measures of bowel function—depends on the individual gut microbiota. The results of this trial will provide insights into the baseline gut microbiota composition of 160 adults living in the Netherlands and how bowel function issues are impacted by different dosages of an intrinsic fiber in the form of dried chicory root particles mediated by the gut microbiota. These insights will help to identify new therapeutic avenues to improve and maintain gut health via gut microbiota-targeted fiber interventions.

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AUTHORS' CONTRIBUTIONS

MLP, HS, WMdV, and EJMF designed the trial and EJMF is the Principal Investigator. MLP and SCCvdZ were responsible for the execution of the study and wrote the first draft. HS, WMdV, and EJMF provided technical support and critically reviewed the draft. All the authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIAL

The data collected during the current study are available from the corresponding author upon reasonable request. Sequencing data obtained from 16S rRNA gene amplicon sequencing will be deposited in the European Nucleotide Archive (ENA).

ETHICS APPROVAL

This study was approved by the medical ethical committee METC Oost-Nederland (protocol ID: NL80274.091.22) and was prospectively registered at ClinicalTrials.gov under NCT05473793.

COMPETING INTERESTS

WMdV is a scientific advisor for WholeFiber BV

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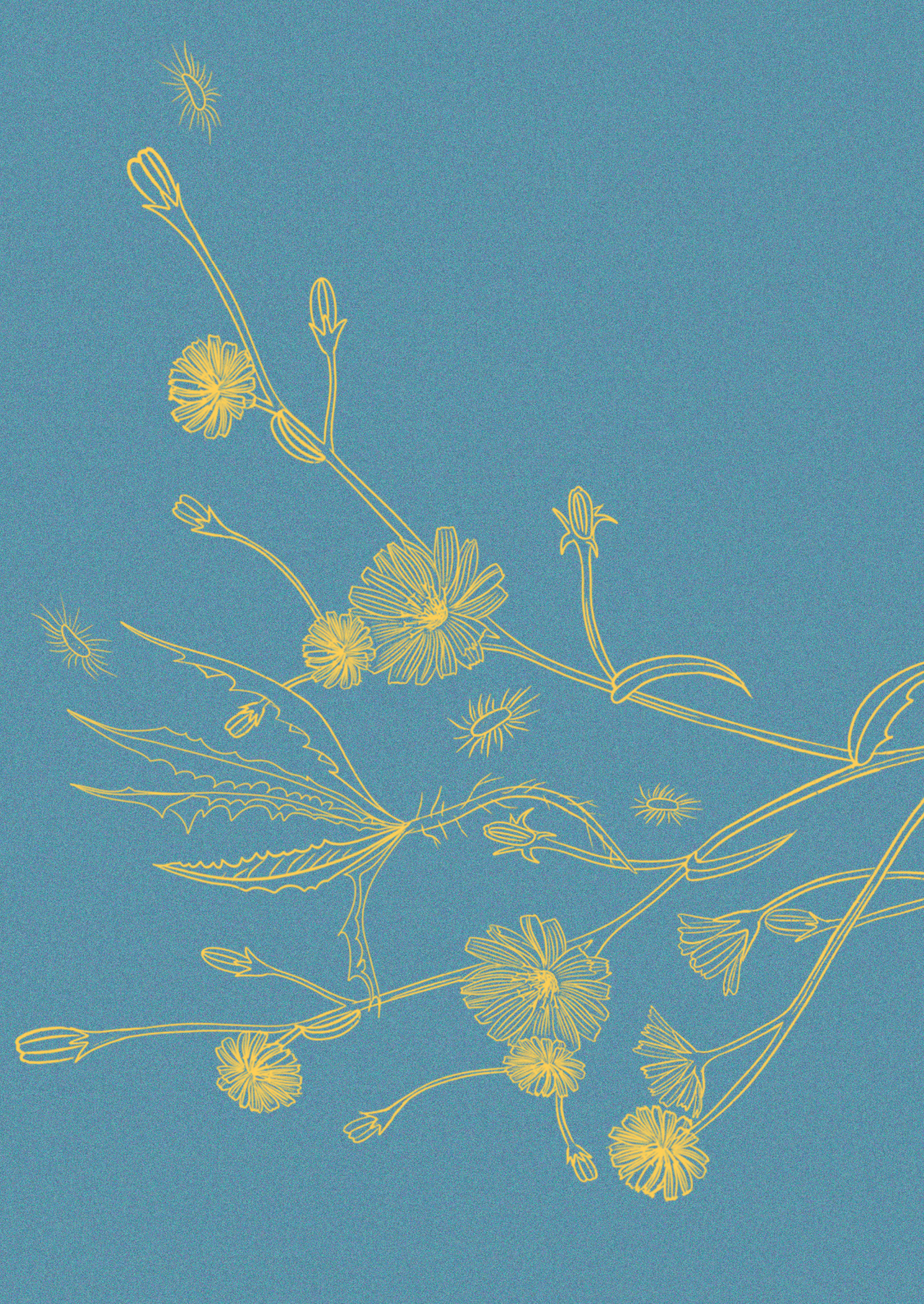

CHAPTER 9

General discussion

"Trowell was disturbed by the studies on isolated polysaccharides because this moved away from the dietary fibre concept of the plant cell wall in foods; however, he was persuaded that the use of isolated polysaccharides provided a powerful experimental tool for studying the mode of action of dietary fibre. Nevertheless, he suggested the term "dietary fibre" should be reserved for cell wall materials and proposed that "edible fibre" be used for other sources (Trowell et al., 1978). This debate still continues and there is strong evidence that the physiological effects of isolated polysaccharides are not identical to those when they are consumed as part of intact cell wall structures; the architecture of the wall confers other properties."

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THE SOCIETAL RELEVANCE OF DIETARY FIBER IN 2024

Of all foods available to humanity, 80% are edible plants (IPPC Secretariat, 2021). These plant foods provide us with essential macro- and micronutrients, including dietary fiber—a component not found in animal-derived foods. Dietary fibers form the backbone of plants, theoretically enabling us to consume fiber in ample quantities. However, reality paints a different picture. Despite the abundance of edible plants, most people in industrialized societies fail to consume enough fiber to meet national guidelines. This shortfall is exacerbated by our modern diet, which provides plenty of refined foods that are palatable, energy-dense, and nutrient-poor—traits commonly found in low-fiber foods. Moreover, these foods are frequently more affordable than whole foods such as vegetables, fruits, and whole-grain products. The consequences of a lifestyle low in fiber may not be immediately apparent. In the short term, individuals might experience bowel function issues. However, it is the long-term consequences, developing slowly over the years, that make a low fiber intake concerning. A higher risk of all-cause mortality, different types of cancer, cardiovascular disease, obesity and type 2 diabetes (T2D) are all related to low fiber intake (Ludwig et al., 1999; Reynolds et al., 2019). Just now, healthcare professionals in the Netherlands are warning of a health crisis that reflects the deterioration of metabolic health linked to our lifestyle choices (Samenwerkende GezondheidsFondsen, 2024). It is now more important than ever to help people make healthy lifestyle choices, and increasing fiber intake is definitively a crucial part of this.

Today, dietary fiber is defined as non-digestible carbohydrate polymers, including synthetic fiber, fiber extracted from plant foods, and fiber as naturally present in whole foods (Joint FAO/WHO Food Standards Programme, 2021). Refined food products can easily be enriched in fiber using synthetic and extracted fibers (Armet et al., 2020; Stephen et al., 2017), but their effects may differ from fibers present in whole plant foods (Augustin et al., 2020). To exemplify this, one can refer to one of the most well-known nutrition experiments conducted by Haber and colleagues (Haber et al., 1977). They compared the consumption of whole apples, apple puree, and apple juice on satiety, postprandial blood glucose, and insulin levels. While this experiment is often cited in the context of sensory science, particularly oral processing, it was actually designed based on the hypothesis that fiber depletion or physical disruption of fiber has negative implications on postprandial metabolism (Haber et al., 1977). The authors confirmed this by demonstrating that insulin concentrations rose more rapidly after consuming apple juice and puree despite the same rise in glucose concentrations when compared to whole apples. The experiment concluded with the hypothesis: "*The removal of fibre from food, and also its physical disruption, can result in faster and easier ingestion, decreased satiety, and disturbed glucose homeostasis which is probably due to inappropriate insulin release. These effects favour overnutrition and, if often repeated, might lead to diabetes mellitus.*"

This experiment demonstrating the impact of the physical structure of fibers in whole foods, and thus the effect of the destruction of the intrinsic plant cell matrix, on human health was carried out almost 50 years ago. Since then, we have advanced our knowledge of dietary fiber types and their interactions with the human body. However, in this process, driven by our increasingly reductionist view, we seem to have lost sight of the physical, three-dimensional structure of dietary fiber. Dietary fibers in whole plant foods are an intrinsic part of the plant cell matrix and form the plant 'skeleton' (**Chapter 2**), and are therefore designated as 'intrinsic fibers' (Augustin et al., 2020). The plant skeleton of intrinsic fibers dictates how fibers are accessible to the gut microbiota, which in turn breaks down dietary fiber, thereby mediating the health benefits of dietary fiber. Consequently, the effect of intrinsic fibers is reflected in their impact on the composition and activity of gut microbes, which we summarized in **Chapter 2**. Increasing awareness and understanding of intrinsic fiber as part of the plant cell matrix will help us understand how dietary fibers benefit human health. By utilizing modern technologies, we can revisit the previously accumulated knowledge and gain deeper insights into the fundamental role of fiber for human health, potentially reversing the looming metabolic health crisis more effectively than focusing solely on synthetic and extracted fibers.

THE DISTAL CONVERSION OF DIETARY FIBER – REVISITING THIS THESIS' WORKING HYPOTHESIS BY ADDRESSING CHANGES IN GUT MICROBIOTA COMPOSITION AND FUNCTION

Why would intrinsic fibers exert their effects differently from synthetic and extracted fiber? The leading hypothesis in this PhD thesis is that intrinsic fibers are broken down gradually along the colon, leading to a slow fiber release and thereby gradually extending fiber fermentation from the proximal to the distal colon. We infer this hypothesis from the structural complexity of the plant cell wall, which hinders the physical accessibility of individual fibers. To test this, we used dried chicory root, a vegetable consisting of more than 85% w/w dietary fiber due to its high (70%) intracellular inulin content enclosed inside the plant cells consisting of pectin (10%) and hemi-/cellulose (5%; **Chapter 3**; Figure 1A**)**. Using different particle sizes of dried chicory root that reflect either an intact (cubes), damaged (powder), or absent plant cell matrix (isolated inulin), we observed that the intactness of the plant cell matrix favored butyrate production at a later stage of the fermentation (**Chapter 4)**. In our human intervention trials (**Chapters 5 and 6**) with dried chicory root, we observed significant and reproducible increases in fecal butyrate levels, along with extensive shifts in gut microbiota composition. Specifically, we saw three-to-four-fold increases in fecal relative abundances of *Bifidobacterium* spp. and *Anaerostipes* spp. (Figure 1B). Following our *in vitro* and *in vivo* insights (**Chapters 4, 5 and 6**), we designed a synthetic tri-culture experiment using strains that represent the canonical functionalities of the impacted genera (*Bacteroides, Bifidobacterium* and *Anaerostipes*) and confirmed that the formation of a butyrogenic trophic chain with only three gut bacterial species from dried chicory root was indeed possible (**Chapter 5**;

Figure 1C). Importantly, this process relied on the presence of pectin-degrading bacteria such as *Bacteroides* spp. and butyrate-producing *Anaerostipes* spp. (Figure 1C).

Translating these findings into an *in vivo* context, we expect that breaking down the plant cell wall in the colon will take time due to the need to liberate cell wall fibers like pectin before inulin is released (Figure 1C). The idea of slow fiber breakdown already emerged from a simple indigestible carbohydrate food intake experiment in healthy volunteers conducted by Cummings and colleagues in 1985. They showed that the fermentation products from pectin, compared to the indigestible disaccharide lactulose, started to appear up to 5 hours later, reached only half the levels observed from lactulose, and continued to appear for a total of 18 hours, necessitating an extension of the monitoring period from 6 to 24 hours (Pomare et al., 1985).

As a consequence of this slow fiber breakdown, dried chicory root continues to travel through the colon and reaches its distal parts, such as the descending and sigmoid colon. Here, the slow release of fibers sustains ongoing microbial activity. The production of butyrate, depending on cross-feeding bacteria, is similarly expected to develop over time. Our observations of butyrate production being favored towards the end of fermentation (**Chapter 4**) aligns with previous findings using models with mixed isolated fibers, whole foods, and wheat bran with large particle sizes (Lu et al., 2020; Rose et al., 2009; Stewart & Slavin, 2009; Tuncil et al., 2017; Yao et al., 2023). Higher butyrate levels in the late fermentation stage have been attributed to reaching a 'fermentation plateau', allowing more time for cross-feeding to produce butyrate (Yao et al., 2023). We hypothesize that *in vivo*, this translates into increased butyrate production in the distal regions, leading to higher levels excreted in feces, as we observed in **Chapters 5 and 6**. These processes of slow fiber release, fermentation, and SCFA production are expected to be influenced by specific gut microbial signatures, such as those identified in **Chapter 6**, for their potential to metabolize pectin or convert inulin, contributing to the shift toward the distal colon.

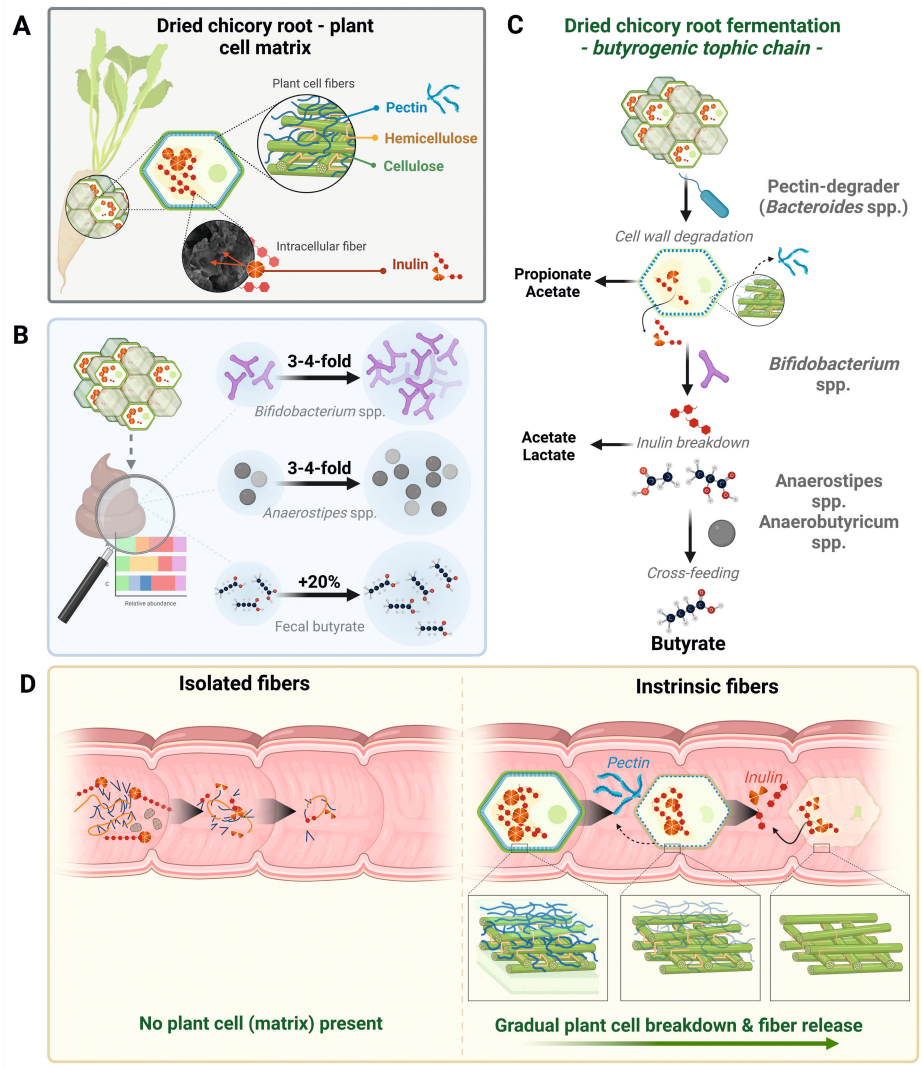

Figure 1. Dried chicory root and its effects on the gut microbiota as observed from *in vivo* fecal samples and *in vitro* experiments. (**A**) Makeup of the plant cell matrix of dried chicory root, where plant cell fibers form the plant cell walls that enclose intracellular inulin. (**B**) Dried chicory root intake in human intervention trials led to an increase in *Bifidobacterium* spp. and *Anaerostipes* spp. fecal relative abundances by three to four-fold, as well as an increase in fecal SCFAs by 20%. (**C**) Proposed butyrogenic trophic chain formed from the dried chicory root particles involving pectin-degrading taxa, such as *Bacteroides* spp., which break open the plant cell wall. Liberated fiber can then be used to form SCFAs such as acetate and propionate. *Bifidobacterium* spp. can use liberated inulin to form acetate and lactate, which in turn, via cross-feeding, are used by *Anaerostipes* spp. and *Anaerobutyricum* spp. to form butyrate. (D) Isolated, single fibers are rapidly fermented in the colon, leading to more proximal fermentation. In contrast, intrinsic fibers, due to the presence of the plant cell wall, lead to a slow release of fiber and fermentation, gradually extending fiber fermentation throughout the colon into the distal colon. Created with Biorender.com

Inferring a meaning from fecal SCFA levels has been criticized because these levels reflect those present after production by the gut microbiota and uptake by colonocytes, rather than reflecting intraluminal levels. However, as early as 1997, Lewis and Heaton demonstrated that alterations in transit time can change fecal butyrate levels. In their study, they showed how speeding up or slowing down transit time using laxatives or anti-diarrheal agents (loperamide) can increase or decrease fecal butyrate levels (Lewis & Heaton, 1997b). The relationship between faster transit time and higher fecal butyrate levels has been confirmed by others in both cross-sectional analyses and changes after dietary interventions (Boekhorst et al., 2022; Procházková et al., 2024; Procházková, Venlet, et al., 2023). This is further affirmed by observations that individuals with long transit times (approximately 72 hours), particularly longer distal (descending) colonic transit, have lower fecal SCFA levels and reduced bacterial richness (Müller et al., 2020). These observations suggest that in controlled intervention trials higher fecal butyrate levels might serve as a potential proxy for feces that are passed more quickly, leading to increased butyrate and SCFA excretion. In our human intervention studies (**Chapters 5 and 6**), we observed increased stool frequency and consistency—both indicative of shorter transit time and potentially related to the higher fecal butyrate levels observed We did not directly measure transit time, but plan to do so in the trial outlined in **Chapter 8**. However, in our second human intervention trial (**Chapter 6**), we did not observe softer stools or increased stool frequency among individuals who had increased fecal butyrate levels and responded strongly to dried chicory root. Additionally, stool moisture/fecal water, indicative of softer stools and often linked to shorter transit time, has not been found to correlate with butyrate levels in both cross-sectional and post-intervention assessments (Procházková et al., 2024; Procházková, Venlet, et al., 2023). Therefore, changes in bowel habits alone may not fully explain the increased fecal butyrate levels observed in our dried chicory root trials, but additional factors might have played a role in distal butyrate production. It is important to note that while the relationship between colonic transit and stool frequency and consistency has been established (Lewis & Heaton, 1997a), individual differences in these factors may still exist (Asnicar et al., 2021).

What factors, aside from gastrointestinal transit, might explain increased distal butyrate levels? An important finding from our dried chicory root trials was the increase in all three fecal SCFAs, acetate, propionate, and butyrate (**Chapters 5 and 6**), which contrasts with inconsistent findings from isolated inulin studies. Recently, higher concentrations of fecal SCFAs have been associated with higher relative (%) levels of butyrate in a cross-sectional analysis of fecal samples from over 150 volunteers (LaBouyer et al., 2022). The authors hypothesized that this increase in SCFAs, particularly butyrate, was favored by the link with a lower fecal pH. This relationship between lower fecal pH and higher butyrate levels has also been consistently demonstrated by others (Boekhorst et al., 2022; Procházková et al., 2024; Procházková, Venlet, et al., 2023). Thus, a lower fecal pH may provide additional insights into luminal conditions favoring butyrate production. We did not measure fecal pH in our intervention studies (**Chapters 5, 6, and 7**) due to limited sample amounts and our sampling protocol,

although we recognize this would have been valuable. However, it is possible torus infer hypotheses based on our *in vitro* findings and existing literature. In **Chapter 4**, we observed higher lactate production at 6 hours from dried chicory root cubes compared to powder or isolated inulin. Subsequently, more butyrate was produced from dried chicory root cubes, and all lactate was consumed (**Chapter 4**). The production of SCFAs and other organic acids, particularly lactate, reduces pH levels. *In vitro* studies indicate that a lower pH, such as 5.5 compared to 6.5, limits the growth of *Bacteroides* spp. and favors that of bifidobacteria and known butyrate producers (Flint et al., 2024). Consequently, the fiber-degrading and SCFA-producing ability of microbes, together with their differences in pH sensitivity, likely facilitates butyrate production, as recently reviewed (Flint et al., 2024).

Butyrate is primarily produced through cross-feeding from acetate along the butyryl-CoA:CoA-transferase route (Louis & Flint, 2017). However, a few specialized gut bacteria can also convert lactate to butyrate using a distinct and conserved set of proteins (Duncan et al., 2004; Sheridan et al., 2022; Shetty et al., 2020). These bacteria include *Anaerostipes* spp. and *Anaerobutyricum* spp., which we found to increase in relative abundance in feces after dried chicory root intake (**Chapters 5 and 6**). In particular, the relative abundances of *Anaerobutyricum* spp. increased with prolonged consumption of dried chicory root, and higher relative abundance of this genus correlated with higher fecal butyrate levels in individuals defined as showing larger metabolic improvements (**Chapter 6**). The importance of these specialized lactate-utilizing bacteria for human (gut) health is gradually being recognized (Louis et al., 2022; Ping Wang et al., 2020). Lactate can be formed by many gut bacteria, including *Bifidobacterium* spp. (Louis et al., 2022). This makes cross-feeding from dried chicory root-derived lactate to *Anaerostipes* spp. and *Anaerobutyricum* spp. plausible (Belenguer et al., 2006), given the observed increased relative abundance of these genera (**Chapters 5 and 6**) and the confirmation of the trophic chain formation in **Chapter 5**. Additionally, our metagenome analysis indicated that the amount of genes encoding the fructose-6-phosphate shunt (also known as bifid shunt (Scardovi & Trovatelli, 1965)) — a pathway of lactate and acetate production characteristic of bifidobacteria — were increased after dried chicory root intake (**Chapter 6**). This increase was more rapid in high responders, along with higher final relative abundances of *Anaerobutyricum* spp. (**Chapter 6**). Consequently, we may hypothesize that lactate might play a role in lowering the intraluminal pH to benefit butyrate production (Flint et al., 2024), also following dried chicory root intake.

In this thesis, we did not consider lactate levels in feces, but a trial administering 20 g of isolated inulin in three daily portions (6-7 g each) over four weeks found lower fecal pH and increased fecal lactate levels (Petry et al., 2012). In this context, I must mention that although a pH-lowering, butyrate-favoring, and health-benefiting effect related to lactate appears plausible, circulating lactate in the blood, notably the D-isomer, is more commonly associated with lactic acidosis (Hove & Mortensen, 1995a; Jin et al., 2023; Li et al., 2022). In this condition, elevated levels of circulating lactate in the bloodstream lower blood pH and lead to detrimental health outcomes. However,

concerning bacterially-produced lactate (i.e. not exercise-related lactate formation), this phenomenon is primarily reported in individuals with specific gastrointestinal conditions such as gastric bypass and short bowel or Inflammatory Bowel Disease (Hove & Mortensen, 1995a, 1995b). In healthy individuals, intraluminal accumulation of lactate in the gut is rare due to the activity of bacteria that utilize lactate and produce beneficial compounds like butyrate or propionate, underscoring their importance for human health as discussed elsewhere (Louis et al., 2022; Ping Wang et al., 2020; Sheridan et al., 2022). Most bacteria can use both L- and D-lactate, but the *Anaerostipes hadrus* type strain has been found *in vitro* to not have the necessary enzyme to convert the isomer and instead uses D-lactate (Allen-Vercoe et al., 2012). Whether this applies to the detected *Anaerostipes hadrus* strains observed to be increased in relative abundance in the interventions (**Chapters 5 and 6**) remains to be determined since considerable metabolic heterogeneity has been found in *Anaerostipes hadrus* strains (Bui et al., 2021).

The results of this thesis all support the notion that dried chicory root promotes the formation of a butyrogenic trophic chain, notably in the distal colon facilitated by the complexity of the plant cell matrix and a likely reduction in intraluminal pH due to the slow and sustained fiber release and SCFA production throughout the colon (**Chapters 2, 3, and 4**). The difference between intrinsic fibers and isolated fibers is summarized in Figure 2A-B, contrasting the evolution of intraluminal processes such as fiber breakdown, pH decrease, and SCFA production (Figure 2C). These differences challenge the prevailing believe that SCFA production primarily occurs in the proximal colon, particularly in the context of intrinsic fibers. While SCFA levels may still decrease and pH rise along the colon for intrinsic fibers, this effect is likely less pronounced than for isolated fibers (Figure 2C). In addition, the location of butyrate formation may also warrant further evaluation, as already suggested for reconsideration two decades ago by Morrison and colleagues (Morrison et al., 2006). Ultimately, the shift towards distal butyrate and overall SCFA production holds significant health implications, which I will elaborate on using the outcomes from **Chapters 5 and 6.**

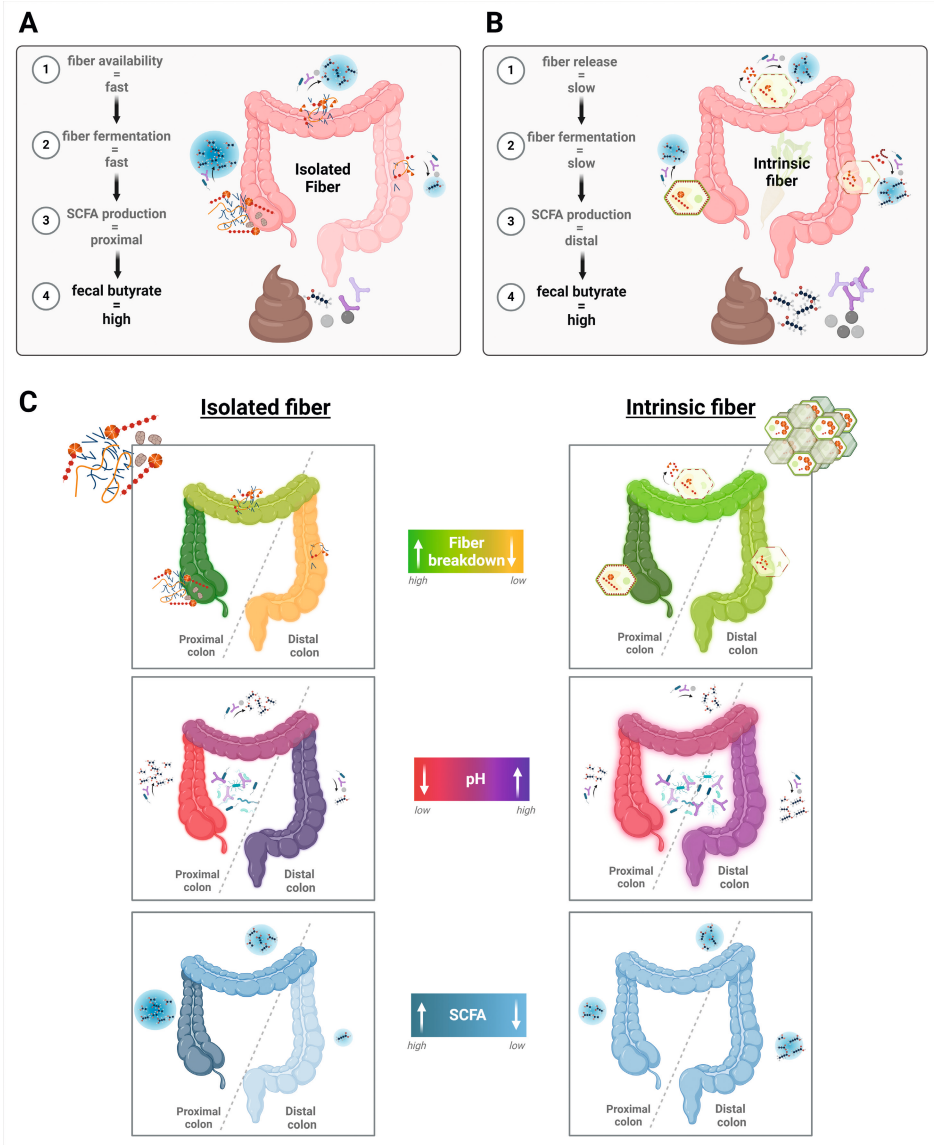

Figure 2. Proposed difference in gut microbial fiber conversion of isolated fibers versus intrinsic fibers. (A) Isolated fibers are readily available, allowing for fast fiber fermentation that primarily restricts it to the proximal colon, resulting in low fecal butyrate levels. (B) Intrinsic fibers lead to a slow fiber release from the plant matrix, resulting in slow fiber fermentation that also still occurs in the distal colon. Consequently, high fecal butyrate levels are found. (C) Differences in fiber breakdown, pH, and SCFA production are separately visualized between isolated and intrinsic fiber, showing that for intrinsic fiber breakdown and pH lowering continue in the distal colon, as does SCFA production. Created with Biorender.com

How else can we study distal fermentation?

While we do not provide direct support for this hypothesis of distal conversion, we infer beneficial effects based on *in vitro* and *in vivo* outcomes and supported by emerging literature underlining the importance of distal gut environment. Recently, a study combining segmental measurements of transit time and pH has shown that distal pH measures (sigmoid, rectal) and fecal pH explain variations in both urinary and fecal metabolomes (Procházková et al., 2024). This, coupled with insights from distal SCFA infusion studies (Canfora et al., 2017; van der Beek et al., 2016), suggests a significant role of distal microbial activity not only in observed fecal outcomes but also in overall metabolism.

How could we study a slow fermentation and the shift to a more distal location to further support our hypothesis? One might think that a straightforward comparison would involve testing isolated fiber derived from dried chicory root and comparing this to dried chicory root particles. This would require extracting single fibers from the same batch of dried chicory root, as the type of hemicellulose and pectin depends on the food source. Furthermore, transitioning from *in vitro* to *in vivo* testing requires extracting these fibers in a food-safe manner, necessitating access to specialized facilities. As an alternative, one might revert to isolated inulin and compare its effects with dried chicory root, as we did in **Chapter 4**. However, repeating such an approach *in vivo* would only reveal potential differences in fecal SCFA levels and fecal microbiota composition, without providing insights into the fermentation kinetics relevant to supporting a shift towards a more distal location.

Alternately, one might attempt to follow fermentation kinetics *in vivo* using ^{13}C-labeled isotopes, a method previously applied with isolated inulin (Deroover et al., 2017; van der Beek et al., 2018), pea flour (Edwards et al., 2002) and pearl barley (Verbeke et al., 2010). We have considered using ^{13}C-enriched dried chicory root pieces with 98% atom percent and comparing this with ^{13}C-labeled inulin extracted from the same batch, available from IsoLife (https://isolife.nl/). Using a cross-over trial, one could gain information on intra-individual differences regarding the kinetics of fermentation product appearance *in vivo* and how this relates to gut microbial baseline composition and fiber utilization. Fermentation could be tracked by assessing fermentation products in breath (e.g., ^{13}C-labeled CO_2) and in blood, urine, and feces (e.g., ^{13}C-labeled SCFAs). Ideally, we would combine this with fecal *in vitro* fermentations using samples collected from our human volunteers (Morrison et al., 2006). Feces could be further used to assess microbial utilization and incorporation of the ^{13}C-labeled fiber by applying techniques such as 16S rRNA-based stable isotope probing (SIP) (Jameson et al., 2017; Kovatcheva-Datchary et al., 2009), nuclear magnetic resonance (Bui et al., 2021; De Vos et al., 2024) or Raman activated cell sorting (RACS) (Alcolombri et al., 2022; Lee et al., 2019; Riva et al., 2023). Such a study will require that individuals adhere to strictly standardized food and water intake before and during the study days. Moreover, the appearance of fermentation products would need to be monitored closely throughout the day via frequent sampling of breath, urine, and postprandial blood. This protocol would

require assessing bowel function, particularly transit time, and ideally be combined with ingestible, real-time monitoring of segmental pH, allowing intraluminal sampling. While such a study would provide valuable insights (Barclay et al., 2008; Butler et al., 2017), its execution would be very labor-intensive. It would demand meticulous pre-testing, assessment of naturally present ^{13}C in food sources, sufficiently sensitive analytical methods, and determining the necessary wash-out and follow-up period, including potential overnight monitoring. Based on previous studies, we estimated needing at least five participants, each consuming 200-500 mg of ^{13}C-enriched dried chicory root or inulin. Combined with quantities needed for potential *in vitro* experiments, we would need investments of approximately 25,000 Euros to purchase 5 g of dried chicory root and 5 g of inulin (https://isolife.nl/). Additionally, there would be the costs of participant reimbursement and routine laboratory and specialized isotope-analytical methods. Clearly, the labor-intensiveness, invasive nature of the measurements for participants, and high costs explain why ^{13}C-isotope studies are not often conducted in humans. For now, a simpler approach would be a cross-over design using emerging ingestible, real-time pH measurement combined with intraluminal sampling.

DIETARY FIBER AND METABOLIC HEALTH

How can dried chicory root and a more distal fermentation benefit human metabolic health? We assessed these questions in two human intervention trials (**Chapters 5 and 6**). Metabolic health encompasses many aspects, but due to inulin's central role in the metabolic response, we focused on insulin-mediated changes in glucose utilization and lipid outcomes in the studies included in this thesis. We combined different measuring techniques, using simple "classic" fasting blood levels of glucose (**Chapters 5 and 6**) and insulin to calculate Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) (**Chapter 5**), as well as dynamic assessment, which were continuous glucose measurement (CGM) (**Chapter 5**) and the hyperinsulinemic-euglycemic clamp technique (**Chapter 6**). Fasting blood metabolite levels can be understood as a 'snapshot' of the metabolic state, reflecting gradual impairments or improvements in metabolic processes over time within the same individual. Similarly, interindividual differences in fasting SCFA levels may reflect the effects of sustained fiber consumption. This allows for the evaluation of treatment effects across individuals, although SCFAs are influenced by endogenous production, rapid clearance, and measurement techniques (Pomare et al., 1985).

In **Chapter 5 and 6,** we recruited individuals who were at risk of T2D and exposed them first to two-week 15 g/day dried chicory root intake followed by either three-week 30 g/day five-week (short-term Trial 1; **Chapter 5**) or longer ten-week 30 g/day dried chicory root intake (long-term Trial 2; **Chapter 6**). The results of both trials are summarized in Figure 3A. In our short-term Trial 1 (**Chapter 5**), we observed small changes in the primary outcome HOMA-IR. Similarly, minimal changes were seen in the "classic" fasting glucose (Figure 3A). However, as both these outcomes represent a 'snapshot' they only limitedly inform us about the dynamics of how the body handles

glucose through insulin. Hence, in Trial 1, using CGM, we observed that individuals consuming dried chicory root showed improved glucose control (**Chapter 5**). This was evidenced by a reduced glycemic coefficient of variation below 20%, which is suggested to reflect stable glucose control in diabetes treatment (Peyser et al., 2018). In Trial 1, individuals with low baseline relative abundances of *Blautia* spp., a genus previously associated with T2D (Gurung et al., 2020), were found to respond positively to the intervention. These subjects showed greater decreases in fasting glucose and increases in fasting SCFAs, along with more rapid improvements in glycemic coefficient of variation (**Chapter 5**). These findings underscore the pivotal role of the gut microbiota in the observed effects. Of note, the decrease in fecal relative abundances of *Blautia* spp. during dried chicory root intake prompted us to speculate that longer interventions beyond the five weeks in Trial 1 may lead to an even more meaningful health impact.

Building on these insights into the modulatory role of the gut microbiota and the improvements in glucose control, our subsequent 12-week intervention trial was designed (Chapter 6) in which individuals consumed the 30 g/day doses of dried chicory root for ten weeks. Individuals in our long-term Trial 2 were slightly younger but appeared more advanced in their metabolic impairment compared to individuals in our short-term Trial 1, indicated by higher baseline HOMA-IR values (Figure 3A). Following prolonged dried chicory root intake, again minimal changes were observed in fasting glucose, but remarkable effects on whole-body and peripheral insulin sensitivity were evident (Figure 3A). This supported our hypothesis that dietary interventions may require extended durations to manifest detectable effects, particularly in insulin-mediated dynamic glucose handling. The need for prolonged dietary interventions to modulate metabolic health aligns with meta-analyses indicating that inulin's effects on glucose homeostasis typically require more than six weeks to manifest (Wang et al., 2019). Dietary fibers likely impact more than one metabolic pathway, and changes in metabolic markers may develop gradually yet yield sustainable outcomes. Therefore, reversing the accumulated impairments in insulin signaling and glucose handling over the years may require extended durations to achieve meaningful reversal.

In addition to improved glucose handling observed in both trials, individuals in our long-term Trial 2 had remarkable reductions in fasting triglyceride levels and a shift in adipocyte size distribution from larger to smaller adipocytes (**Chapter 6**). The effects were particularly pronounced in individuals classified as strong responders, meaning they had more than a 15% improvement in insulin-mediated glucose disposal (**Chapter 6**). Additionally, strong responders had increased fasting high-density lipoprotein (HDL) cholesterol levels (**Chapter 6**). All these changes in adipocyte size and lipid profiles signify metabolic improvements. Smaller adipocytes are associated with improved insulin sensitivity, while decreased triglyceride levels and increased HDL levels indicate improvement of dyslipidemia in T2D (Ginsberg et al., 2005; Stenkula & Erlanson-Albertsson, 2018). In the short-term Trial 1, we observed expectedly only slight changes in fasting triglycerides and HDL cholesterol levels; although the minimal decrease in triglycerides mirrored that observed in Trial 2 (Figure 3A).

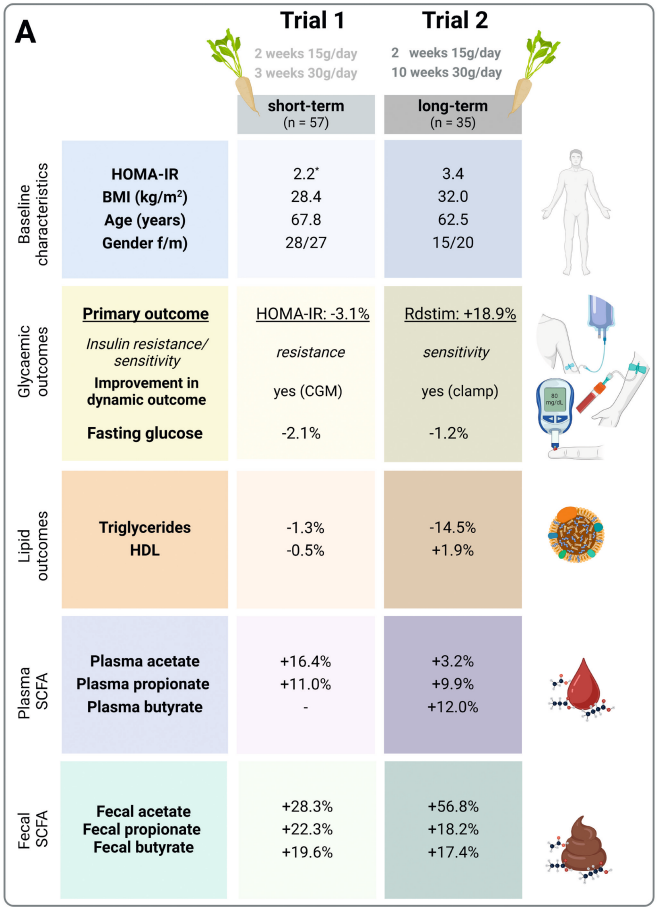
A
Trial 1
Trial 2
2 weeks 15g/day
3 weeks 30g/day
2 weeks 15g/day
10 weeks 30g/day
short-term
(n = 57)
long-term
(n = 35)
Baseline characteristics
HOMA-IR
BMI (kg/m²)
Age (years)
Gender f/m)
2.2*
28.4
67.8
28/27
3.4
32.0
62.5
15/20
Glycaemic outcomes
Primary outcome
Insulin resistance/ sensitivity
Improvement in dynamic outcome
Fasting glucose
HOMA-IR: -3.1%
resistance
yes (CGM)
-2.1%
Rdstim: +18.9%
sensitivity
yes (clamp)
-1.2%
Lipid outcomes
Triglycerides
HDL
-1.3%
-0.5%
-14.5%
+1.9%
Plasma SCFA
Plasma acetate
Plasma propionate
Plasma butyrate
+16.4%
+11.0%
-
+3.2%
+9.9%
+12.0%
Fecal SCFA
Fecal acetate
Fecal propionate
Fecal butyrate
+28.3%
+22.3%
+19.6%
+56.8%
+18.2%
+17.4%
B
XS−VLDL CE (mmol/L)
XS−VLDL C (mmol/L)
XL−HDL PL (mmol/L)
XL−HDL P (µmol/L)
XL−HDL L (mmol/L)
XL−HDL FC (mmol/L)
XL−HDL CE (mmol/L)
XL−HDL C (mmol/L)
L−HDL FC (mmol/L)
IDL−FC (mmol/L)
VLDL
HDL
−0.04 −0.02 0.00 0.02 0.04 0.06
Median Change
C
XL−VLDL TG (%)
XL−VLDL FC (%)
XL−HDL TG (%)
XL−HDL PL (%)
S−HDL FC (%)
L−HDL FC (%)
IDL FC (%)
−5 0 5 10
Median Change
Intervention groups
Placebo
Treatment
D
Size (nm)
Placebo
Treatment
VLDL
LDL
HDL
0.027
-0.013
-0.036
-0.076
-0.018
0.005
VLDL - decrease in size and TG content?
HDL - changes in composition?
E
R = 0.053, p = 0.84
R = −0.54, p = 0.024
Change in plasma triglycerides (mmol/L)
1
0
-1
-4 -2 0 2 4
Change in fecal butyrate (mmol/kg)

Figure 3. Summary of metabolic changes observed in the two dried chicory root human intervention trials. (A) Overview of observed changes in both trials expressed as % change of baseline due to different measurement techniques. HOMA-IR was calculated using the HOMA-IR2 model (Levy et al., 1998; Matthews et al., 1985) and is changed here for comparison to HOMA-IR1 (Turner et al., 1979). Plasma butyrate could not be assessed in Trial 1 as levels were below the detection limit. (B-D) Changes in lipid markers following dried chicory root intake in Trial 1. (B) Median changes in lipoprotein lipid content and HDL size and (C) in lipoprotein relative lipid composition were identified to differ ($p < 0.05$; unadjusted p-values obtained from non-parametric testing) after treatment and placebo intake. FC = free cholesterol. PL = phospholipids. L = total lipids. CE = cholesteryl esters. C = cholesterol. TG = triglycerides. P = lipoprotein particle concentrations. (D) Median changes in VLDL, LDL, and HDL particle size after treatment and placebo intake and summary of observed lipid marker changes in Trial 1. (E) Pearson correlation between fecal butyrate changes and fasting triglyceride changes in Trial 2. Created with Biorender.com

Hence, we hypothesize that, similar to glucose homeostasis, alterations might have occurred but are not yet all detectable, requiring deeper-level investigation. To explore this hypothesis, we conducted lipid metabolomics (Figure 3B-C) using the Nightingale platform (Nightingale Health Ltd., Helsinki, Finland, (Ala-Korpela et al., 2022; Soininen et al., 2015)), which will be published elsewhere (Puhlmann et al., 2025). This analysis revealed that in our short-term Trial 1, changes in lipoprotein particles and their subclasses were indeed noticeable and were also primarily related to HDL particles (Figure 3C-D).

HDL particle subtypes have recently been associated with T2D risk (Sokooti et al., 2021). Specifically, reduced risk of T2D was found to relate to larger HDL particles, higher HDL-cholesterol levels, HDL particle concentration, and HDL size—all of which increased after dried chicory root intake in Trial 1 (Figure 3B-C). Therefore, the observed changes in HDL may reflect metabolic improvements that are not yet visible in overall HDL and triglyceride levels. Additionally, we observed notable decreases in both the relative triglyceride content and the size of VLDL particles in Trial 1 (Figure 3C-D). VLDL particles are formed by the liver and primarily contribute to fasting triglyceride levels (Gill & Sattar, 2011). These findings were particularly intriguing since fasting triglyceride levels in Trial 2 correlated with fecal butyrate levels, suggesting potential mechanisms by which dried chicory root-derived SCFAs may exert metabolic benefits (Figure3E).

In both trials, dried chicory root intake increased fecal levels of acetate, propionate, and butyrate (Figure 3A). In Trial 1, individuals with high baseline relative abundance of *Blautia* spp., linked to non-response, had lower baseline fecal SCFA levels compared to those with low *Blautia* spp. relative abundances. However, after five weeks of dried chicory root intake, individuals in the high *Blautia* group were observed to develop fecal SCFA levels similar to those found in the low *Blautia* group (unpublished observations). This demonstrated again the potential of dried chicory root to promote the formation of a butyrogenic trophic chain that might require more sustained exposure in certain individuals to be established.

In addition to increases in fecal SCFA levels, we also observed increases in plasma acetate and propionate in both trials (Figure 3A). Notably, the increase in plasma acetate was higher in responders in Trial 1 and in high responders in Trial 2 (**Chapters 5 and**

6). A key observation from the prolonged dried chicory root intake in Trial 2 was that an increase in fecal butyrate was associated with increased levels of fasting acetate. Additionally, fecal butyrate correlated with smaller adipocytes and decreased fasting triglycerides (Figure 3E). Combining insights from **Chapters 5 and 6**, we propose that dried chicory root-derived colonic SCFAs, particularly butyrate, along with changes in fasting acetate, may mediate the metabolic effects observed on insulin-mediated glucose and lipid profiles.

Fiber-derived SCFAs as mediators of fibers' effect on metabolic health

How would SCFAs produced in the colon influence peripheral processes in adipose and muscle tissue? In the 1970s and '80s, Cummings and colleagues demonstrated in humans using rectal dialysis techniques that SCFAs could be absorbed by the colon (McNeil et al., 1978). This discovery challenged the prevailing belief that SCFAs were solely excreted in feces, where they were thought to retain water and contribute to stool bulk. It was known already at that time that SCFAs can have direct effects on metabolic processes in animals. For instance, during the 1960s and '70s, the insulin-stimulatory effects of propionate and butyrate were demonstrated in sheep by injecting SCFAs directly into the arterial blood supply to the pancreas of live animals (Manns et al., 1967). Consequently, Cumming's finding sparked interest in assessing the direct effect of SCFAs in humans. Over the past decade, we have considerably advanced our understanding of the mechanisms underlying the modulatory role of SCFAs in metabolism through studies using *in vitro* cell lines and rodent models, as extensively reviewed elsewhere (Blaak et al., 2020; Canfora et al., 2015; van Deuren et al., 2022).

However, to exert a direct systemic effect, SCFAs produced in the colon must enter the bloodstream (Figure 4A). Cummings demonstrated that this uptake does not occur in a one-to-one ratio. In a study involving sudden-death victims, he and colleagues observed a rapid decline in SCFA levels from the gut lumen to the portal vein, hepatic vein, and peripheral circulation (Cummings et al., 1987). Consequently, it was established that butyrate is primarily utilized by colonocytes as an energy source, while propionate is predominantly metabolized in the liver (Cummings et al., 1987). Therefore, acetate remains the SCFA that is most likely to reach systemic circulation, explaining also its highest levels following dried chicory root intake in **Chapters 5 and 6**. The efficient extraction of SCFAs from the colon also explains why fecally excreted SCFAs rarely correlate with circulating systemic levels (Müller et al., 2019), which we also observed in **Chapter 6**. Circulating SCFA levels, however, correlated with measures of insulin sensitivity, lipolysis, and circulating gut hormones (Müller et al., 2019). Therefore, to elicit these systemic effects, SCFAs must 'escape' the 'filtering' processes by colonocytes and the liver.

The extent of a potential SCFA 'escape' may depend on their site of production in the colon, as evidenced by the metabolic effects associated with the distal colon. Infusion studies with SCFAs have shown that distal, rather than proximal, SCFAs affect energy substrate utilization and lipolysis (Canfora et al., 2017; van der Beek et al., 2016).

Also, in **Chapter 6**, we observed such a relationship between systemic improvements (adipocytes, fasting triglycerides, plasma acetate, and propionate levels) and increased fecal butyrate levels, which are linked to more distal production and subsequent excretion. Additionally, the impact of distal pH on urinary profiles further indicates that distal microbial metabolite formation seems to affect systemic outcomes more than proximal production (Procházková et al., 2024). The hypothesis that distal SCFA uptake benefits their effects intriguingly aligns with the differences in blood drainage between the distal and proximal colon. I illustrate this using a figure from Tun & Ehrenpreis (Tun & Ehrenpreis, 2021) and data from Neis et al. (Neis et al., 2019) in Figure 4B. Blood is drained from the proximal colon by the superior mesenteric vein, which carries only half the SCFA concentrations found in blood from the inferior mesenteric vein draining the distal colon (Neis et al., 2019). This higher drainage of distal SCFAs challenges the commonly held assumption of lower distal SCFA production inferred from lower intraluminal SCFA levels and higher distal pH (Cummings et al., 1987).

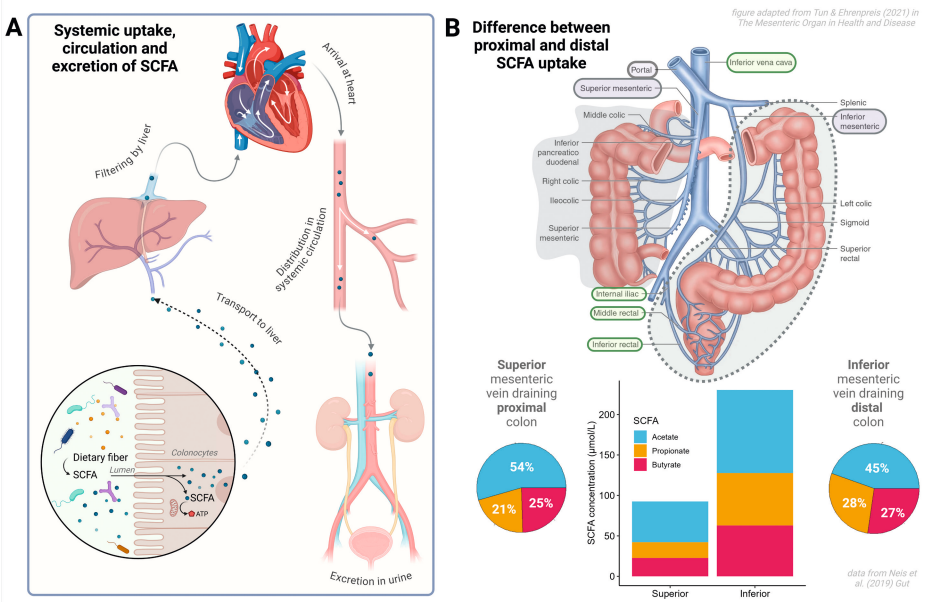

Figure 4. Fiber-derived SCFAs in the human body. (A) Schematic representation of the proposed uptake of SCFAs from the lumen into colonocytes, subsequent metabolism in the liver, and distribution through systemic circulation via the heart before excretion of any remaining SCFAs via the urinary system. SCFA concentrations decrease significantly from the lumen to the heart, with butyrate serving as an energy source for colonocytes and propionate being metabolized in the liver, making acetate the SCFA most likely to reach systemic circulation. (B) Variation in blood vessel distribution and subsequent SCFA drainage from the proximal and distal colon based on schematic representations by Tun & Ehrenpreis (2021) and data from Neis et al. (2019).

The reasons behind this two-fold difference in SCFA concentrations in blood from the distal colon remain unclear, reflecting our still limited understanding of SCFA

transporters in colonocytes (Blaak et al., 2020; van Deuren et al., 2022). Intriguingly, the relative abundance of one of these SCFA transporters in humans has been observed to steadily increase along the colon, reaching its peak in the distal colon (Gill et al., 2005). However, this concerns only the transport from the lumen into colonocytes (apical side), and little is known about the transport into the bloodstream (basolateral side) (Blaak et al., 2020; van Deuren et al., 2022). One could hypothesize that blood vessels in the rectum contribute to higher systemic SCFA levels. The rectum contains blood vessels that drain directly into systemic circulation via the internal iliac vein, which connects to the inferior vena cava leading to the heart (see Figure 4B). This mechanism is utilized in the rectal administration of medications such as fever-reducing drugs. Despite Cummings' demonstration of SCFA uptake in the colon through rectal infusion (McNeil et al., 1978), the rectum is likely a minor contributor to SCFA levels. This is due to the fact that the rectum is thought to primarily serve during the defecation process as a repository for feces and this explains why it is empty in most individuals, while its microbial load is highly variable (Chanderraj et al., 2022; McNeil & Rampton, 1981).

How dried chicory root-derived SCFAs affect metabolic health

Although we do not fully understand how proximal and distal colonic SCFAs differ in reaching systemic circulation, their differences in metabolic health effects are evident. Considering a distal production of SCFAs, particularly butyrate, from dried chicory root and our observation that fecal butyrate levels correlate with plasma acetate and propionate, a role of distal colonic butyrate from dried chicory root can be hypothesized (**Chapter 6**). This effect may be exerted through direct local colonic or hepatic effects, as well as indirect effects via gut hormones, plasma acetate and propionate, which is summarized in Figure 5.

Locally in the colon, butyrate benefits gut barrier function by serving as an energy source for colonocytes, strengthening the colonic lining, reducing oxidative stress, and attenuating inflammation (Hamer et al., 2008). We did not study gut barrier function *in vivo*, but gut barrier function has been reported to be impaired in metabolic diseases like T2D and obesity. Consequently, a sustained supply of butyrate through fiber fermentation may reverse the damage observed in these conditions (Régnier et al., 2021; Riedel et al., 2021).

Given the potential for high delivery of distally-derived butyrate to the liver and the crucial role of the liver in human metabolism, it is conceivable that butyrate modulates hepatic function. Prolonged butyrate exposure can enhance hepatic insulin sensitivity, improve glucose regulation, and reduce lipid accumulation, as indicated by mechanistic models summarized elsewhere (Blaak et al., 2020; van Deuren et al., 2022). In both our dried chicory root trials in individuals at risk for T2D, we observed effects on lipid homeostasis that we can relate to the liver (**Chapters 5 and 6**). In Trial 2 (**Chapter 6**), long-term dried chicory root intake reduced liver fat content, a finding to be detailed elsewhere (Omary et al., 2024). This change occurred together with a decrease in fasting triglyceride levels, primarily attributed to VLDL lipoprotein production in the liver. In

Trial 1 (**Chapter 5**), we found that short-term dried chicory root intake decreased the particle size of these VLDL lipoproteins and their relative triglyceride content (Figure 3). Consequently, dried chicory root may exert effects via changes in lipid homeostasis. As discussed in **Chapter 6**, it remains unclear whether these metabolic changes precede improvements in peripheral insulin sensitivity and adipose tissue function or result from them. For comparison, another human intervention study investigating the effects of eight-week resistant starch intake reported improvements in insulin-mediated glucose disposal in peripheral tissues and adipose tissue function but no changes in liver fat content or fasting triglycerides (Robertson et al., 2005). Resistant starch is likely fermented more proximally than dried chicory root. Therefore, differences in SCFA production location may again contribute to the observed differences.

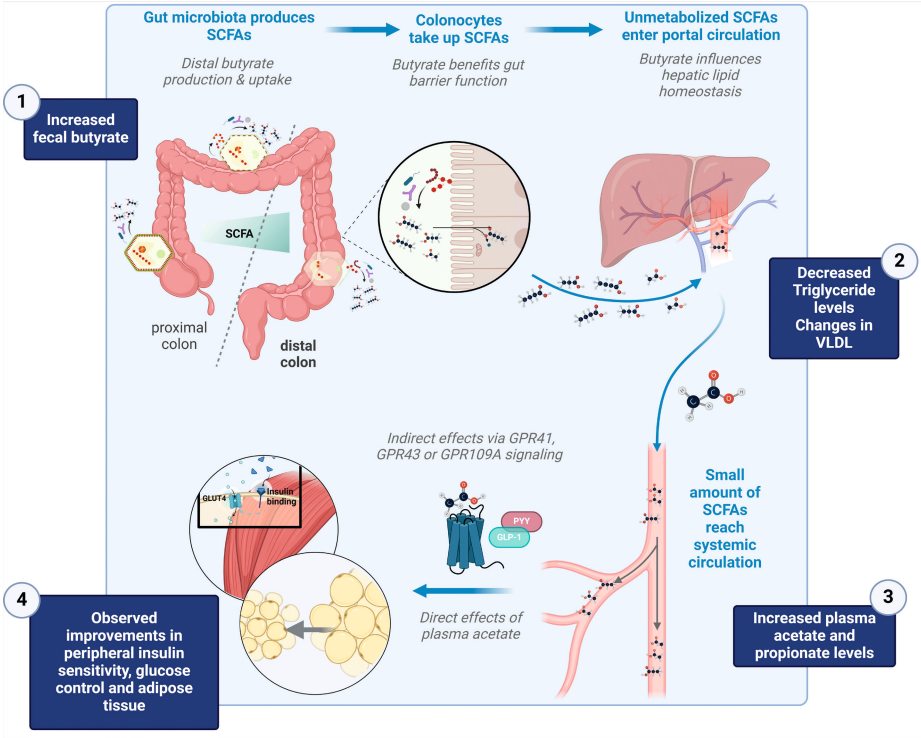

Figure 5. Proposed mechanism of action of dried chicory root-derived SCFAs on metabolic health. (1) Dried chicory root undergoes fermentation, leading to distal production of butyrate. SCFAs enter colonocytes, contributing to gut barrier function.(2) SCFAs influence liver function by modifying lipid metabolism, reflected in decreased fasting triglyceride plasma levels and VLDL size and composition alterations. (3) SCFAs that escape the liver enter systemic circulation and can exert indirect effects via signaling through GPR41/43 or GPR109A. (4) Systemic effects are reflected in improvements in insulin sensitivity, glucose control, and adipocyte sizes.

Systemic effects, such as changes in adipocyte size and improvements in peripheral insulin sensitivity observed in **Chapter 6**, are likely indirect effects of dried chicory

root-derived butyrate and mediated through alterations in circulating acetate levels or signaling pathways. While the correlations between increased fecal butyrate levels and smaller adipocyte size, as well as between increased fecal butyrate and increased plasma acetate in **Chapter 6**, do not establish causality, they can aid in generating hypotheses. Increases in plasma acetate following colonic SCFA infusion have been shown to link with increased fat oxidation *in vivo*, and in cell line models acetate affects adipogenesis and intracellular lipid handling (Canfora et al., 2017; Jocken et al., 2018). Therefore, increased plasma acetate levels could potentially explain the changes in adipocyte size related to fecal butyrate levels in **Chapter 6**, despite no observed changes in weight or fasting free fatty acids.

Many of the effects of both colonic and plasma SCFAs are mediated by SCFAs binding to G-protein-coupled receptors, including GPR41, GPR43, and GPR109A. One widely discussed effect of such signaling is the stimulation of the gut hormone Glucagon-like Peptide 1 (GLP-1). GLP-1, produced by L-cells, plays a crucial role in the postprandial response by stimulating insulin secretion (Holst, 2007). Therefore, the beneficial effects of butyrate would theoretically translate into increased postprandial GLP-1 levels. In our human trials with dried chicory root, we did not measure postprandial GLP-1, but fasting GLP-1 levels. These levels did not significantly change across intervention groups, as detailed in forthcoming publications (Omary et al., 2024; Puhlmann et al., 2025). However, individuals with low relative baseline abundance of *Blautia* spp. had decreased fasting GLP-1 levels in Trial 1, and decreased fasting GLP-1 levels correlated with increased fecal butyrate levels in Trial 2 (Omary et al., 2024; Puhlmann et al., 2025). The biological significance of decreased fasting GLP-1 levels remains unclear, but studies have reported higher fasting GLP-1 levels in individuals with impaired glucose tolerance and type 2 diabetes compared to healthy individuals (Huber et al., 2024). Although the precise implications of these effects are yet to be fully understood, it is noteworthy that L-cell density increases from the proximal to the distal colon in both animal and human colonic samples, suggesting potential benefits of distal fiber fermentation. Additionally, intake of fructo-oligosaccharides, similar to short-chain fructans released from dried chicory root, has been shown to augment L-cell numbers in rat colons (Kaji et al., 2011). Hence, in conclusion, there are several mechanisms through which dried chicory root-derived butyrate may influence metabolism, including enteric routes via the gut-brain axis, many of which require further exploration.

What should we assess in the future?

Insights from this thesis (**Chapters 5 and 6**) suggest that high fiber intake through dried chicory root has a remarkable potential to improve metabolic health, notably in the long term. However, our understanding of the underlying mechanisms that mediate the microbiota-mediated effects of these fibers on systemic health outcomes is still limited. ^{13}C-labeled isotopes, as previously mentioned, can assist us herein. However, to further elucidate how SCFAs are drained from the colon and affect metabolic outcomes, we would need invasive methods. Invasive sampling of abdominal blood vessels, as

performed by Cummings and colleagues on sudden-death victims (Cummings et al., 1987) or by Neis and colleagues during abdominal surgery (Neis et al., 2019), have provided us with fundamental insights but remain exceptional approaches.

If we wish to elucidate underlying mechanisms further, we will most likely continue to rely on assessments of tissue specimens, *in vitro* studies, and cell lines, making wise use of animal models. However, these assessments should preferentially follow from controlled human intervention studies. Additionally, reporting both fasting and postprandial hormone and plasma SCFA levels will help us give biological meaning to observed fasting effects, as seen in **Chapters 5 and 6**. In this context, human intervention studies with prolonged exposure to fiber treatment, as described in **Chapter 6**, appear more logical since the effects of fiber on human health most likely develop not overnight but from prolonged exposure. This is also illustrated by our *ex vivo* assessments of butyrate-containing dried chicory root fermentation supernatants on human colonic biopsies in **Chapter 4**. There, we did not find a uniform effect on gut barrier integrity after acute exposure that we attributed to effects of a personalized microbiota. This would be similar to variable findings with sodium butyrate solutions (Tabat et al., 2020). However, repeated *in vivo* colonic supply of butyrate using colonic enemas has shown beneficial effects on mucosal health in line with butyrate well-reported benefit on gut barrier function (Hamer et al., 2009). Intriguing mechanistic insights may also be obtained from colonic infusion studies focusing exclusively on acetate and propionate, excluding butyrate, or vice versa (Canfora et al., 2017; van der Beek et al., 2016). These studies could be combined with assessments of colonic biopsies before and after dried chicory root intake to elucidate how these interventions influence gut barrier function. However, none of these fiber-derived studies will advance our understanding if we do not consider the location of fermentation. This involves measuring transit time and stool pH, which could include using ingestible sensors, and I will elaborate on this later.

Although the mechanism by which fiber-derived colonic SCFAs, particularly butyrate, affect metabolic health is not fully understood, the outcomes of **Chapters 5 and 6** on glucose control, insulin sensitivity, and notable gut microbiota changes are promising. Ready-to-use intrinsic high-fiber products like dried chicory root cubes can assist in increasing fiber intake in metabolically impaired individuals. Based on the outcome of this thesis, we hypothesize that prolonged intake of such fiber has the potential to reverse metabolic declines. Hopefully, future studies using intrinsic fiber and assessing the location of fermentation through measuring colonic environmental aspects will raise awareness of the everyday benefits of fiber intake on our metabolic health, which, though not immediately felt, will benefit our future selves.

DIETARY FIBER AND BOWEL FUNCTION

While the long-term effects of increased dietary fiber intake on human metabolism will take time to develop and may not be directly noticed by the individual, improvements in bowel function can be experienced immediately. Bowel function entails the various processes related to the formation, movement, and elimination of stools. However, what

is normal bowel function, and how can we assess it to measure the impact of fiber on it? The fiber enthusiast and physician Heaton, living in Bristol in the 20th century, already asked this question (Heaton et al., 1992). 'Normal' bowel function was established based on the most prevalently experienced outcomes. Most individuals defecated every 24 hours, meaning once a day, in the morning between 7 and 8 o'clock, and had, on a scale from 1 (hard) to 6 (soft), the stool type 4 (Heaton et al., 1992). Based on his work and that of his colleague, Lewis, the Bristol Stool Form Scale (BSFS; score 1-7) was developed and published five years later (Lewis & Heaton, 1997a). They demonstrated how the BSFS could be used as a proxy for whole gastrointestinal transit time and potentially replace invasive transit time measures such as radio-opaque markers to measure changes in bowel function. Consequently, we now primarily assess bowel function by evaluating stool consistency, indicating the softness of stools, and stool frequency, indicating how often stools are passed in a specific time period.

In this thesis, we used these two parameters to evaluate the impact of dried chicory root particles (**Chapter 5**) and cubes (**Chapter 6**) on aspects of bowel function and metabolic health outcomes. The rationale behind this approach is that changes in bowel function reflect fiber-mediated alterations in the intestinal environment due to gut microbiota modulation. In Trial 1, remarkable effects of both 15 g and 30 g daily dried chicory root intake were observed, and these findings were confirmed in Trial 2, albeit with smaller magnitudes. Combining their results (see Figure 6A-D), dried chicory root increased stool frequency by up to one additional bowel movement every other day and stool consistency by up to one BSFS unit. As individuals were not included based on specific bowel habit patterns, participants in both studies reported various baseline stool consistencies and frequencies. When comparing the average baseline values in both trials, it became evident that individuals in Trial 1 initially had slightly harder stools and lower frequencies, resulting in a larger impact of dried chicory root on bowel habits. Plotting the change in stool consistency of both trials based on baseline stool consistency (Figure 6E) shows that individuals with harder stools (type 1-2) experience a larger improvement with both dosages, while those with normal stools (type 4) experienced minimal change, and individuals with softer stools (type 5-6) may even revert towards type 4, a possibly more normalized state. This suggests that dried chicory root could facilitate stool normalization, benefiting individuals with harder stools more and potentially aiding those with very soft stools as well.

While we understand what constitutes a normal bowel habit in terms of common frequency and consistency of bowel movements, it does not address how individuals experience these movements or whether such experiences are significant to them. One of Heaton's peers, Walker, articulated this concern in his exploration of the significance of bowel function habits across different ethnicities: "*The primary public health issue is, is it of real significance to health whether feces are voided frequently or infrequently, are soft or hard, large or small, unformed or formed, and passed with ease or with pain?*" (Walker et al., 1982). He emphasized that considering frequency alone was insufficient in relation to bowel diseases.

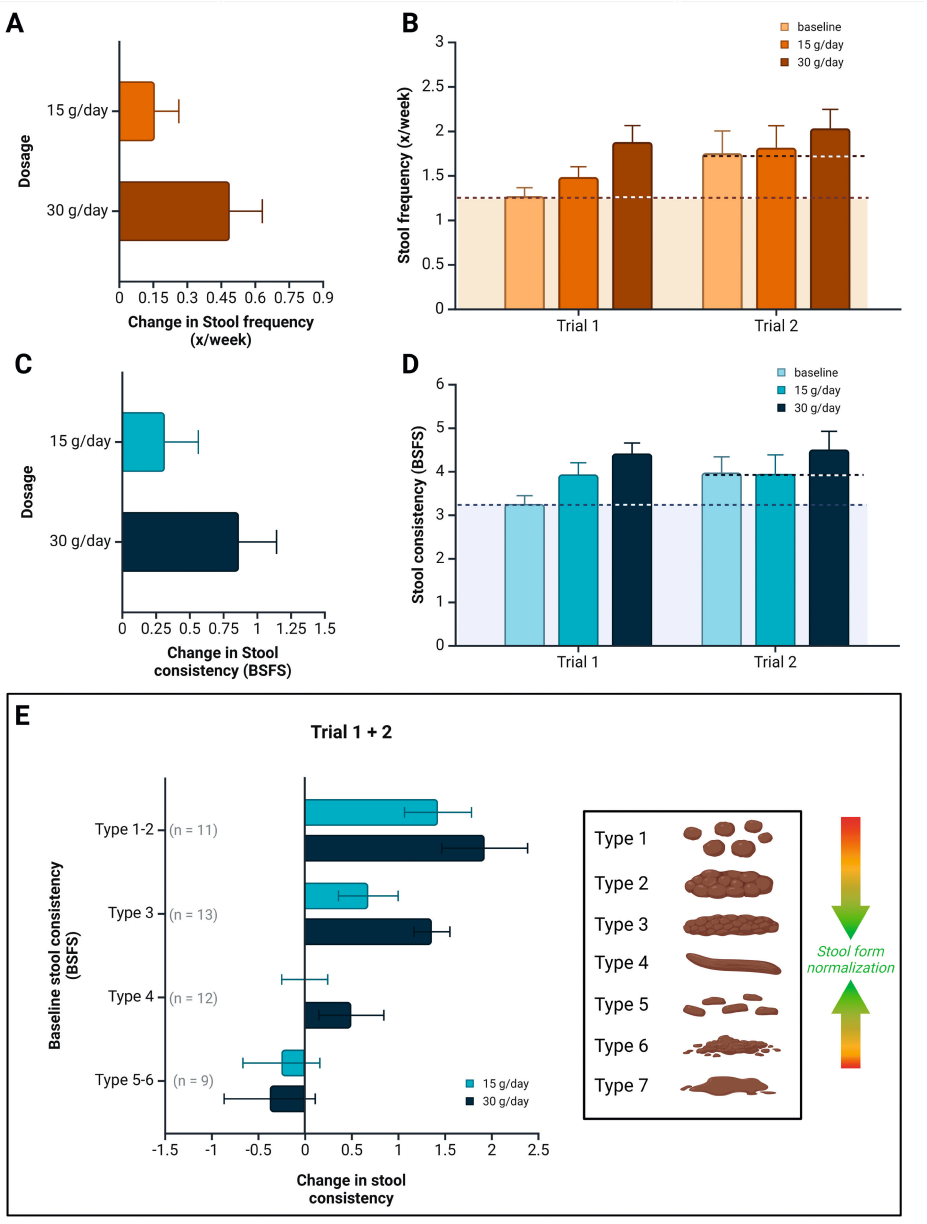

Figure 6. Effect of dried chicory root on bowel function. (A) Observed change in stool frequency based on the combined results of both trials and (B) increases in stool frequency observed in our human short-term trial (Trial 1) and our long-term trial (Trial 2). The shaded area represents the baseline levels of Trial 1, and the horizontal dotted lines represent the baseline levels of each respective trial for comparison. (C) Observed change in stool consistency based on the combined results of both trials and (D) increases in stool consistency observed in our human short-term trial (Trial 1) and our long-term trial (Trial 2). (E) Changes in bowel function appear to depend on baseline bowel habits, as evidenced by changes in stool consistency based on the baseline

Bristol Stool Form Scale (combined results of Trial 1 & 2), indicating that dried chicory root can assist in stool form normalization. Given the longer period of daily 30 g dried chicory root intake in Trial 2 (four, seven, and ten weeks), we compared here the four-week outcomes of Trial 2 to the three-week intake in Trial 1. Created with Biorender.com

Whether normal or not, painful bowel movements and related symptoms can significantly disrupt daily life. Gastrointestinal conditions lacking an underlying anatomical cause but characterized by an individual's experience of painful symptoms are classified as disorders of gut-brain interaction (DGBI), guided by the Rome Foundation (Drossman & Hasler, 2016; Drossman & Tack, 2022). This international organization develops guidelines for diagnosis (the Rome criteria) and treatment of these conditions (Drossman & Hasler, 2016). Formerly known as 'functional gastrointestinal disorders', these conditions are now referred to as DGBI to reduce stigma associated with their psychosocial aspects (Drossman & Tack, 2022). In line with this effort, the Rome Foundation has recently advocated the criterion of 'bothersomeness' — the interference of symptoms with daily life — in clinical assessments of these disorders (Drossman & Tack, 2022). While the etiology of DGBI is still not understood, the gut microbiota is recognized as a key player as it mediates the intraluminal factors (Drossman & Hasler, 2016). Consequently, efforts have focused on profiling the microbiota in conditions like constipation, irritable bowel syndrome, and inflammatory bowel diseases (Lloyd-Price et al., 2019; Mancabelli et al., 2017; Mars et al., 2020). Ultimately, the aim is to find potential avenues to treat these conditions by addressing the gut microbiota.

In **Chapter 7**, we investigated isolated inulin as a potential alternative to pharmacological treatments for functional constipation. Functional constipation is one of these DGBI characterized by painful and hard-to-pass stool. Participants in that trial started with a baseline stool consistency of BSFS type 2 and a frequency of just two stools per week—equivalent to one bowel movement every three to four days. After four weeks of 12 g daily isolated inulin intake, participants had softer stools (half a BSFS unit) and a bowel movement nearly every other day. However, it was particularly compelling that inulin positively impacted emotional and social well-being and reduced abdominal symptoms and discomfort. The outcomes of this trial underscored the importance of prioritizing the individual's perception of bowel function issues in treatment success, highlighting the need to evaluate subjective aspects alongside stool frequency and consistency.

In the context of increased dietary fiber intake, bloating and flatulence are commonly seen as negative outcomes (Mysonhimer & Holscher, 2022), yet they indicate microbial activity resulting from fiber fermentation. In both of our dried chicory root trials (**Chapters 5 and 6**) and **Chapter 7** we observed increased bloating and flatulence. None of these and other gastrointestinal symptoms led to drop-outs in our dried chicory root trials (**Chapters 5 and 6**). In Trial 1, bloating notably increased among participants with baseline fiber intake above the group median (>20 g/day), while flatulence levels were similar between those with high and low (<20 g/day) intake (Puhlmann et al., 2025). These changes are often measured numerically as values, but their significance is

ultimately defined by the individual and the individual's experience. In this context, it is also relevant to note that there were no dropouts in Trial 1 and 2 elated to gastrointestinal symptoms. The results from **Chapter 7** underscore the importance of such a distinction; inulin effectively alleviated abdominal symptom severity despite reported adverse events of flatulence and bloating.

Drawing insights from **Chapters 5, 6, and 7**, we designed a new intervention trial (**Chapter 8**) to assess the effects of up to 15 g of dried chicory root on bowel function in individuals reporting dissatisfaction. Since we observed that this dosage can soften stool consistency by up to one BSFS unit in individuals with hard stools (type 1-2), and given that 15 g of dried chicory root contains 70% inulin, similar to isolated inulin, we expect beneficial effects on bowel habits and their perception in these individuals. We will assess the occurrence and severity of gastrointestinal symptoms. Additionally, we aim to understand how shifts in gut microbiota composition translate into tangible changes in bowel function by implementing a study design that includes increasing increments of dried chicory root and weekly sampling.

How inulin and dried chicory root affect bowel function

Historically, the effect of fiber on bowel function has been attributed to the idea that fibers retain water in the colon. Water-holding softens stools, contributes to fecal mass and bulk, and accelerates colonic transit, potentially increasing stool frequency. Synthetic polymers like macrogol (polyethylene glycol) or fiber such as psyllium exert their laxative or stool-normalizing effects through this mechanism and thereby prevent water reabsorption from the colon (McRorie & McKeown, 2017; Van Der Schoot et al., 2022). However, when water-holding fibers are (partly) fermentable, they are degraded in the colon by gut bacteria and consequently do not hold water anymore. In addition, other fibers dissolve in water but do not bind it. How, then, can other fibers like inulin and dried chicory root improve bowel function? This is best understood by considering other properties apart from water retention, notably fibers' ability to impact the gut microbiota.

Most of our understanding of fibers' modulatory effect comes from studying individual fibers and are summarized in Figure 7A (McRorie & McKeown, 2017; So et al., 2021; Stephen et al., 2017). Combining these insights with the results of **Chapters 5, 6, and 7** shed more light on the differences and similarities in the mechanisms by which inulin and dried chicory root improve bowel function (Figure 7B). Inulin, an easy-to-dissolve, simple, single-chain fructose polymer, does not hold water but is fermentable. Its microbial breakdown contributes to microbial mass and the production of gases and SCFAs, which together increase stool bulk and mass. Additionally, these gases and SCFAs are thought to stimulate colonic motility. Together, these effects make stool easier to pass, speed up colonic transit, and result in the reported increase in stool frequency for which inulin is known (EFSA, 2015), as confirmed in **Chapter 7** for individuals with functional constipation. Although, in **Chapter 7**, we did not find a significant effect of inulin on stool consistency when compared to placebo in the cross-over trial, there were indications for an improvement based on the first-period

analysis (**Chapter 7**). Variability in inulin's stool-softening effect has been reported before (Reimer et al., 2020; Watson et al., 2019) and is most likely explained by the lack of water-holding capacity and proximal fermentation location, in addition to the food matrix inulin is delivered in (Jackson et al., 2022).

Dried chicory root contains four types of fiber: cellulose, hemicellulose, pectin, and inulin (Figure 1A). Consequently, dried chicory root likely exerts its effect on bowel function through a synergistic combination of the physicochemical properties of these fibers, with their three-dimensional organization adding additional complexity to the effect. The plant cell matrix of dried chicory root rapidly absorbs water in the stomach and small intestine, as demonstrated *in vitro* in an upper gastrointestinal digestion experiment (see Figure 7C). Small amounts of inulin can 'leak' from the cut surfaces of dried chicory root particles **(Chapter 4**). Additionally, soluble pectin, likely originating from the intercellular space (middle lamella), may leak out, as evidenced *in vitro* (**Chapter 4)**. Small amounts of fermentable inulin and pectin can serve as initial substrates for gut bacteria but likely contribute minimally to the overall effect. Most of the effect is attributed to the continuous and slow release and fermentation of fiber from the plant cell matrix and intracellular contents (Figure 7B). Consequently, increases in stool bulk through microbial mass and the production of gases and SCFAs will continue in the distal colon. As a result, we most likely see higher fold-changes in *Bifidobacterium* spp. and *Anaerostipes* spp. relative abundances compared to those from isolated inulin, along with increased fecal SCFA levels, which have not always been reported for inulin (**Chapter 5**). It is possible that remnants of the cell matrix may mechanically irritate the intestinal lining and retain water if they are not degraded. Mechanical irritation stimulates fluid secretion and motility and is the mechanism to which the effects of the dense cellulose network of wheat bran are primarily attributed (McRorie & McKeown, 2017; So et al., 2021). In our *in vitro* experiments, we observed that chicory root cube structures remained intact after 48 hours of fermentation (see Figure 7D). This is again compatible with our hypothesis of a slow fiber release and distal effect, leading to both increased stool frequency and softer stools. Whether dried chicory root remains intact in the distal colon remains to be elucidated, potentially using microscopic assessments of fecal material as employed before (Ellis et al., 2004).

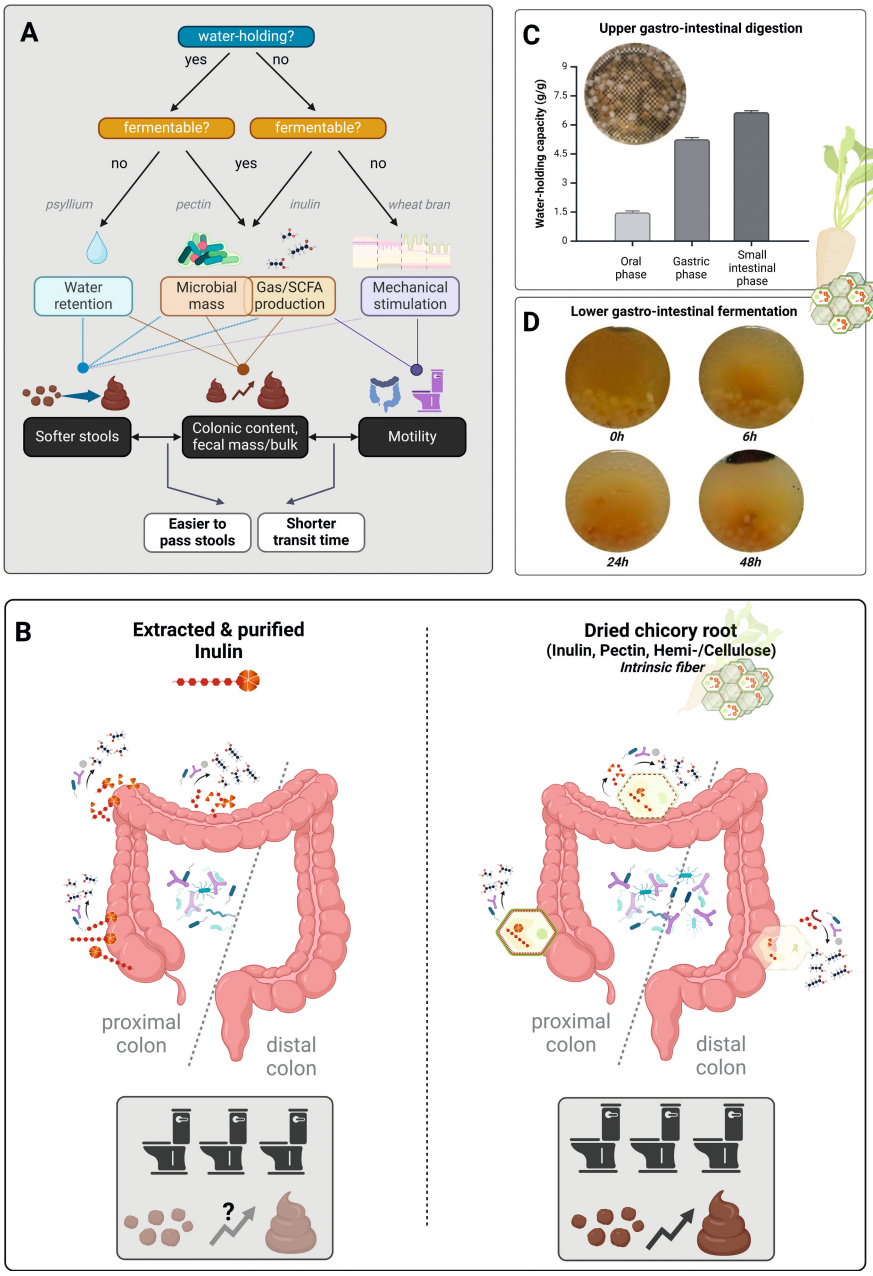

Figure 7. Modulation of Bowel Function by Dietary Fiber. (**A**) Schematic representation of how different fibers affect bowel function based on their water-holding capacity and fermentability. This leads to bulkier, softer stools that are easier to pass, and together with increased motility, results in shorter transit times. (**B**) Proposed mechanisms by which inulin or dried chicory root impact bowel function. Inulin is primarily fermented in the proximal colon, while dried chicory root extends fermentation into the distal colon. (**C-D**) Potential maintained water-holding capacity of dried chicory root as evidenced by (**C**) *in vitro* upper gastrointestinal digestion and (**D**) the visual presence of dried chicory root cubes throughout lower gastrointestinal *in vitro* fermentation (personal communication FS Kaper, WholeFiber BV). Created with Biorender.com

In conclusion, inulin without the physical shield of the plant cell wall benefits bowel function through its microbial conversion as a readily accessible fiber in the proximal colon. Within the plant cell wall of dried chicory root, we can expect a synergistic effect of inulin and the plant cell wall fibers, which most likely extends from the proximal to the distal colon. This difference in colonic location might explain the smaller increase in stool frequency and consistency with inulin (**Chapter 7**) compared to the change observed from dried chicory root in individuals with a baseline BSFS score of 1-2 (Figure 6E). Both fibers offer advantages over common laxatives by potentially relieving bowel function issues and benefiting the gut microbiota, albeit with varying efficiencies, thereby creating simultaneous health effects beyond the colon.

What should we assess in future?

Insights from this thesis (**Chapters 5, 6 and 7**) suggest that fiber-induced changes in gut microbiota composition and function mediate the effects these fibers have on bowel function. Over the last decade, we have succeeded in assessing the relationships between bowel function and specific gut microbiota compositional and functional signatures using next-generation sequencing (Asnicar et al., 2021; Boekhorst et al., 2022; Müller et al., 2020; Roager et al., 2016; Vandeputte et al., 2016). The emerging picture suggests that harder stools and longer transit times correlate with higher gut microbial richness. This vis likely indicative of denser, moisture-depleted feces. Hard stools are also associated with specific gut microbial taxa, including *Akkermansia* spp. and *Methanobrevibacter* spp. The former might reflect increased mucin degradation due to lower nutrient flux and potential expulsion in the feces due to straining. *Methanobrevibacter* spp. are Archaea known to grow slowly, which is promoted by longer transit times, meaning feces reside longer in the colon. Interestingly, in **Chapter 7**, we observed that individuals who showed greater improvement during inulin intake—and drove the placebo response observed in the entire trial —had lower baseline relative abundances of this taxon. We interpreted this as a potential difference in the intestinal environment that might make individuals more inclined to improve. Similar observations were made for lower relative abundances of *Bifidobacterium* spp. and higher relative abundance of butyrate-producing genera, uniquely marking individuals with larger improvements.

While we did not directly explore the relationships between gut microbial taxa changes and bowel function in **Chapters 5 and 6**, we plan to delve into this with data from the HappYFiber study (**Chapter 8**). Ultimately, the combined information from **Chapters 5, 6, and 8** will provide insights into how dried chicory root, specifically the innate qualities of its intact plant cell matrix, can benefit bowel function in both individuals with and without complaints. These insights might also help develop further applications for how isolated inulin can be used in specific clinical settings. Therefore, ongoing research into the influence of dietary fiber on bowel function through microbiota modulation remains crucial in 2024.

Despite the long-established relationships between stool consistency and transit time, as well as stool frequency and moisture (Lewis & Heaton, 1997a), future studies

should still explore other aspects of the gut environment to understand fiber-mediated gut microbiota modulation. Factors such as stool moisture, pH, and transit time not only influence but also reflect the activity and composition of the gut microbiota (Procházková et al., 2024; Procházková, Falony, et al., 2023). Analytical methods for assessing stool moisture and fecal pH are relatively simple to implement. However, they pose biohazard concerns due to potential pathogens, and the distinctive odor of fecal matter affects the feasibility of these measurements. In the past century, total stool mass was often assessed, and the effect of fiber was quantified as grams of stool per gram of fiber produced (Cummings, 2001; Cummings et al., 1978). Currently, the King's scale is used in healthcare settings to assess fecal output. It may be worth considering whether such a measurement is also valuable in a research setting (Whelan et al., 2008).

Low-cost transit time measurements that avoid invasive radio-opaque techniques have made a comeback in study designs. The measurement of transit time using fecal dye methods (or "first appearance" markers) has gained popularity since its implementation in a large cohort study (Asnicar et al., 2021). In the same manner sweet corn has been previously applied (Keendjele et al., 2021; Procházková et al., 2024; Procházková, Venlet, et al., 2023). However, the use of non-absorbable markers such as carmine, brilliant blue, or charcoal is not new and dates back to before the 1960s (Lutwak & Burton, 1964). Revisiting old literature and integrating that knowledge with our current analytical tools might help us advance our understanding of the interplay between fiber, microbiota, and bowel function. For example, cross-over experiments where transit time is artificially altered within the same individual using laxatives or antidiarrheal drugs, combined with analysis of changes in microbiota composition, metabolites, intraluminal and fecal pH, and stool moisture, can help us understand why our predecessors observed changes in butyrate levels with altered transit time (Lewis & Heaton, 1997b) and link that to the gut microbiota (Asnicar et al., 2021). Modern real-time ingestible capsules that measure segmental changes along the proximal and distal colon would be a great addition for this purpose (Procházková et al., 2024). Ultimately, we could avoid what Cummings stated: *"Since then, much of the early work on fiber has inevitably been repeated, and many papers do little if anything to advance our knowledge or understanding of the mode of action"*(Cummings, 2001).

METHODOLOGICAL CHALLENGES AND CONSIDERATIONS IN FIBER-GUT MICROBIOTA RESEARCH

In this thesis, we investigated the impact of dietary fiber on human gut and metabolic health by studying changes in the gut microbiota. To analyze the gut microbiota composition and function, we relied on relative compositional data (relative abundances) obtained through DNA sequencing techniques in fecal samples. It is important to note that fecal microbiota composition may differ from that of the gut lumen microbiota and relative abundances from absolute abundances. Despite the difference between the fecal and luminal samples, I have used 'gut microbiota' and 'fecal microbiota' interchangeably in this thesis (**Chapters 5, 6, 7, and 8**). In the future, if we succeed in

sampling intraluminally through ingestible devices, it will become even more important to distinguish these terminologies and relate their outcomes to each other. Our data was primarily obtained through human intervention studies (**Chapters 5, 6, and 7**), which have the advantage of providing real-life insights into the *in vivo* conversion of dietary fiber. We preferred these studies over extensive *in vitro* testing, though we acknowledge the usefulness of *in vitro* models to pre-screen treatments of interest and develop a hypothesis as we did in **Chapter 4**. Since human intervention studies are prone to large variability in outcomes due to heterogeneous *in vivo* responses, we employed post-hoc subgroup analysis to address these differences (**Chapters 5, 6, and 7**). All these methods have their advantages and limitations, which are extensively discussed elsewhere (Assmann et al., 2000; Isenring et al., 2023; Lloréns-Rico et al., 2021). Here, I will summarize the findings from DNA sequencing, post-hoc subgroup analysis, and *in vitro* models and discuss considerations for future research directions.

Assessment of gut microbiota composition using 16S rRNA gene amplicon versus shotgun metagenomic sequencing

In this thesis, we mainly relied on 16S rRNA gene amplicon sequencing to assess fiber-induced changes in gut microbiota composition at genus level (**Chapters 5, 6, and 7**). However, differences in species and even specific strains have been shown to play a role in fiber-induced gut microbiota changes (Chung et al., 2016; Deehan et al., 2020; G. Wu et al., 2021; Zhao et al., 2018). Currently, 16S rRNA gene amplicon sequencing approaches are restricted by short read lengths, which hinders such species-level annotation (Johnson et al., 2019). Additionally, these methods involve preprocessing steps such as PCR and library preparation, introducing additional biases (Abellan-Schneyder et al., 2021). Consequently, sequencing techniques that circumvent these problems are rising in fiber-gut microbiome research, such as shotgun metagenomics. In **Chapter 6**, we therefore applied 16S rRNA gene amplicon sequencing alongside shotgun read-based metagenomic profiling to gain deeper insights into the species level. To analyze the generated metagenomic data, we utilized the MetaPhlAn4 workflow along with its integrated database for species-level resolution (Blanco-Míguez, Beghini, et al., 2023).

Very similar insights emerged overall from 16S rRNA gene amplicon and shotgun metagenomic outcomes, with differences primarily related to updated taxonomic identification not yet implemented in the SILVA database used for taxonomic assignment of 16S rRNA gene sequences. For instance, this concerned the reclassification of *Prevotella* 9 spp. to *Segatella* spp. or *Eubacterium hallii* group to *Anaerobutyricum* spp. (Blanco-Míguez, Gálvez, et al., 2023; Hitch et al., 2022; Shetty et al., 2018). However, we also observed differences in the prevalence and abundance of certain taxa when aggregating MetaPhlAn4-derived profiles at higher taxonomic levels, such as genus level. In this context, I would like to highlight that MetaPhlAn4's criteria for including reference genomes of only species 'reconstructed from isolate sequencing or single cells' available in the National Center for Biotechnology Information (NCBI) database (Agarwala et al., 2018; Blanco-Míguez, Beghini, et al., 2023). If a genome in the NCBI

database is obtained from Metagenome-Assembled Genomes (MAGs) rather than isolation sources, it is not included (Blanco-Míguez, Beghini, et al., 2023). In contrast, 16S rRNA gene reference databases for amplicon sequencing, like the SILVA database (Glöckner et al., 2017), may contain numerous entries at the species level, with many of them representing yet uncultured microorganisms.

It is important to consider inherent limitations and differences when interpreting outcomes from 16S rRNA gene amplicon and shotgun metagenomic sequencing in the context of their ongoing technological development. Resolving database limitations to transition from the genus to species or even strain level will require time to generate and compile more data. Moreover, the computational resources, pipelines, and bioinformatics expertise necessary for shotgun metagenomic analyses remain more complex and demanding compared to 16S rRNA gene amplicon-based analysis. Consequently, bioinformatically skilled experts are crucial for integrating such data meaningfully until these interfaces become more user-friendly. In essence, while shotgun metagenomics is valuable, it currently remains labor-intensive. At the same time, 16S rRNA gene amplicon sequencing continues to improve in quality (Johnson et al., 2019). At this stage, relying exclusively on species and strain levels derived from shotgun metagenomics may not universally address fiber-gut microbiota research questions. Instead, we might need to concurrently evaluate 16S rRNA gene amplicon data alongside shotgun metagenomic data as done in **Chapter 6** to advance the field in the interim.

Individual gut microbiota compositions using subgroup analysis

Human intervention studies, especially those involving dietary and pharmacological interventions, are and have always been prone to variability in individuals' responses to the treatment of interest. In the last few decades, this heterogeneity has spurred interest in better understanding individual responses, leading to the research field of personalized or precision nutrition and medicine (Bailey & Stover, 2023; Zeisel, 2020). Given that the gut microbiota composition is as unique to each individual akin to a fingerprint - a characteristic demonstrated over 25 years ago (Zoetendal et al., 1998) - the gut microbiota likely mediates observed heterogeneity (Zeevi et al., 2015). Evidence supports this notion, as seen in data analysis controlling for metformin intake in T2D, highlighting the microbiota's pivotal role in mediating the effect of the drug (Pedersen et al., 2016; H. Wu et al., 2017). Consequently, there is a shift away from relying solely on group averages toward assessing individuals and subgroups (Bailey & Stover, 2023; Mannion et al., 2023). In this thesis, we explore variations in treatment responses by investigating differences in individual gut microbiota composition through post-hoc subgroup analysis in **Chapters 5, 6, and 7**.

Using a straightforward approach of dividing individuals in the treatment group based on relative changes in the primary outcome, we tried to understand how treatment success related to baseline gut microbiota differences. In **Chapter 5**, in our short-term dried chicory root trial, we observed that improvements in insulin sensitivity assessed by HOMA-IR were reflected in the baseline difference in *Blautia* spp. relative abundance.

As high levels of *Blautia* spp. are linked to negative metabolic health outcomes and refined diet consumption (Bolte et al., 2021; Gurung et al., 2020; Le Roy et al., 2017), we hypothesized that different levels of this taxon mirror a gut environment adapted to such diets, requiring prolonged dietary perturbations to observe systemic effects. Building on these insights, in **Chapter 6**, we applied a similar rationale in our long-term dried chicory root trial. Improvements in insulin-sensitivity assessed by insulin-mediated glucose disposal were reflected in baseline differences of pectin-degrading taxa, including *Bacteroides* spp. and *Monoglobus* spp., and the pectin-degrading potential (pectate lyase genes). High levels of *Bacteroides* spp. have been linked to lower success in weight-loss trials, increased metabolic activity, and reduced stool energy contents and high pectate lyase levels to insulin-resistance (Boekhorst et al., 2022; Hjorth et al., 2018; Kolmeder et al., 2015; Procházková, Venlet, et al., 2023). Consequently, we hypothesized again that differences in these taxa mirror a gut environment that is less responsive to dietary perturbation due to differences in speed and resulting location of dried chicory root conversion and SCFA uptake. In **Chapter 7**, we used a similar approach not to determine treatment success but to understand the observations related to potential carry-over. Here, we observed that the notable placebo response in the primary outcome was reflected in baseline differences in putative butyrate producers and *Bifidobacterium* spp. in one of the intervention sequences. We hypothesized that the levels of these taxa mirror a gut environment that is more predisposed to lasting improvements. Collectively, our subgroup analyses in **Chapters 5, 6, and 7** demonstrated that post-hoc evaluations of baseline gut microbiota signatures in relation to clinical outcomes can assist in generating hypotheses about individual treatment responses.

Nevertheless, it seems currently unrealistic to expect that we can define a single, universal, and biologically meaningful gut microbiota signature that predicts the success of all fiber interventions for every individual. Instead, different gut microbiota signatures likely exist on a continuum. This idea can be illustrated in the concept of enterotypes. Enterotypes represent clusters of gut microbiota compositions characterized by the predominance of specific bacterial taxa such as *Bacteroides*, *Prevotella*, and *Ruminococcus* (Arumugam et al., 2011). However, instead of forming distinct categories, enterotypes are understood to exist along a gradient where the dominance of one bacterial taxon gradually merges into that of another (Costea et al., 2017). This means that these taxa are not mutually exclusive, but coexistent. While none of these approaches are perfect, they provide valuable insights into gut microbiota characteristics. Critics of post-hoc subgroup analysis may advocate for a priori determined subgroup definitions – either based on the clinical outcome or in relation to gut microbiota signatures (Assmann et al., 2000; Boshuizen & te Beest, 2023; Mannion et al., 2023; Schandelmaier & Guyatt, 2024). It is questionable whether such an approach is currently feasible in gut microbiome research, as specific clinical outcome changes and gut microbiota signatures also depend on the applied analytical methods and may be unique to particular subpopulations, which are not yet understood. For instance, *Blautia* spp., despite their clear link with the available literature, did not re-emerge as

a responder signature in our long-term dried chicory root trial, although the relative abundances of this genus were found to decrease in both trials (**Chapters 5 and 6**). In contrast, both **Chapters 5 and 7** demonstrated a higher fold-change in individuals with low compared to higher baseline *Bifidobacterium* spp. relative abundances following the intake of dried chicory root and inulin, respectively. This observation was consistent despite being assessed in two different populations: older-aged individuals at risk for T2D and middle-aged females with functional constipation. These discrepancies exemplify the limitations of such approaches. It is possible that the observations related to *Blautia* spp. in **Chapter 5** were linked to specific species, which we could not assess due to the limitations of 16S rRNA gene amplicon-based gut microbiota analysis. As more data accumulates, reanalyzing datasets will be crucial to assess, which gut microbiota signatures are broadly applicable. This process requires that datasets are easily discoverable, accessible, and shareable (see also the FAIR principles) (Rodriguez et al., 2023; Wilkinson et al., 2016). It will also involve revisiting taxonomies using updated databases and rigorously evaluating preprocessing techniques. Conducting such data analyses would greatly benefit from future randomized controlled trials with larger sample sizes, enhancing the reliability and validity of subgroup assessments (Burke et al., 2015). Ultimately, these efforts will enhance our understanding of how the individual gut microbiota contributes to explaining inter-individual variation, benefiting precision nutrition and medicine.

Fiber-induced gut microbiota modulation assessed by in vitro models

Assessing the intraluminal processes of food digestion and fermentation in the human body heavily relies on using *in vitro* models. These models enable the study of interindividual differences and, in advanced settings, aspects such as particle size, transit time, and meal timing (De Paepe et al., 2017, 2019; Minnebo, De Paepe, et al., 2023; Minnebo, Delbaere, et al., 2023). However, the conclusions drawn from these models depend significantly on the specific protocols employed, including factors such as pretreatment of feces, inoculum concentrations, pretreatment conditions, and nutrient density, all of which influence observed changes in gut microbiota composition (Minnebo et al., 2021; Poppe et al., 2023). Therefore, it is crucial to interpret findings within the context of the model's choices and limitations.

In **Chapter 4**, we applied two *in vitro* fecal batch fermentation models – one with a low inoculum concentration and another with a high inoculum concentration, following established protocols from our collaborators. While the model with a higher inoculum amount exhibited a faster rate of butyrate production, both models confirmed the production of butyrate during later stages of fermentation. These findings supported our hypothesis of slow fiber release and distal fermentation and influenced our decision to proceed with dried chicory root cubes instead of finer particles in future human trials (**Chapters 6 and 8**).

Many of these models do not allow for the assessment of macroscopic differences in particle size due to the need to control for microbial contamination and maintain

anoxic conditions. In the future, developing unified protocols that address these aspects and include the often omitted upper gastrointestinal digestion steps will help us study intrinsic fiber more extensively (Pérez-Burillo et al., 2021). Ultimately, findings from these models require validation in human studies and should not be used to exclusively claim health benefits, particularly in asserting a prebiotic effect.

FOOD PROCESSING AND DIETARY FIBER

The presence of intrinsic fiber in our diet is fundamentally impacted by the level of food processing, which can disrupt the plant cell matrix of edible plants, determining whether intrinsic fiber remains intact (**Chapter 2**). The food choices we make and the level of processing we accept or apply directly influence the amount of intrinsic fiber we consume and, consequently, our overall health. While the impact of food processing on health seems like a recent 21st-century concern, Burkitt and his colleagues proposed this in their fiber hypothesis over six decades ago. They posited that the consumption of a refined, westernized diet was the primary driver behind the rise in non-communicable diseases (Cummings & Engineer, 2017; O'Keefe, 2019; Southgate, 1992). At that time, a 'refined diet' lacked a definition, but it was evident that it correlated with the absence of fiber rather than the presence of excessive sugars and refined carbohydrates, as these could not coexist without the removal of fiber from foods (Cummings & Engineer, 2017). Many of these insights stemmed from historical comparisons between the 19th and 20th centuries, where industrialization and modern food processing techniques coincided with dietary changes (Walker, 1980). For example, the shift from brown to white bread due to advances in milling techniques was associated with increased constipation rates, whereas a return to less refined products during World War II reduced such incidences (Walker et al., 1982). As food processing appears to go hand in hand with human health deterioration, one may wonder: what is the role of food processing in the future for human health?

Food processing plays a vital role for humanity; many of our edible foods would not be digestible or safe without domestic or industrial processing (Carmody et al., 2016; Wrangham et al., 1999). It enables us to increase the availability of energy in the form of macronutrients and to access essential micronutrients necessary for various bodily functions, along with other bioactive compounds (Carmody et al., 2011; Gibson et al., 2006; Groopman et al., 2015; Palermo et al., 2014; Platel & Srinivasan, 2016). However, over the past century, it appears that we have exploited food processing to such an extent that it has shifted from benefiting to potentially harming human health. Today, we have highly palatable, easily digestible foods made from ingredients extensively extracted from their source crops to provide quick energy and a long shelf life. These refined foods are labelled today as 'ultra-processed foods' to distinguish them from less processed foods. For this purpose, the NOVA classification system was developed (Monteiro et al., 2019). Despite its positive intentions, the system has faced extensive criticism for not being straightforward and universally applicable and for casting food processing in a negative light (Astrup & Monteiro, 2022; Monteiro, 2009; Monteiro et al., 2018; Yeo, 2024). Unfortunately, the discussion surrounding the health implications of

highly refined foods in the context of the NOVA classification system appears to have divided scientists into opposing camps as they debate the definition.

Regardless of the NOVA system's usefulness, we continue to consume high amounts of refined foods that are low in dietary fiber, even though Burkitt and colleagues demonstrated the consequences over 50 years ago. Accordingly, we must explore how food processing can be a solution and driver in reversing the global metabolic health crisis rather than merely debating its role in its origin. Current efforts are increasingly focused on studying the incorporation of fiber into our food products, utilizing established and newly discovered fibers (Haque et al., 2023; Lai, 2023). Simultaneously, valorizing food waste through upcycling waste streams and preventing them in the first place has emerged as a pivotal strategy to combat food loss and advance sustainability goals (Aschemann-Witzel et al., 2023; Papargyropoulou et al., 2014; Punia Bangar et al., 2024; Sandström & Kummu, 2023). While these efforts will undoubtedly help enrich the fiber content in our Westernized and refined diets, they also appear ironic. Initially, we eliminated fiber from our diets through processing, only to now seek ways to reintroduce fiber into our fiber-depleted foods. In essence, we are addressing the consequences of our own dietary and food processing choices through further processing - an attempt to repair the damage while perpetuating it.

How can we reconsider our current food processing practices? Moving away from a reductionist perspective that views fiber solely as isolated molecular structures with distinct physicochemical properties toward recognizing fiber as an integral part of plant cells whose full value we have yet to fully comprehend? In **Chapter 2**, we propose reducing the extent of processing of our edible food crops to safeguard the intrinsic plant cell matrix. Milder food processing choices, such as preserving the natural structure of chicory roots by drying the plant tissue instead of extracting inulin, and using coarse structures instead of fine powder, can enhance fibers' beneficial effects on human health. Although we do not compare the effects of different processing degrees *in vivo*, we used the *in vitro* outcomes (**Chapter 4**) to guide our choices for the human intervention trials we executed using dried chicory root particles (**Chapter 5**) and later cubes (**Chapter 6**). We provide evidence that such minimal food processing methods of dietary fiber also benefit gut and metabolic health by altering intracolonic processes, as observed from changes in the gut microbiota composition and SCFA levels in fecal samples (**Chapter 5 and 6**).

The concept of minimal food processing providing greater health benefits is not new. As mentioned earlier, Haber et al. demonstrated through their apple experiment the benefits of preserving the plant cell wall intactness for the insulin response (Haber et al., 1977). Similarly, O'Donnell et al. showed that bread made from coarse flour reduced both glucose and insulin responses compared to fine-flour bread, advocating it as a 'simple alternative' (O'Donnell et al., 1989).It remains to be explored how these processing differences also affect the gut microbiota, as current insights are limited to *in vitro* and animal models (Carmody et al., 2019; Lerma-Aguilera et al., 2023; Pérez-Burillo et al., 2018; Rajakaruna et al., 2024; Smith et al., 2022). Aside from the potential impact on health, less food processing will also necessitate a reevaluation of how intrinsic

fiber can be used in food formulations. Fibers in their intrinsic form do not provide the same physicochemical properties as their isolated single components, and each step of food processing can further alter this. Moreover, the type of food processing needed to safeguard the plant cell matrix will depend on the type of food (hard/soft, ripe/unripe). It is compelling to reimagine how food processing could transform fiber from a bland, off-tasting ingredient into an attractive food component that enhances both health benefits and sensorial appeal without the need for isolating and purifying fibers. Investigating how food processing techniques could achieve this and how they positively could affect the gut microbiota, gut health, and metabolic outcomes will provide new perspectives on leveraging food processing to mitigate the unintended consequences of our dietary choices. The process may not be straightforward, but hopefully, nutritionists and food technologists in academia, industry, and governmental bodies will move beyond the scientific debate on the usefulness of classifications and definitions like the NOVA system to collectively address our self-created refined-diet issues.

CONCLUSION AND FUTURE CONSIDERATIONS

This thesis explored how dried chicory root, a fiber-rich food product notably high in inulin, benefited human metabolic and gut health and thereby revisited the added benefits of the plant cell matrix in enhancing the effects of dietary fiber. We demonstrated that dried chicory root is an intrinsic fiber product, exceptionally high in fiber compared to other food products and with an intact plant cell matrix encapsulating inulin. The presence and intactness of this matrix fundamentally influenced the microbial breakdown of dried chicory root and promoted the production of butyrate, a crucial SCFA for host health. Dried chicory root rapidly, significantly and reversibly altered the gut microbiota composition, promoting bacterial taxa involved in butyrogenic trophic chains, particularly those capable of converting lactate into butyrate. These microbiota changes coincided with improved bowel function, warranting investigation into the potential benefits of dried chicory root for individuals with reported bowel function issues. Furthermore, dried chicory root positively impacted systemic metabolic outcomes, as evidenced by reduced glycemic variability and improved insulin sensitivity in individuals at risk for T2D. These changes were notably influenced by the individual gut microbiota composition, highlighting it key role in determining treatment success. Ultimately, the insights from this thesis will help us raise awareness that dietary fibers in their intrinsic form enhance their health benefits and are essential to consider in our current efforts to address the self-created fiber gap and strive to make the healthy choice the easy choice.

Learning from the past

Like many scientists, this thesis has drawn inspiration from the most recent scientific findings, that is typically defined over the period of the last 5-10 years. During this period, we have gained valuable insights into the composition and function of gut microbiota. However, our understanding of how this intricate microcosm in the human gut influences overall health is still limited. While it is acknowledged that more

studies are needed in the future, focusing solely on recent developments will enforce a reductionist view rather than widen our horizons. Revisiting early research on fiber, as suggested by Cummings, and integrating this with our modern technologies holds more promise in advancing our knowledge of its effects on human health (Cummings, 2001). To achieve this, we should advocate for incorporating 'old-school' measures such as fecal pH, water content, bacterial load, and possibly total fecal mass and remnants of fiber. Microscopic assessment of food residues, as demonstrated by Ellis et al. two decades ago, could also provide valuable insights (Ellis et al., 2004). Furthermore, as Cummings noted, studies should integrate the physiology of the large bowel. Combining traditional fecal dye methods with modern technologies such as real-time ingestible sensors or magnetic resonance imaging can merge old and new insights into a unified understanding. By building upon the work of our predecessors rather than starting anew, we could possibly avoid what Cummings already stated: *"If these [earlier] studies had received greater attention, much that is mundane and repetitious in current nutritional research would have been obviated."*(Cummings, 2001).

When it comes to our modern food choices, the question arises: should we focus on new functional foods to lower blood sugar levels, or should we prioritize consuming whole foods? In other words, should we create new food formulations to address gut and metabolic diseases symptomatically, or should we tackle the root cause of the issues? Drawing from insights in this PhD thesis, I support Burkitt and Trowell's stance that many of our modern diseases stem from fiber deficiencies. We need to address this deficiency by rethinking our food choices and food production processes.

Our current fiber intake guidelines

For nearly 20 years, the Health Council of the Netherlands has recommended consuming 14 g of dietary fiber per 1,000 kilocalories (Health Council of the Netherlands, 2006). This translates to 30-40 g per day based on an average daily intake of 2,100 to 2,800 kcal, suitable for a sedentary to lightly active lifestyle (Health Council of the Netherlands, 2022). The Netherlands Nutrition Centre suggests a slightly lower intake of 25 g per day for women and 30 g per day for men, aligning with 12 g per 1,000 kcal (2,000 kcal for women, 2,500 kcal for men) (The Netherlands Nutrition Centre, n.d.). Of course, dietary fiber needs vary depending on the individual, lifestyle, and energy requirements. However, using this recommendation as an example, we find that 25-30 g per day aligns closely with the dietary fiber intake of 25 to 29 g per day, which has been identified as a minimum to reduce various health risks in a meta-analysis involving 4,635 individuals (Reynolds et al., 2019). Nevertheless, is consuming the bare minimum enough? Probably not. In another meta-analysis the same authors, including data from over 10,000 individuals with type 1 diabetes, T2D, and prediabetes, concluded that increasing daily fiber intake by 15 to 35 g significantly reduces the risk of diabetes-related mortality (Reynolds et al., 2020).

In the process of reviewing old literature, I discovered that Burkitt was reported to advocate for a daily fiber intake of 50 g (O'Keefe, 2019). I also noted that previous

research on bowel function frequently compared outcomes to fiber consumption in pre-industrialized eras (Walker et al., 1982). To illustrate this, references were made to the daily intake of whole-meal bread in the 19th century, where individuals consumed up to 600 g. This intake decreased to 450 g at the beginning of the 20th century, 300 g by mid-century, and 150 g by the end of the century (Walker et al., 1982). Today, the recommended intake of whole-meal bread is 90 g, as advised by the Health Council of the Netherlands (Health Council of the Netherlands, 2015). Commonly, whole-meal bread in the Netherlands contains approximately 7 g of fiber per 100 g (RIVM, 2023). Extrapolating this to a daily intake of 600 g of bread suggests that individuals in the past effortlessly consumed 42 g of fiber daily from bread alone. However, we should not forget that other foods, like fruits and vegetables, were less accessible back then. Today, we are advised to complement our 90-gram portion of whole-meal bread with 200 g of vegetables and 200 g of fruit. Estimates suggest that pre-industrial fiber intake may have exceeded 100 g per day (Jenkins et al., 2001; Jew et al., 2009). Moreover, processing methods of previous centuries likely provided more intrinsic fiber than modern practices. Notably, the stone-ground wheat of the 19th century retained its fiber-rich bran layer, contrasting with today's reconstituted whole wheat bread. In modern processes, the bran is initially separated, milled, and subsequently mixed with refined flour after milling, thereby omitting the inherent benefits of intrinsic fibers (**Chapter 4** and (Van Der Kamp, 2012; Walker et al., 1982)).

Given these considerations, should we increase our fiber intake recommendations with a focus on intrinsic fiber? Drawing insights from this thesis using dried chicory root, historical literature, and intervention studies like the African-American-African diet switch, I believe we should aim to meet or exceed the threshold set by the Health Council of the Netherlands, emphasizing intrinsic fiber (Jenkins et al., 2001; O'Keefe et al., 2015). However, as early as 2006, 90% of the Dutch population did not meet the recommended intake of 14 g per 1,000 kcal. Changing dietary habits is challenging, despite everyone's desire for a healthy and fulfilling life, because the immediate benefits are often not apparent until later years.

Moving fiber research forward

How can we change our view on dietary fiber to transition to better food choices? In the field of nutrition science, dietary fiber is currently not classified as an essential nutrient due to the 'absence of a deficiency state' (Kohn, 2016). Reframing gut and metabolic diseases as deficiencies in fiber intake and thereby establishing dietary fiber as an essential nutrient could lead to a paradigm shift in our perception of fiber. However, it might also promote 'fiber enrichment', where the focus shifts towards adding extracted fibers, potentially encouraging rather than preventing food refinement. Consequently, this approach might defeat the intended purpose. Furthermore, we should let go of the notion that food induces changes as rapidly as pharmaceutical interventions. While drugs efficiently target specific pathways, dietary changes can simultaneously affect multiple metabolic functions (de Vos et al., 2006). Therefore, although food interventions

require more time, they may yield broader and more balanced outcomes. I believe as scientists, we need to rediscover fiber for what it inherently offers. Understanding individual fibers and their microbial breakdown by considering cross-feeding, functional redundancy, and metabolite production will help us comprehend molecular processes, but it will not provide more insights beyond these individual fibers. Since we consume foods rather than nutrients, we need to delve deeper.

Fibers are ubiquitous as the backbone of our plant foods. They fundamentally benefit human health through their effects on food intake and satiety in the upper gastrointestinal tract and through their impact on the gut microbiota in the lower gastrointestinal tract. However, many of us find consuming fiber in the form of cereals, fruits, nuts, pulses, and vegetables a nuisance. Rediscovering the benefits of the forgotten vegetable chicory root in its minimally processed form, as demonstrated in this thesis, will facilitate making the healthy choice the easy choice. If we were able to recognize fiber as essential for human health, particularly in nourishing our gut microbiota, we might start making food choices that benefit both us and the planet. Perhaps in 150 years, parents won't urge their children to “Eat your vegetables” but will instead say, “Feed your gut microbes.” This shift would embody the recognition of fiber as an essential nutrient in the human diet, not as an isolated component, but as an integral part of edible plants. And possibly in German, dietary fiber will still be called “Ballaststoffe”, not because they are considered economically worthless, but as essential “Ballast” that keeps our human bodies stable and buoyantly.

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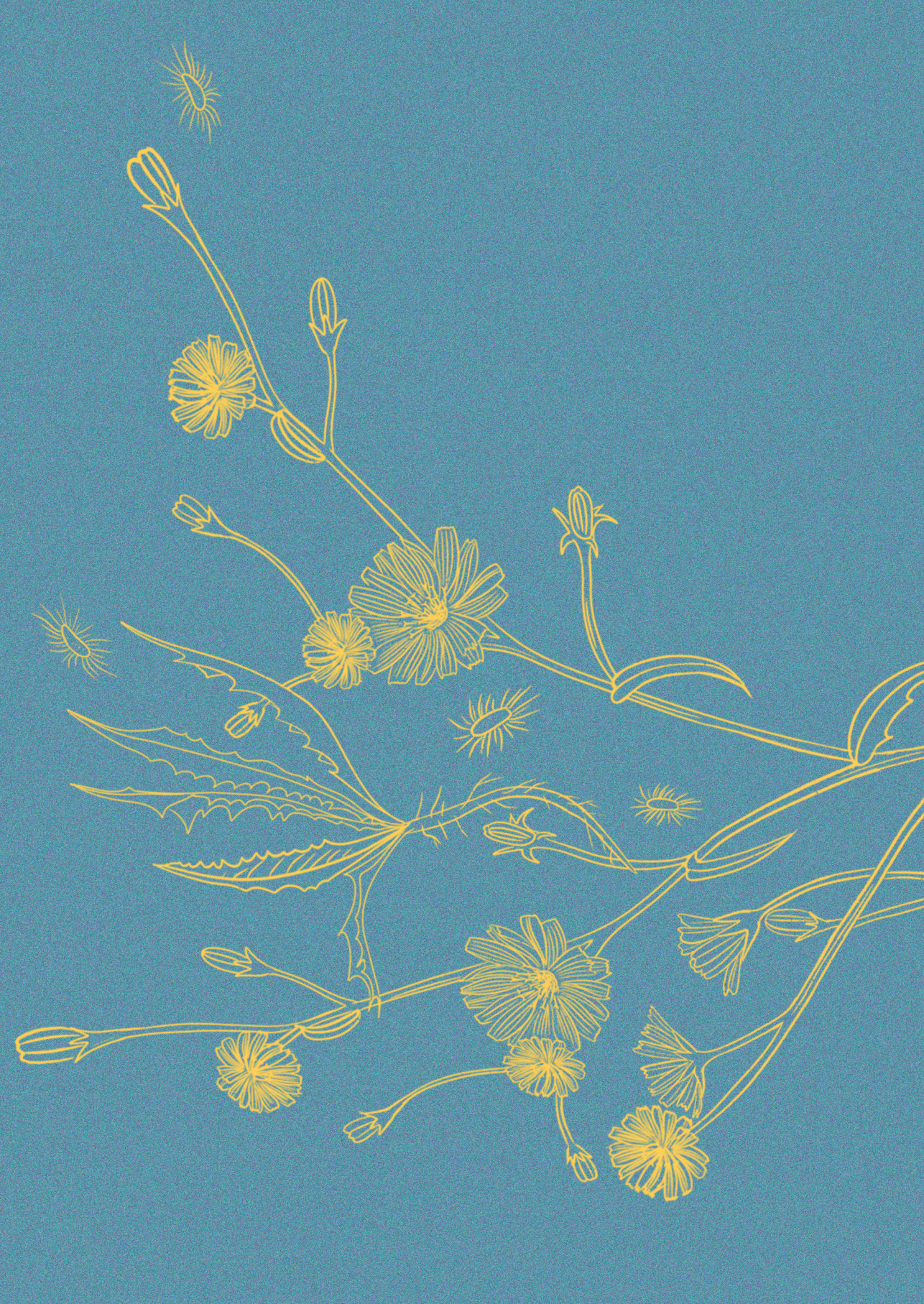

SUMMARY

In the past 150 years, humanity has witnessed an unprecedented rise in technological advances and, at the same time, a decline in metabolic health. Poor metabolic health evolves over the course of several years and is closely linked to the low consumption of dietary fibers. Dietary fibers fundamentally impact human health, particularly through their interaction with the gut microbiota. Plant foods are our primary source of dietary fibers. In plant foods, fibers are not merely isolated compounds entering the human digestive tract as loose entities but are intrinsically part of the complex, intertwined, three-dimensional plant tissue structure. These intrinsic structures determine how the gut microbiota can access and metabolize fibers, likely affecting both local gut and peripheral metabolic health outcomes. The aim of this thesis was to explore how dried chicory root, a fiber-rich food product notably high in inulin, can benefit human metabolic and gut health and to revisit the added benefits of the whole plant matrix in enhancing the effects of dietary fibers.

In **Chapter 1**, I provided a general introduction to this thesis by reviewing the current understanding of dietary fiber from a historical perspective. While dietary fiber was traditionally understood in the context of plant cells until the end of the 20th century, our increasing molecular insights and reductionist view have narrowed our concept of dietary fiber to single molecular structures. I advocated for a return to an integral understanding of dietary fiber in its intrinsic form in future research. I presented the leading research hypothesis of distal fiber conversion of intrinsic fiber, outlined the aim of the thesis, and described our approach to studying the effects of dried chicory root on human gut and metabolic health using literature reviews, *in vitro* studies, and *in vivo* human intervention trials.

In **Chapter 2,** we explained the differences between intrinsic and isolated fibers and discussed their distinct impacts on digestion. In edible plant tissue, fibers are entangled in a complex network of plant cell walls that encapsulate and shield intracellular fibers, such as fructans, from immediate breakdown by the gut microbiota. We explained how food processing can fundamentally affect this plant cell matrix and thereby determine whether a fiber is an intrinsic fiber or not. However, the effect of food processing is not uniform; it impacts the intactness of the plant tissue differently depending on the type of plant food (botanical part of a plant, hardness, ripeness) and the harshness of food processing. An important difference lies in whether the overall cell matrix (the plant tissue) is maintained, despite reduced coherence between plant cells or damage to the cell wall in the form of cracks or holes, compared to tissue where the plant cells are destroyed, broken open, and then further processed to extract single fibers. Considering that the intactness of the plant cell matrix is rarely addressed in fiber-gut microbiome studies but might limit the gut microbiota's ability to access and ferment dietary fibers, we summarized human intervention studies that used intrinsic

fibers. To date, few studies have included changes in gut microbiota composition along with their metabolites, such as short-chain fatty acids (SCFAs), bowel function, and metabolic health markers. However, the available data clearly indicate that intrinsic fibers can induce small to moderate effects on the gut microbiota, which, if assessed, are concomitant with changes in markers of metabolic health or bowel function. Particle size appears to be an important factor here, as evidenced by the few studies that investigated this aspect. We postulated that instead of further processing already extensively processed foods to create new products, we should minimize this processing to exploit the intrinsic health benefits associated with the original cell matrix of plant tissues. Understanding and recognizing the effect of food processing on plant tissue will help us comprehend how the gut microbiota interacts with plant fibers and possibly explain the variability in study outcomes, as observed from *in vivo* and *in vitro* assessments.

In **Chapter 3**, we highlighted how chicory root, as a forgotten food, is rarely used as a culinary ingredient today. The taproot of the chicory plant (*Cichorium intybus* L.) is a vegetable high in fiber due to its significant inulin content. Especially in their dried form, chicory roots contain nearly 90% fibers, a level not found in other foods when correcting for water content. Chicory roots contain four fiber types: cellulose, hemicellulose, and pectin, which form the plant cell matrix, enclose inulin, and include complex phytochemicals such as sesquiterpene lactones. Although chicory roots enjoyed broad popularity as medicinal and culinary food for over 2000 years, they became increasingly associated with famine food over the last three centuries. Today, chicory roots are mainly used as a source for extracting inulin, which is used as a prebiotic food ingredient. With the recent renewed recognition of the importance of dietary fibers for improving and maintaining human health, fiber-rich chicory roots offer an attractive option to close the gap between fiber recommendations and consumption. Revisiting chicory roots in the 21st century as a rich source of intrinsic dietary fibers opens up opportunities to improve metabolic and gut health problems stemming from our current fiber-poor diets.

As intact plant cells form a physical barrier that hinders immediate access to fibers by gut microbiota, **Chapter 4** investigated how the presence of the plant cell wall matrix in dried chicory root influences its breakdown in the human gut. We compared an intact plant cell matrix in the form of dried chicory root cubes, a damaged matrix due to reduced particle size in the form of dried chicory root powder, and the absence of the plant matrix using isolated inulin. To explore this, we employed a series of *in vitro* digestion and fermentation models alongside an *ex vivo* gut barrier integrity model. Following upper gastrointestinal digestion *in vitro*, we used scanning electron microscopy to visualize the intactness of the plant cell matrix. Despite the harsh conditions (pH 2-3) in the gastric phase, the plant cell matrix in dried chicory root cubes remained intact. We detected minor amounts of pectin, possibly originating from the intercellular space

(middle lamella), and short and long-chain inulin in digestive juices during the gastric and small intestinal phases, likely from the cut surfaces. Next, we assessed the lower gastrointestinal breakdown of inulin, dried chicory root powder, and cubes using an *in vitro* fecal batch fermentation model with stools from a donor low in bifidobacteria. We analyzed the kinetics of pH changes, gas production, and SCFA production. Changes in gut microbiota composition, assessed by 16S rRNA gene amplicon sequencing, revealed that fermentation of dried chicory root resulted in higher final relative abundances of pectin-degrading *Monoglobus* spp. and butyrate-producing *Roseburia* spp. compared to incubations with inulin. Incubations with dried chicory root cubes produced similar total SCFA levels but higher final butyrate levels compared to chicory root powder or isolated inulin, and also led to less gas production. Notably, dried chicory root cubes with their intact plant cell matrix differed by leading to more lactate production and consumption and butyrate production peaking towards the end of the 48-hour fermentation period. Furthermore, we explored the impact of fermentation supernatants produced from dried chicory root on gut barrier integrity using an Ussing chamber model with fecal inocula and colonic biopsies from four healthy individuals. The effect on gut barrier integrity was variable and donor-dependent, exemplifying the high individuality of the gut microbiota. Combining the outcomes of all four donors, we found a remarkable seven-fold increase in the relative abundance of *Bifidobacterium* spp. and confirmed butyrate production following lactate consumption at a later stage during fermentation. Our findings highlighted how the physical presence and intactness of the plant cell matrix affect its breakdown kinetics by the human gut microbiota, rationalizing a more distal production of butyrate, which could benefit human health *in vivo*.

In **Chapter 5**, we transitioned from *in vitro* to *in vivo* assessments, studying for the first time how dried chicory root affected the human gut microbiota in adults at risk for type 2 diabetes. In this randomized, placebo-controlled parallel trial, 55 subjects consumed 15 g of dried chicory root particles per day for the first two weeks, followed by 30 g per day for the next three weeks. Dried modulated gut microbiota composition rapidly, and at the genus level, we observed 3-4-fold increases in the relative abundances of *Anaerostipes* spp. and *Bifidobacterium* spp. based on 16S rRNA gene amplicon sequencing. These changes were concomitant with changes in stool softness and frequency as well as increases in the concentration of all three fecal SCFAs, namely acetate, propionate, and, notably, butyrate. These changes appeared in a dose-dependent and reversible manner. We further demonstrated that a synthetic community, including selected *Bifidobacterium* and *Anaerostipes* strains representing the canonical functionality of these genera together with a pectin-degrading *Bacteroides* strain, generated a butyrogenic trophic chain from dried chicory root. The changes induced by dried chicory root were not restricted to local effects in the gut but also extended to systemic changes. Fasting circulating levels of acetate and propionate increased, and the glycemic coefficient of variation, assessed using continuous glucose measurement, decreased. We observed that subjects with more than a 10% improvement in the

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (n = 8) had low baseline relative abundances of *Blautia* spp. Using this gut microbiota signature of high (n = 14) compared to low (n = 13) *Blautia* spp. baseline relative abundances, we observed that individuals with low baseline *Blautia* spp. relative abundances experienced a more rapid decrease in glycemic variation, lower fasting glucose levels, and higher plasma SCFA levels. This first *in vivo* human trial demonstrated to us the potential of dried chicory root as an intrinsic dietary fiber product to benefit human gut and metabolic health through modulation of the gut microbiota.

In **Chapter 6**, we continued our work by assessing gut microbiota changes after prolonged dried chicory root cube intake of 10 weeks. For this randomized, placebo-controlled, parallel trial, 35 individuals with obesity were recruited who were at risk for type 2 diabetes and who were tested for tissue-specific insulin sensitivity using a two-step hyperinsulinemic-euglycemic clamp. Changes in microbiota composition were followed using 16S rRNA gene amplicon sequencing combined with in-depth taxonomic and functional profiling using shotgun metagenomics. In line with our previous findings, we observed a rapid 4-5-fold increase in relative abundances of *Bifidobacterium* spp. and *Anaerostipes* spp. by the dried chicory root product based on 16S rRNA gene amplicon sequence data. Metagenome analysis revealed that these changes were mainly attributed to species-level modulations in *B. adolescentis, B. bifidum, B. longum*, and *A. hadrus*. Individuals who metabolically responded to the dried chicory root by a large improvement of insulin-mediated glucose disposal (> 15% improvement, high responders, n = 9) had lower relative abundances of potential pectin-degrading bacteria and pectate lyase genes throughout the study compared to those who had a low response (≤ 15% improvement; low responders, n = 8). Moreover, high responders had a more rapid increase in relative abundances of *Bifidobacterium* spp. and associated bifid shunt pathway encoding genes and higher final relative abundances of known propionate- and butyrate-producers, notably *Anaerobutyricum* spp. that, like *Anaerostipes* spp. can convert lactate and acetate into butyrate. These compositional and functional changes aligned with higher average fecal butyrate, propionate, and plasma acetate levels in high responders compared to low responders. For all individuals consuming dried chicory root cubes, increases in fecal butyrate levels were related to decreased fasting triglyceride levels, increased proportion of small adipocytes as well as increased plasma fasting acetate levels. These observations suggested a potential mediating role of fiber-derived colonic butyrate on systemic metabolic health outcomes. In this study, we confirmed that intake of dried chicory root reproducibly modulates the gut microbiota to increase relative abundances of bacteria involved in butyrogenic trophic chains, and that prolonged intake can benefit the metabolic health of overweight individuals or those at risk for type 2 diabetes.

In **Chapter 7**, we shifted our focus from the impact of gut microbiota changes on metabolic health to the effects on gut health in individuals experiencing bowel function

problems. In the study described in this chapter, we examined data from a randomized, placebo-controlled, cross-over trial involving 39 mainly female individuals suffering from functional constipation. Functional constipation is a common disorder of the gut-brain interaction, characterized by infrequent and difficult bowel movements, which significantly affects patients' quality of life. While the cause of functional constipation is not fully understood, the gut microbiota is recognized as a crucial factor in the disorder, making microbiota-targeted fiber interventions an intriguing alternative to laxatives. In this study, isolated inulin was used. After individuals ingested 12 g of inulin per day for four weeks, we observed substantial improvements compared to the placebo in stool frequency, abdominal symptoms, and aspects of quality of life. Notably, the improvement in quality-of-life aspects related to social and emotional well-being was evident only during inulin intake, not during placebo intake. Additionally, relative abundances of potential butyrate-producing bacteria, including *Anaerostipes* spp. and *Coprococcus* spp., were higher after inulin intake than after placebo. However, as this was a cross-over trial, which is prone to potential carry-over effects, we noted bias related to carry-over despite the four-week wash-out period. Further investigation hereof revealed that half of the subjects who initially received inulin showed a remarkable placebo response in the subsequent period. These individuals had the largest improvements in all outcomes during inulin intake, with higher baseline and washout relative abundances of butyrate-producing *Faecalibacterium* and *Roseburia* spp., and initially lower but more responsive relative abundances of *Bifidobacterium* spp. This suggested a potential role of the gut microbiota in the lingering improvement following inulin intake in these individuals. To mitigate the bias induced by potential carry-over, we also analyzed the first period separately as a parallel trial, which confirmed the positive impact of inulin intake observed from the cross-over analysis. This trial demonstrated that fiber interventions targeting the gut microbiota in disorders of the gut-brain interaction can indeed alleviate constipation-related low stool frequency and abdominal symptoms, as well as social and emotional aspects of quality of life. The modulation of the gut microbiota after inulin intake and the differences in individuals affected by the seemingly lingering effect of inulin driving the carry-over-related bias indicate a potential role of butyrate-producing microorganisms in alleviating functional constipation.

Based on the observed effect on bowel function in our two dried chicory root studies and the potential of inulin to improve the quality of life in individuals with functional constipation, we designed a new study presented in **Chapter 8**. In this study, we will assess the impact of dried chicory root cubes on bowel function and the gut microbiota in healthy individuals with self-reported bowel function issues. The study will include a total of 160 adults aged between 20 and 80 years and is designed as a double-blinded, randomized, placebo-controlled, parallel trial with four arms. The subjects will be exposed to a four-week intake of three different dosages of dried chicory root particles or placebo. The study is centered around the individual, meaning we will assess not only objective outcomes of stool frequency and consistency but also subjective measures,

including defecation ease, feeling of complete bowel emptying, and satisfaction. We expect to observe that changes induced by dried chicory root in these outcomes are dose-dependent and associated with time-dependent modulations of the gut microbiota and its activity. Our findings will contribute to a better understanding of the role of gut microbiota in bowel function issues and highlight potential therapeutic interventions using intrinsic fibers to treat nonmedical bowel function issues.

In **Chapter 9**, I synthesized all our findings. I reiterated the importance of dietary fibers in 2024 for human health and extensively reflected on how dried chicory root-induced gut microbial and functional changes observed *in vitro* and *in vivo* support our working hypothesis. Following this, I summarized the metabolic effects of dried chicory root intake and speculated on how dried chicory root-derived colonic SCFAs might have facilitated these effects. Additionally, I summarized the effects of dried chicory root on bowel function, explained how inulin or dried chicory root can affect bowel function, and discussed the potential of dried chicory root in treating bowel function issues, drawing insights from inulin's effect on functional constipation. Both of these sections conclude with my suggestions for future studies. I then reflected on the methodologies used, including DNA sequencing, subgroup analyses, and *in vitro* models. Before concluding the thesis, I debated the role and future of food processing in the impact of dietary fiber on human health. Finally, I evaluated our current dietary guidelines and shared my hopes for the future of dietary fiber research.

In conclusion, with the studies described in this thesis, I demonstrated how dried chicory root, as an exceptional example of intrinsic fibers, can improve human gut and metabolic health through gut microbiota modulation. This forgotten vegetable, with an intact plant cell matrix where hemi-/cellulose and pectin encapsulate high levels of inulin, fundamentally affects how fibers are accessed by the gut microbiota and, thereby, the production of SCFAs associated with human health. Notably, the higher levels of butyrate produced at a later stage in fermentation might translate to a more distal location of butyrate production in the colon. *In vivo*, we observed higher fecal butyrate levels and a reproducible increase in bacteria involved in butyrogenic trophic chains. Considering our observations in light of recent insights into the importance of the distal colon, we propose that the positive effects on bowel function and metabolic health are driven by dried chicory root derived SCFAs, notably butyrate. In the future, we should reassess our current reductionist approaches to dried chicory root and food processing choices to use dietary fibers in their most optimal form to benefit human health and reverse the self-made human metabolic crisis.

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ABOUT THE AUTHOR

Marie-Luise was born in Luckenwalde, East Germany, exactly one month before the fall of the Berlin Wall in 1989. After experiencing life abroad at the age of 17, she moved to France for a gap year after high school. Fascinated by the curriculum of the Bachelor's program in Human Nutrition & Health at Wageningen University, she relocated to the Netherlands in 2010 to pursue her studies. There, she learned about the effects of food on the human body but wanted to understand what food is actually composed of. To achieve this, she included food technology courses in her Bachelor's program (graduated *cum laude*) and ultimately pursued double master's degrees in Human Nutrition & Health and Food Technology. While completing both master's programs, she sought research topics that would combine these disciplines and eventually discovered the field of food digestion.

After her master's, Marie-Luise worked for over two years as a research assistant, engaging in both laboratory work and human intervention trials. It was during one of these projects that she met her PhD supervisors, Prof. Dr Willem M. de Vos and Prof. Dr Edith Feskens. Initially convinced that dietary fiber was a "boring" subject ("we already know everything about them"), she soon realized that this area was still underexplored. In early 2019, she expanded her research focus to include a new discipline, Microbiology and embarked on her PhD journey under the supervision of Prof. Dr. Willem M. de Vos, Prof. Dr. Edith Feskens, and Prof. Dr. Hauke Smidt.

The PhD project was a collaborative effort, financially supported by both the Laboratory of Microbiology and the Division of Human Nutrition & Health. In this project, Marie-Luise continued and expanded upon her work as a research assistant on the VEZEL study, the first human intervention trial using dried chicory root (WholeFiber™). During her PhD, she supervised nine BSc and MSc students, as well as four students during her time as a research assistant. As part of her PhD project, Marie-Luise spent four months at the Nutrition-Gut-Brain Interactions Research Centre within the School of Medical Sciences, Faculty of Medicine and Health, Örebro University, Sweden. She presented her research at several national and international conferences. Together with her colleagues M. Fassarella, M. Edinka, Z. Liu, Marie-Luise organized an online minisymposium on *in vitro* studies of the human intestinal microbiota (2020). Beyond her research, Marie-Luise was actively involved as a PhD representative, serving as a member and later co-chair of the VLAG PhD Council, as well as the secretary of the Wageningen PhD Council and a member of the Microbiology PhD Board.

Having lived in several countries, Marie-Luise does not consider any one place her ‘home.’ For her, home is where her husband Koen and their (retired research cat) Skye are. After 14 years in the Netherlands and 15 years away from Germany, Marie-Luise will soon move to Denmark to explore how ‘home' feels there. She will join the Microbiome & Metabolomics research group at the Department of Nutrition, Exercise and Sports at the University of Copenhagen under the supervision of Dr Henrik Munch Roager. Speaking four languages imperfectly (including her mother tongue with a Dutch accent and Dutch with a German accent), Marie-Luise will soon begin learning Danish. She expects that this will come at the expense of her Dutch, make her German even more amusing, and add yet another accent to her English. (She hopes her French will remain unchanged.) Marie-Luise is grateful for her PhD journey and looks forward to embracing what the future holds.

LIST OF PUBLICATIONS

PUBLISHED

Puhlmann, M.-L., van de Rakt, E., Kerezoudi, E. N., Rangel, I., Brummer, R. J., Smidt, H., Kaper, F. S., & de Vos, W. M. (2024). Analysis of the fermentation kinetics and gut microbiota modulatory effect of dried chicory root reveals the impact of the plant-cell matrix rationalizing its conversion in the distal colon. Microbiome Research Reports, 3(3), 28. https://doi.org/10.20517/mrr.2024.04

Tagliamonte, S., Puhlmann, M.-L., De Filippis, F., Guerville, M., Ercolini, D., & Vitaglione, P. (2024). Relationships between diet and gut microbiome in an Italian and Dutch cohort: does the dietary protein to fiber ratio play a role? European Journal of Nutrition, 63(3), 741–750. https://doi.org/10.1007/S00394-023-03308-4

Puhlmann, M.-L., & de Vos, W. M. (2022). Intrinsic dietary fibers and the gut microbiome: Rediscovering the benefits of the plant cell matrix for human health. Frontiers in Immunology, 13, 16. https://doi.org/10.3389/fimmu.2022.954845

Puhlmann, M.-L., Jokela, R., Van Dongen, K. C. W., Bui, T. P. N., Van Hangelbroek, R. W. J., Smidt, H., De Vos, W. M., & Feskens, E. J. M. (2022). Dried chicory root improves bowel function, benefits intestinal microbial trophic chains and increases faecal and circulating short chain fatty acids in subjects at risk for type 2 diabetes. Gut Microbiome, 3, e4. https://doi.org/10.1017/gmb.2022.4

Pol, K., Puhlmann, M. L., & Mars, M. (2022). Efficacy of L-Arabinose in Lowering Glycemic and Insulinemic Responses: The Modifying Effect of Starch and Fat. Foods, 11(2), 157. https://doi.org/10.3390/foods11020157/S1

Puhlmann, M.-L., & de Vos, W. M. (2020). Back to the Roots: Revisiting the Use of the Fiber-Rich Cichorium intybusL. Taproots. Advances in Nutrition, 11(4), 878–889. https://doi.org/10.1093/advances/nmaa025

SUBMITTED OR IN PREPARATION

Omary, L., Canfora, E. E., Puhlmann, M.-L., Gavriilidou, A., Rijnaarts, I., Holst, J. J., Bruls, Y. M. H., de Vos, W. M., & Blaak, E. E. (2024). Intrinsic chicory root fibers modulate colonic microbial butyrate-producing pathways and improve insulin sensitivity in individuals with obesity. *Submitted to Cell*

Puhlmann, M.-L., van der Zalm, S. C. C., Smidt, H., de Vos W. M., Feskens, E. J. M. (2024). The impact of dried chicory root on bowel function and the gut microbiota in adults with bowel function issues in the Netherlands: a study protocol for a double-blinded randomized controlled trial (HappYFiber study). *In Preparation*

Puhlmann M.-L., Wegh, C. A. M., van der Zalm, S. C. C., Dam, V., Doolan, A., Meyer, D., Benninga, M. A., Belzer, C., Smidt, H., Vaughan, E. E. (2024). Findings from a randomized, double-blind, placebo-controlled study to evaluate the effects of inulin on bowel habit and fecal microbiota in adults with functional constipation. *In Preparation*

Puhlmann, M.-L., Rosendaal, P., Smidt, H., de Vos, W.M., Feskens, E.J.M. (2025). Exploring the effect of dried chicory root on lipid metabolites, gut hormones, feelings of satiation and gastrointestinal symptoms: secondary analysis of a randomized, placebo-controlled, parallel trial in subjects at risk of type 2 diabetes. *In Preparation*

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Category	Organizing institute(s)	Place, Year
Discipline-specific activities		
Mering Symposium - Diabetes Technology and Prevention	EASD	Cottbus, D, 2019
37th International Symposium on Diabetes and Nutrition	EASD (DNSG)	Kerkrade, 2019
TIFN Personalized Nutrition Symposium	TiFN	Wageningen, 2019
Darmen Dag (Gut Day)	Amsterdam UMC	Amsterdam, 2019
Virtual Microbiome Summit	Lucy Mailing	online, 2020
Minisymposium: *in vitro* studies of the human intestinal microbiota	MIB-WUR	online, 2020
ILSI Vahouny Symposium	IAFNS	online, 2020
13th International Gut Microbiology Symposium	University of Aberdeen/Rowett Institute, INRAE	Aberdeen, UK, 2023
TNO 'Optimising Food and Fibre Composition'	TNO	Leiden, 2023
EWUU Methodological Challenges in Microbiome Multi-omics	Alliantie EWUU	Utrecht/Wageningen, 2023
Buikbelang	MDL Stichting	Wageningen/ Amersfoort, 2023/2024
Applied Statistics	VLAG	Wageningen, 2019
Chemometrics (Multivariate Statistics)	VLAG	Wageningen, 2019
Mixed Linear Models	PE&CR	Wageningen, 2019
Stable Isotope Methods in Nutrition Research	VLAG	Wageningen, 2019
Workshop Data Exploration and Analysis in Gut Microbiome Profiling Studies	Developmental Psychobiology Lab (Nijmegen)	Nijmegen, 2019
Intestinal Microbiome of Humans and Animals	WUR	Wageningen, 2019
PhD Course on Basal Metabolism & Molecular Mechanisms in Diabetes	Danish Diabetes Academy	Nyborg, DK , 2021
EWUU Microbiome Analysis Course	Alliantie EWUU	Utrecht, 2023
General Courses		
Introduction to R	VLAG	Wageningen, 2019
PhD Workshop Carousel 2019	WGS	Wageningen, 2019
Supervising BSc & MSc thesis students	WGS	Wageningen, 2019
Competence assessment	WGS	Wageningen, 2019
VLAG PhD week	VLAG	Baarlo, 2019
Rmarkdown Course	VLAG	Wageningen, 2020
Staying visible ("Hoe blijf ik in beeld in de online wereld")	NAV	online, 2020
Scientific Artwork	WGS	Wageningen, 2019
Rothman Lunches	HNH	Wageningen, 2020-2021
PhD Workshop Carousel 2021	WGS	Wageningen, 2021
Project and Time Management	WGS	Wageningen, 2021
Popular Science Writing	WASS	Wageningen, 2021

Category	Organizing institute(s)	Place, Year
PhD Workshop Carousel 2022	WGS	Wageningen, 2022
Scientific Writing 2024	WGS	Wageningen, 2024
Other activities		
Preparation of research proposal	VLAG	Wageningen, 2018/19
Group meetings MolEco	WUR (MIB)	Wageningen, 2019-22
Journal club MIB or HNH	WUR (MIB/HNH)	Wageningen, 2019-20
AIO/Postdoc meeting MIB	WUR (MIB)	Wageningen, 2019-22
PhD Board	WUR (MIB)	Wageningen, 2019-21
Group meetings HNH-GN	WUR (HNH)	Wageningen, 2019-22
Gut group meeting HNH	WUR (HNH)	Wageningen, 2019-22
VLAG Council - member and co-chair	VLAG	Wageningen, 2019-21
VLAG Council coaching (organization & participation)	VLAG	Wageningen, 2021
Wageningen PhD Council - Internal Secretary	WUR	Wageningen, 2019-20
Assisting in teaching and supervision activities		
HNE-30706 Food Digestion: Nutrient Breakdown and Absorption		2020/2022
FHM-30806 Advanced Fermentation Science		2020/2021
Supervising BSc & MSc thesis students		2019-2024

ABOUT THE COVER

Have you ever noticed the light-blue flowers blooming along roads and paths in late summer? They might remind you of cornflowers, but upon closer inspection, you'll see they are different. These flowers belong to the wild *Cichorium intybus* L. – the chicory plant. Whether in old or new botanical books, you'll find it documented under various local names, such as "Gemeine Wegwarte" ("lookout along the road" in German). This plant, long cultivated by humans, is known for its large taproots, commonly referred to as "chicory roots." This PhD thesis explored how these once-familiar but now often overlooked roots benefit the human body today, aptly titled "Back to the Chicory Roots." In doing so, classic concepts such as dietary fibers are revisited within a modern context, blending past and present.

The cover design harmoniously combines historical and contemporary elements to reflect the thesis's exploration of both past and present. The line drawing by Marion Buso is inspired by antique botanical illustrations of the wild chicory plant found in textbooks from the 17th to 19th centuries, which Marie-Luise referenced in Chapter 3. The central drawing of a cultivated chicory root from the past centuries complements this historical nod. Further inspiration comes from the cover designs of old books, specifically *Tales of the Marvellous and News of the Strange*. The blue background mirrors the chicory flower's hue, while the golden accents echo the classic gilded embossing of old book covers. To merge the historical with the modern, traditional drawings and colors are paired with a contemporary font influenced by book cover designer Elisha Zepeda. Throughout the golden-yellow line drawing, tiny bacteria are subtly illustrated, hinting at their essential role in the beneficial effects of dietary fiber for the human body. Although Marie-Luise initially explored AI for the cover design, she and Marion discovered that AI struggled with specific scientific concepts like botanical illustrations and gut bacteria. As a result, this cover is a product of human creativity, inspired by existing works and brought to life through Marion's artistic skills.

COLOPHON

The research described in this thesis was a collaborative PhD research project between the Laboratory of Microbiology and the Division of Human Nutrition and Health, and financially supported by the Netherlands Organization of Scientific Research (NWO) via the 2008 Spinoza award and the SIAM Gravitation Grant 024.002.002 of Willem M de Vos. In addition, Chapter 4 was supported a VLAG fellowship grant 2.0 2022/23 to Marie-Luise Puhlmann, Chapter 5 was supported by the 2018 Innovation Program Microbiology of Wageningen University, Chapter 6 was supported by EFSD/Lilly European Diabetes Research Program 2019 as well as Topconsortia voor Kennis en Innovatie (TKI)/Health Holland (LSHM19050), Chapter 7 was supported by Sensus (Royal Cosun, Roosendaal, The Netherlands), and Chapter 8 by the 2022 Innovation Program Microbiology of Wageningen University. Finally, the research in Chapters 4, 5, 6, and 8 was supported by in-kind contributions of WholeFiber BV.

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