LIGNAN INTAKE & OBESITY

DIET-DISEASE ASSOCIATION AFFECTED BY PROCESSING, BIOACCESSIBILITY AND CONVERSION

Umi Kalsum Hussain Zaki

Propositions

1. Food fermentation improves the conversion of lignans to enterolactone in the human body.
   (this thesis)

2. Lignan intake induces weight loss in humans.
   (this thesis)

3. The widespread interest in traditional medicine is not based on solid science.

4. Nutrient fortification of infant milk formulas is insufficient in improving the intelligence of babies.

5. Social media are more effective at spreading knowledge in society than scientific journals.

6. The goal of food sharing is cultural exchange.

Propositions belonging to the thesis, entitled

Lignan intake & obesity: Diet-disease association affected by processing, bioaccessibility and conversion

Umi Kalsum Hussain Zaki

Wageningen, 8 November 2022

LIGNAN INTAKE & OBESITY:

Diet-disease association affected by processing, bioaccessibility and conversion

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This thesis was conducted under the auspices of the Graduate School VLAG (Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

LIGNAN INTAKE & OBESITY:

Diet-disease association affected by processing, bioaccessibility and conversion

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University,
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 8 November 2022
at 1.30 p.m. in the Omnia Auditorium.

Umi Kalsum Hussain Zaki

Lignan intake & obesity: diet-disease association affected by processing, bioaccessibility and conversion, 204 pages

PhD Thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summary in English

ISBN: 978-94-6447-336-0
DOI: https://doi.org/10.18174/574557

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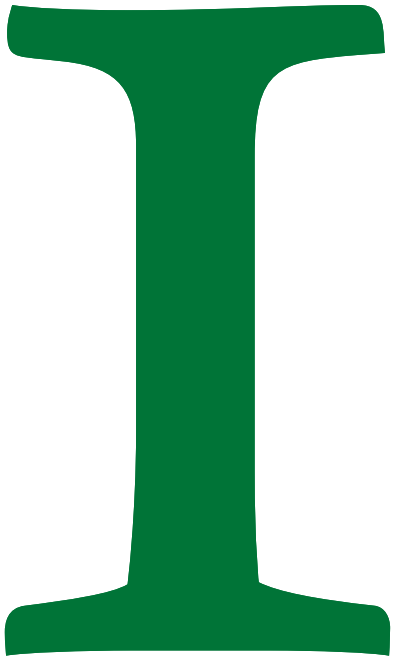

CHAPTER 1

General introduction

Obesity has become a global epidemic and a public health crisis, particularly in recent decades, and the prevalence of obesity is still increasing at an alarming rate[1]. The worldwide growth in being overweight or obese is largely due to dietary and lifestyle changes. As such, defining diets and dietary habits that mitigate obesity risk is of great public health importance. Recently, the intake of foods rich in phytoestrogens, including lignans, has emerged as an interesting dietary strategy for combating obesity[2,3]. Moreover, the effect of different processing techniques on the bioaccessibility of lignans and conversion to gut microbial metabolites is relatively nascent. In this chapter, a general introduction to the thesis is provided by addressing the definition of phytoestrogens and lignans, the effect of processing on lignan content, their bioaccessibility and conversion into microbial metabolites in the human large intestine, the occurrence of obesity globally and specifically in Malaysia, as well as the relationship of lignan intake and obesity. Finally, the objectives of this thesis will be presented.

PHYTOESTROGENS: DEFINITION AND MAIN CLASSES

In recent years, research has focused on the characterization of phytoestrogens and their metabolites in various biological fluids as well as on their potential health-promoting properties[4-6]. Phytoestrogens are phytochemicals that exhibit weak oestrogenic activity and are found in plants or derived from plant precursors. They comprise four major chemical compounds (lignans, isoflavones, coumestans and prenylflavonoids) that are structurally similar to endogenous oestrogens; hence they can have both oestrogenic and anti-oestrogenic effects[7-11].

LIGNANS:

Chemical structure, biosynthesis, and food sources

Lignans are phenolic secondary metabolites derived from the phenylpropanoid biosynthetic pathway and are also known as monolignol-derived dimers. Lignans are derived from the oxidative dimerization of two coniferyl alcohol (CO) molecules, forming an 8,8' bond, as presented in Figure 1.

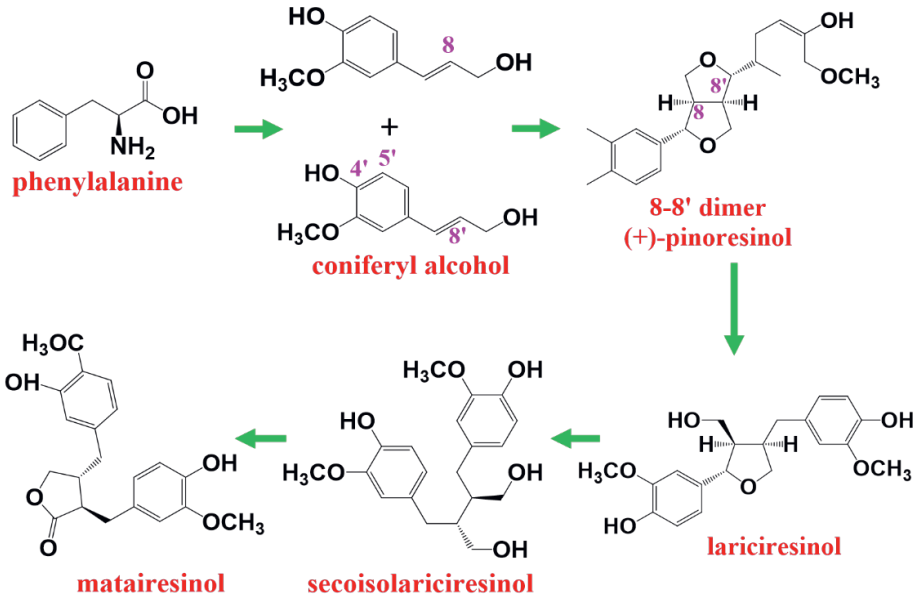

Figure 1: Schematic of the biosynthesis of lignans

The coupling of the two coniferyl alcohol (CO) residues by enantioselective dimerization with the aid of dirigent protein leads to pinoresinol (PINO) formation. PINO is believed to be a central intermediate leading to the different synthesis of lignans[12]. Pinoresinol is then reduced by pinoresinol/lariciresinol reductase via lariciresinol (LARI) to secoisolariciresinol (SECO), which is in turn oxidized to form matairesinol (MATA) by secoisolariciresinol dehydrogenase[13-15].

Lignans occur in edible plants[16] where they have been detected in different parts, including roots, leaves, wooden parts, flowers, fruits, and seeds[17]. Oilseeds (flaxseed, sesame, linseed, and sunflower) are particularly rich in lignans, and flaxseed is the richest dietary source of lignans, with an average content of 300 mg/100g. Other foods contain moderate to low amounts[5]. Dietary surveys have found that whole grains (particularly rye), legumes, fruits, vegetables, nuts, and some beverages, such as tea and coffee, are important dietary sources of these compounds[18,19].

Effect of bioprocessing

Bioprocessing refers to technologies to extract and/or produce food or ingredients using enzymes or living systems such as microorganisms. Food bioprocessing research encompasses a wide range of topics, including the characterization of modifications induced by food bioprocessing and their effect on their functionality and quality[20]. Pickling, germination, and fermentation are bioprocessing techniques that have recently attracted consumers seeking more "natural," health-promoting, and sustainable food products[21, 22]. The content of lignans in foods may be affected by the food processing techniques[23], and germination, fermentation, and pickling have been shown to influence lignan content[24].

Germination is the physiological process by which seeds develop an embryonic root and stem within a set time frame[25]. It can improve the nutritional properties of edible seeds[26], and germination is highlighted as a potential bio-processing trend in food science for producing functional germinated foods[27].

Fermentation is a process that relies on microorganisms' biological activity to produce a variety of metabolites that can inhibit the growth and survival of undesirable microflora in foodstuffs[28]. It is a traditional bioprocessing method that increases food shelf-life, food nutritional properties[29], the bioactivity of phytochemicals, flavour, stability, and digestibility[30,31]. Fermented foods are produced by the controlled growth and enzymatic activities of microorganisms via four main processes, i.e., lactic, acetic, alcoholic, and alkaline[32, 33].

Pickling is a natural fermented food of food preservation and used as fermentation method of fruits and vegetables[34]. It has been a viable method of preserving foods, particularly seasonal foods, prior to the invention of modern preservative equipment such as the refrigerator. It preserves foodstuffs using acid without refrigeration in two ways: anaerobic fermentation in brine, or immersion in vinegar and salt[35, 36]. Naturally occurring bacteria grow over 1 to 2 weeks to produce lactic acid, inhibiting the growth of food poisoning bacteria and other spoilage microorganisms[36]. However, a detailed description of the metabolic changes associated with pickling, particularly after prolonged storage, is still lacking.

Absorption, metabolism, distribution, and excretion of lignans in the human body

Digestion comprises a series of physiological, mechanical, and biochemical processing steps that break down the food structures in order to release its constituents, allowing for nutrient absorption and utilization by the body[37,38]. The composition and structure of foods play an important role in the kinetics of food digestion[39-41].

The gastrointestinal tract (GI) is complex, with multiple layers of control involving four distinct compartments, namely the mouth, stomach, small intestine, and large intestine[37]. The disintegration mechanism varies by food and is related to the original food structure and how it changes during digestion. Although most of the disintegration of solid foods occurs in the mouth, the remainder occurs during transit through the digestive system, particularly in the stomach[42,43]. The kinetics of food disintegration are influenced by physiological conditions such as mechanical forces, pH, temperature, level of digestive enzymes, as well as food/meal properties such as composition, amount, texture, structure, and viscosity[42,44,45].

The health effect of food often depends on the fraction that can be absorbed. Bioavailability refers to a relative amount of phytochemicals that are digested, absorbed, and becoming available to reach the circulatory system and act on specific sites of the body[46,47]. Food technology research has increased the attention to designing various foods to modulate food digestibility and release rate of active compounds[48]. Several post-harvesting processing procedures, such as de-branning, milling, bran fractionation, grinding, and crushing, can impact bioaccessibility[49,50]. Bioaccessibility is the release of an active compound from its matrix so it can be absorbed in the intestine[51]. However, as far as we know, there are relatively few reports on the effect of various processing techniques on lignans' bioaccessibility from food.

The large intestine has an anaerobic environment and is colonized by a community of microorganisms (approximately 10^{11}/g), primarily composed of obligate anaerobic microbiota. The gut microbiota has a key role in the metabolism of lignans. It has been shown that the intestinal microbiota metabolizes lignans to enterolignans, namely enterodiol (END) and enterolactone (EL)[52-54].

The metabolism process of lignans includes deglycosylation of glycosylated lignans and the subsequent demethylation and dehydroxylation of SECO to produce END. Then END is presumed to be oxidized to produce EL[55]. No metabotypes can be defined, but a gradient of END and EL allows distinguishing among producers, which are produced to varying degrees and allow classification of low or high producers of END and EL in human[56-58]. *Ruminococcus bromii*, *Bacteroides ovatus*, and *Eggerthella lenta*,

among many other bacterial species, have been identified as being involved in the metabolism of lignans, through *in-vitro* approaches[59-62].

Lignan aglycones and enterolignans are efficiently absorbed in the small intestine or colon. Then, they are conjugated to glucuronides and/or sulfates by enterocytes[55]. Finally, the lignan aglycones, enterolignans, glucuronides, and sulfates are detected in blood and excreted via urine, enterohepatic circulation, and feces[55]. Figure 2 describes the absorption, metabolism, distribution, and excretion of dietary lignans.

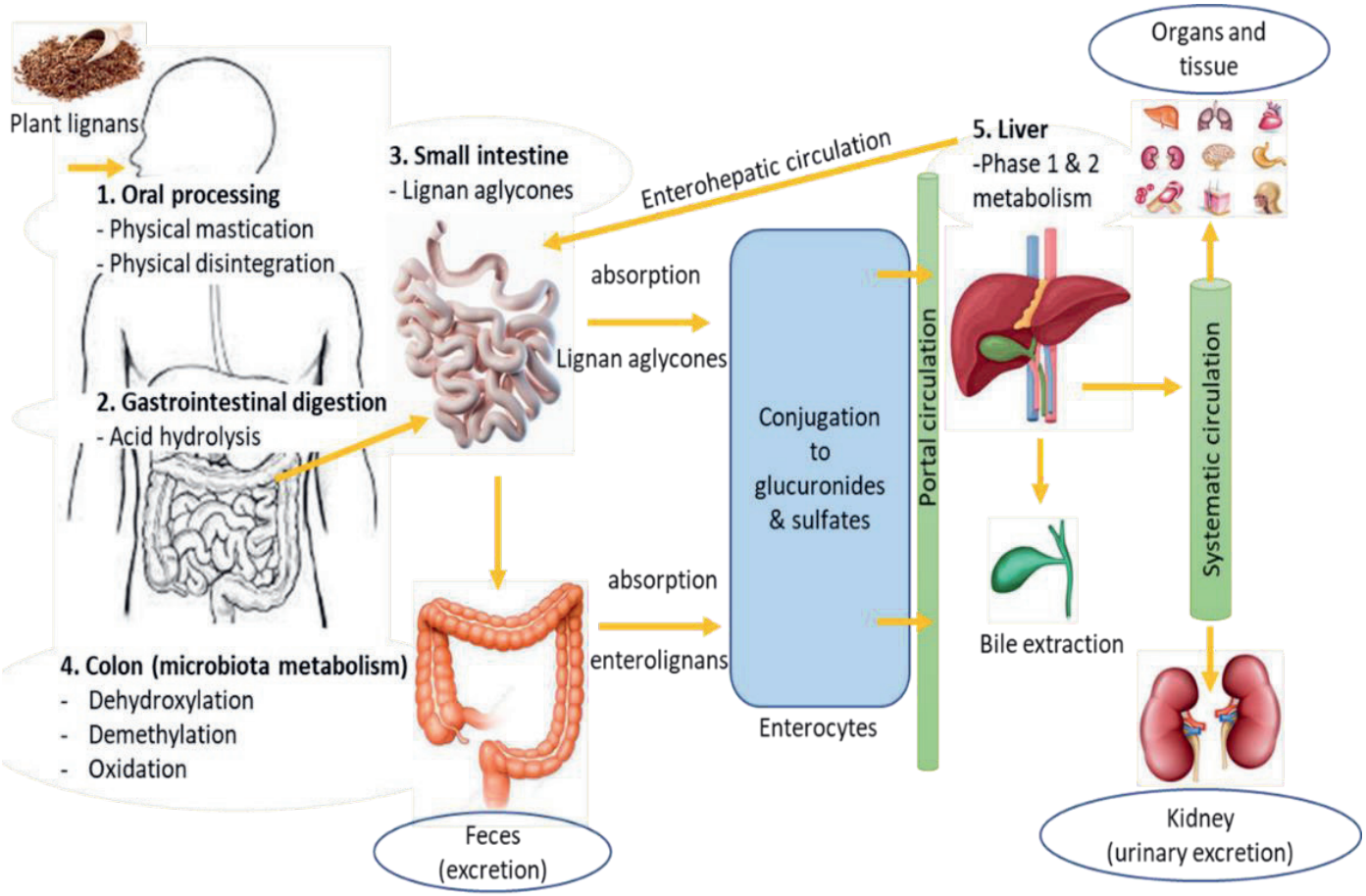

Figure 2: Schematic representation of absorption, metabolism, distribution and excretion of dietary lignans

The role of the food matrix and the original source of plant lignans should not be underestimated since they may also explain differences, mainly in the production yield of enterolignans[63]. Mechanically disrupting food structure such as particle size reduction allows surface area increase for interactions with digestive enzymes, which may improve its bioaccessibility[64]. A study showed that the crushing and milling of flaxseed significantly increases enterolignans production compared to ground flaxseed[50]. An *in-vitro* fermentation study reported that the production of enterolignans varies among plant foods such as berries, vegetables, and potatoes. Berries showed the highest enterolignans production compared to other foods[65]. Thus, food structures and composition influence the production of enterolignans.

Individual variability in the conversion of plant lignans

Classification of humans in metabotypes or stratification in high/low producers is a way to represent inter-individual differences in microbiota functionality[66, 67]. Metabotypes is a broad concept that can refer to microbial and host metabolism in the gut and subsequent biological activity (e.g., synthesis, transport, and receptor interaction)[66, 68-70]. The metabolism of lignans is highly variable between individuals, as evidenced by the presence of a metabolite production gradient that gives rise to "high producers" and "low producers" of enterolignans[71,72]. An *in-vitro* fermentation study of flaxseed extract using human feces from low and high enterolignan producers showed a clear distinction in EL production[73]. Similarly, another study showed that the individual gut microbiota could influence the lignan metabolism as microbiota diversity was significantly associated with EL production in premenopausal women[74].

The differences in enterolignan production can be explained by various factors, including gender, age, genetics, lifestyle, physiological status, gut microbiota, and dietary factors such as food matrix and processing and dietary patterns[68,75,76]. Thus, the conversion rate and maximal yield as a result of microbiota metabolism must be carefully considered when investigating the gut conversion of plant lignans into enterolignans.

OBESITY

In 2016, more than 1.9 billion or 39% of adults aged 18 years and over were overweight[77]. Obesity has been shown to reduce health-related quality of life[78], as well as overall life expectancy[79]. In 2010 a worldwide study showed that obesity accounted for 0.7% to 2.8% of each country's total health care expenditures[80]. Type 2 diabetes[81], hyperlipidemia, hypertension, coronary artery disease[82], gallbladder disease[83], and several cancers[84] are among the most common obesity-related comorbidities. In addition, it has become clear that obesity is also a risk factor for an increased risk of hospitalization, admission to intensive care units, and an increased need for invasive mechanical ventilation, due to COVID-19[85].

Obesity is primarily caused by excessive energy consumption and/or insufficient energy expenditure. Excessive food/energy intake causes adipose tissue expansion, including an increase in fat cells (adipogenesis) and the size of individual adipocytes (hypertrophy). Mammals have at least three types of adipocytes: white, beige, and brown adipocytes[86]. White adipose tissue (WAT) depots are widely distributed intra-abdominally or subcutaneously and are composed of WAT with a large unilocular lipid droplet for energy storage in triglycerides form, which is their primary function. Lipolysis and energy release from WAT as free fatty acids are triggered by appropriate systemic stimuli of starvation or lack of energy. WAT also secretes adipokines such as adiponectin and leptin to modulate organismal energy homeostasis[87, 88].

On the other hand, brown and beige adipocytes have multilocular intracellular lipid droplets and are rich in mitochondria that stimulate heat production. Thus, they are also called thermogenic adipocytes[86]. Due to the energy-consuming feature of thermogenic adipocytes, it has been suggested that promoting brown/beige adipogenesis would be an advantageous approach to treat obesity[87].

Relation of dietary lignans with obesity

Phytochemicals in foods may stimulate adipogenesis and exhibit anti-obesity activity[87]. Phytoestrogens are one of the potential phytochemical groups that affect lipid metabolism by promoting adipogenesis[8,89]. The ability of phytoestrogens to activate or inhibit PPAR signaling in adipocytes determines whether they are pro- or anti-adipogenic[90]. Since phytoestrogens can influence adipose tissue distribution, their intake may reduce the amount of visceral fat and, as a result, the risk of metabolic complications associated with obesity[91]. Several mechanisms have been proposed that support the role of lignans in combating obesity[92,93]. Flaxseed is an excellent source of

lignans[17, 94, 95] and has been used to reduce obesity-related disease conditions such as hypertension, cardiovascular disease, and diabetes[96,97].

Recent studies investigated the association between lignan intake and obesity. A cross-sectional study showed a significant negative correlation between lignan intake and body mass index (BMI) (-1.85 kg/m^2 per mg/d; CI:-12.10,-0.40) in 404 Iranian women[98]. Another study on sesame lignans reported that 3 μg/ml of lignans could decrease serum triglycerides and alleviate cellular oxidative stress. In addition, it was demonstrated that lignans participated in hepatic lipid metabolism via the AMPK and PPAR signalling pathways, which regulated various cellular activities such as fat synthesis and fat oxidation[99]. Thus, both human studies as well as molecular studies suggest that dietary lignans can affect obesity and related lipid metabolism.

ZOOMING ON THE DIETARY INTAKE AND OBESITY SCENARIO IN MALAYSIA

In Malaysia, approximately 15.7% of adults are obese[100]. According to World Health Organization (WHO) statistics from 2016, more than 42% of Malaysians are overweight. Furthermore, Malaysia has the second-highest obesity rate among children and adolescents in Asia, at around 12.7%[101]. Globalization affects Malaysia, causing a change from traditional diets and lifestyles to more Westernized versions.

Despite three nationwide lockdowns, Malaysia's confirmed COVID-19 cases continued to rise. Restricted movement, unhealthy eating habits, and a decrease in moderate-to-vigorous intensity physical activity due to limited access to exercise amenities during the lockdowns can significantly impact body weight and body composition[102-104]. A recent study found a marginal increase in the prevalence of overweight/obesity among Malaysian adults after a year of the COVID-19 pandemic (pre-pandemic 48.4%, versus pandemic 49.2%)[105].

Many developing countries, including Malaysia, fail to ensure that their populations consume adequate amounts of fruits and vegetables[106]. The Malaysian Non-Communicable Disease Surveillance highlighted the rapid rise of obesity[107] due to the low intake of fruits and vegetables. Vegetable intake, for example, decreased by around 5% between 2003 and 2014[108]. On top of that, research reported that education, income, and residence location are significant predictors of fruits and vegetable intake[109]. Additionally, 68.1% reported consuming high sugar-sweetened beverages and calorie-dense local foods, depending on gender and ethnicity[110].

Recognizing the importance of the growing obesity problem, the Ministry of Health, the Academy of Medicine, and several specialist medical societies collaborated to develop and publish Clinical Guidelines for the Management of Obesity[111]. The Malaysian Association for the Study of Obesity published an obesity prevention strategy in 2005. Aside from elucidating the role of diet and physical activity, the report acknowledged that obesity management should be a collaborative effort involving government, industry, professional bodies, non-governmental organizations, communities, and individuals[112].

It was also concluded that a national approach needs to be in place to help prevent, treat and manage obesity in Malaysia, and to fill research gaps, potential new foods or compounds should be investigated to help prevent obesity. Lignans are a good candidate for this, although there is currently no data on lignan intake in Malaysia, particularly in relation to obesity. Furthermore, the knowledge gained on the bioavailability of compounds such as lignans from, e.g., Malaysian foods, could be applied more globally.

AIM AND THESIS OUTLINE

Evidently, expanding the data on the lignan content of foods could improve dietary assessment in future studies and help to elucidate the mechanisms of actions that underpin the health benefits of dietary lignans. A better understanding of the role of the food matrix, as modulated by processing, on the fate of lignans in the gastrointestinal tract would help to further explore the relationship between different food properties and differences in microbiota. In addition, the relationship between food properties and microbiota would help better interpretation of the associations between lignan intake and health in population-based studies and, most importantly, design proper strategies to fully exploit the potential of lignans in combating obesity. The research described in this thesis aims to assess the bioaccessibility and conversion of lignans by using simulated *in-vitro* digestion experiments. Subsequently, a human population-based study investigates lignan intake in association with obesity.

The sequence of the research is presented in Figure 3. In **Chapter 2**, a systematic review of the literature is performed on the potential beneficial role of dietary phytoestrogens regarding weight loss. In **Chapter 3**, the effect of bioprocessing on the lignan content of various foods consumed in Malaysia is investigated. In addition, flaxseed is included since it is known as a major lignan source. The lignans were measured by a validated extraction method and quantified by LC-MS/MS. In **Chapter 4,** the bioaccessibility of flaxseed that was bio-processed by different techniques and

particle sizes and tea brew are assessed by the simulated gastro-intestinal *in-vitro* digestion (INFOGEST). Then, the effect of the food matrix on the conversion of lignan to enterolignan is evaluated by the *in-vitro* batch fermentation. In **Chapter 5**, lignan intake in the Dutch population is assessed in the NQplus study using multiple 24-hour recalls, and associations between dietary lignan intake and obesity indicators are evaluated. Finally, in **Chapter 6**, the main outcomes across all studies are synthesized and discussed, placed in a larger context, and recommendations for further research are being made.

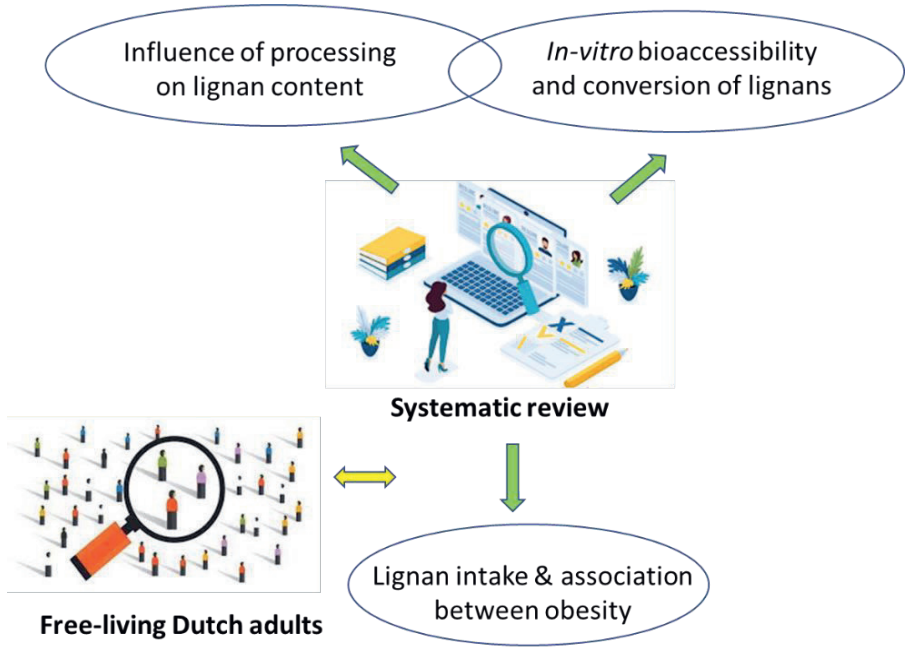

Figure 3: Schematic overview of the different goals addressed in this thesis and their interconnection

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2

CHAPTER 2

Beneficial role of dietary lignans and isoflavones in weight reduction: A review

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(Submitted)

Record searching, n=4698
2010 to April 2022

CAB Abstract, n=1048
Scopus, n= 3266
Pubmed, n= 384

Included for analysis

LIGNANS
15 reviews

ISOFLAVONES
13 reviews

ABSTRACT

The epidemic of obesity represents a rapidly growing global trend, with an estimated 38% of the world's adult population will be overweight, and another 20% will be obese by 2030. Phytochemicals have been shown to affect energy metabolism and possibly weight loss. Lignans and isoflavones are two major phytoestrogens classes, basically being biologically active plant-derived compounds with a chemical structure similar to estrogen. They exist in the plant as glycosides and are converted to active forms by intestinal microbiota in the colon. The present work describes the occurrence of lignans and isoflavones in foods, as well as their bioavailability and metabolism, including absorption in the gut. In addition, a systematic review of dietary interventions that address the link between lignans and isoflavones intake and weight loss and the proposed underlying mechanisms is presented. The systematic review included 28 observational and experimental studies. Significant beneficial effects of dietary lignans and isoflavones on weight reduction were observed in both human and animal studies and in normal weight and obese subjects. This effect is mediated by nuclear transcription factors, such as PPARs and SREBPs, which are the central modulators of lipid metabolism. These nuclear transcript factors possibly suppress adipose tissue growth, thus inhibiting adipogenesis and increasing energy expenditure, reducing adipose tissue mass. In conclusion, lignans and isoflavones could be a potential alternative dietary source in preventing and treating obesity. Future studies that focus on how processing impacts lignan content and its bioaccessibility and conversion are needed to understand the role of lignan associated with obesity.

Keywords: lignans, isoflavones, weight reduction, microbiota, bioavailability

INTRODUCTION

The continuous rise in average body mass and its associated prevalence of obesity in adults and children has been one of the most dramatic changes in modern society[1]. The WHO Expert Consultation held in 1997 cautioned this epidemic would escalate, and the prevalence statistics have increased rapidly since that date in almost all countries[2]. An estimated 38% of the world's adult population will be overweight, and another 20% will be obese by 2030[3]. The large increase in obesity has been mainly attributed to the change in dietary patterns characterized by high-energy foods in combination with reduced physical activity[4].

Searching for effective dietary strategies for mitigating excessive weight gain has become essential. Certain phytochemical classes have gained more attention, i.e., phytoestrogens. Lignans and isoflavones are two major groups of phytoestrogens with estrogenic properties. They occur in the plant as glycosides and are converted to active forms by intestinal microbiota in the colon[5]. Some studies found that lignan-rich diets increase energy expenditure in mice[6,7]. Furthermore, soy-based isoflavones were shown to have favorable effects on reducing weight and fat composition, lower triglycerides, and positively affecting glycemic control[8]. These compounds were reported to correlate positively with weight reduction observed in healthy and experimental animals, healthy humans, and obese subjects[9-12].

The bioavailability of lignans and isoflavones, i.e., the fraction of ingested bioactive compounds absorbed by the intestinal epithelium and transported to the active sites, probably modulates the health effect of these compounds. This bioavailability depends on the food matrix wherein phytochemicals occur and on the host's physiological factors, including the gut microbiota's metabolism[8]. The colonic microbiota are responsible for converting the parent compounds into more bioactive metabolites that can be absorbed by the colon epithelium[13]. The gut microbiota composition, in turn, is affected by several factors such as genetics[14], gender[15], and age[16], including long and short-term habitual dietary intake[17].

Based on the existing evidence, the mechanism of action of lignans and isoflavones on body weight and fat metabolism remains unclear. Therefore, this review scrutinizes the information on the possible role of dietary lignans and isoflavones in weight loss. Then, the chemistry and biochemistry, availability and food source, and the effect of processing will be summarized. In addition, the bioavailability, including the fate due to microbial metabolism in the gut as well as the putative mechanisms responsible for weight reduction, will be discussed.

MATERIAL AND METHODS

This review aims to summarise information on phytoestrogens occurrence in foods, their bioavailability, and metabolism, as well as the role of gut microbiota metabolism on their health effect. Finally, we investigate the association of these phytoestrogens with weight reduction. For this latter part of the aim, a literature search was conducted within three databases: CAB Abstracts, Scopus, and PubMed. Title-abstract-keywords were included in three different search strings, one on obesity-related terms; ("losing weight" OR "weight reduction" OR obesity OR overweight OR "weight management" OR "overnutrition" OR "body mass index"), the second including food-related terms (food OR beverages OR "raw food" OR "cooked food" OR diet OR "processed food") and the third, on phytoestrogen related compounds (phytoestrogens OR lignan OR coumestan OR isoflavone OR polyphenol OR flavonoid). The operator AND combined the search strings. The searches were limited to English journal articles and publications from 2010 to April 2022. Initially, 4,698 articles were found (1,048 CAB Abstract, 3,266 Scopus, Pubmed 384); after deduplication, 3,762 articles were included (Figure 1).

Identified publications were screened based on the title and abstract by the 2009 Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines[18]. The exclusion criteria were: not related to weight reduction, and subsequently: 1: an active compound that is not associated with weight loss, 2: broad and general discussion on weight loss, 3: food without compound mentioned or only a single compound without the food being analyzed, 4: not a phytoestrogen compounds (vitamin, fatty acids, mineral, acetic acid, etc.) and 5: other programmes related to weight loss (physical activity, diet regimes) with no active compound being investigated.

After the title-abstract screening, 102 articles remained for full-text reading using the mentioned exclusion criteria. After reading the full text, 28 articles were selected for further evaluation, 15 and 13 on lignans and isoflavones, respectively. Data were extracted on the phytoestrogen content of foods, chemical structure, microbial activity, bioavailability, and mechanisms of action on weight reduction. The flow diagram in Figure 1 describes the process for the literature selection of the review on the phytoestrogens associated with weight reduction.

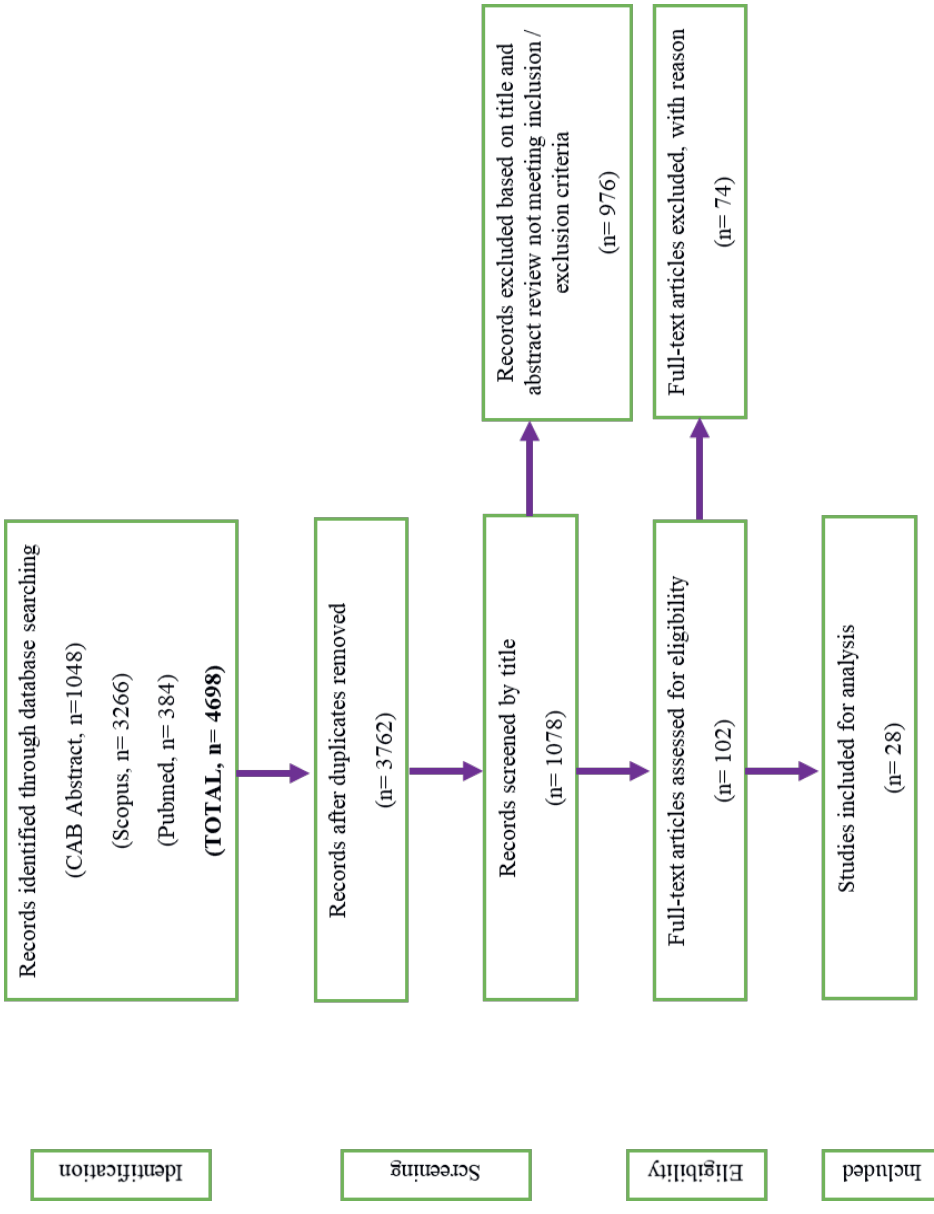

Figure 1 Flow diagram of literature selection for the review of phytoestrogens associated with weight reduction

RESULTS

LIGNANS

Chemistry and biochemistry of lignans

Lignans are a class of polyphenolic substances derived from phenylalanine through dimerization of substituted cinnamic alcohols, found abundantly in plants as esters, aglycones, or glycosides. Lignans are phenylpropanoid dimers linked by the central C8 carbon of their side chains[6]. Lignans can be categorized into eight chemical groups and several subgroups based on how oxygen is integrated into the skeleton and the cyclization pattern[19]. Secoisolariciresinol (SECO) is the most abundant lignan, whereas matairesinol (MATA), pinoresinol (PINO), and lariciresinol (LARI) occur as minor components[20]. LARI and SECO are derived from PINO, while SECO is the common precursor of MATA[8].

Occurrence in food, dietary intake, and effect of processing of lignans

Lignans occur in edible plants[21] where they have been detected in different parts, including roots, leaves, wooden parts, flowers, fruits, and seeds[22]. Table 1 reports the content of the main lignans in different food sources. The foods shown were selected based on the availability of occurrence data for the main lignans and are listed in descending order of total lignan concentration. Flaxseed is the richest source of lignans, with an average content of 300 mg/100g. Sesame seeds have the second-highest concentration of lignans with approximately 40 mg/100g. Brassica vegetables such as cabbage, brussels sprouts, and kale also have a high level of lignans ranging from 1-2 mg/100g. The richest lignan source among fruits is noni[23], widely cultivated in tropical areas[24].

Furthermore, food processing is known to affect the content of lignans[30]. Simbalista et al. (2012) found that 89% of the original lignan content of defatted flaxseed flour was maintained in bread after baking[31]. Lignans are stable in the bread-making process because they are relatively heat stable[32]. Another study showed that the amount of lignans in doughs, breads, cookies, and muffins remain stable during baking at 225^{o}C, with recovery ranging from 73-99% and relatively stable during storage[32]. In addition, processing affected the bioavailability of lignans. For example the bioavailability of lignans was 28% and 43% higher in ground and crushed flaxseed, respectively, compared to whole flaxseed[15]. All in all, there are relatively few reports on the effect of

various processing and storage conditions on lignan stability, and further investigation is required.

The median lignan intake in a representative sample of the Dutch population was calculated to be 1.0 mg/day, based on the sum of four lignans (PINO, LARI, SECO, and MATA)[27]. Moreover, in a French study (58,049 women), the median total lignan intake was 1.1 mg/day[33]. In addition, a study was conducted to estimate the lignan intake in five European countries (Denmark, Finland, Italy, Sweden, United Kingdom) among men and women using a lignan database by Milder et.al[22]. The result showed that the dietary lignan intakes ranged from 1 to 2 mg/day; Swedish men had the highest lignan intake (1.95 mg/day); and Finnish women had the lowest lignan intake (1.04 mg/day)[34]. Similarly, the EPIC-Spanish cohort study found that lignan intake was 1.0 mg/day.

In most western countries, cereals are the major sources of lignans because of their regular daily consumption[3]. The different dietary habits of total lignan intake vary among countries[35]. For instance, the main sources of lignan intake in U.S. women were fruit, berries, and bread[36], while in German women, breads, nuts, and seeds contributed the most to lignan intake[37]. Furthermore, oilseeds, nuts, cereals, vegetables (*Brassica* species), fruits, and beverages (tea, coffee, beer, and wine) are the major sources of lignans in the European diet[38].

Table 1 Concentration (mean value) of the main lignans in different food sources

Food source	Total lignans	PINO*	LARI*	SECO*	MATA*	References
Seeds, cereals, legumes (mg/100g wet weight)						
Flaxseed	300	3.3	3.0	294	0.5	22
Sesame seed	40	29	9	0.1	0.5	22
Flaxseed bread	13	0.2	0.4	12	0.03	22
Sunflower seed	8	6.8	1.1	0.01	0.1	2
Rye bran	4	1.6	1.5	0.5	0.7	3
Oat bran	2	0.5	0.7	0.1	0.4	3
Wheat bran	2	0.1	0.7	0.9	0.4	3
Chickpea	0.4			0.4		18
Barley bran	0.3	0.07	0.1	0.04	0.04	25
Peas	0.1			0.1		18
Fruits (mg/100g wet weight)						
Noni	1.3					23
Yuzu	1.0	0.7	0.2	0.03		26
Apricot	0.5	0.1	0.3	0.03		27, 28
Dried apricot	0.4	0.2	0.1	0.1	0.001	2
Dried dates	0.3	0.1	0.1	0.1	0.003	2
Kiwi	0.2			0.2	0.001	29
Vegetables (mg/100g wet weight)						
Brassica vegetables	2	1.7	0.6	0.002	0.012	22
Asparagus	1	0.1	0.09	0.7	0.014	26
Garlic	0.6	0.5	0.05	0.04	0.005	2

Carrot	0.2	0.02	0.06	0.09	0	27
Drinks and beverages (mg/100 mL)						
Black tea	0.1	0.04	0.03	0.01	0.002	22
Earl grey black tea	0.06	0.03	0.03	0.001	0	22
Soy milk	0.04	0.03	0.01	0.001	0	27
Green tea	0.04	0.01	0.02	0.01	0	22
Coffee	0.03	0.002	0.01	0.02	0.01	27

*PINO = Pinoresinol, *LARI = Lariciresinol, *SECO = secoisolariciresinol, *MATA = matairesinol

Bioavailability of lignans and microbial metabolism in the gut

The bioavailability of lignans varies between individuals and the study population[39]. At least 40% of lignans starts to be absorbed and appears in the blood circulation 8-10 hours after ingestion[40]. Hydrolysis of the glycosides by the de-glycosylating activity of membrane-bound enzymes in the small intestine contributes to the increase of bioavailability of flavonoids in the upper intestine. Still, specific information on the bioavailability on lignans is missing[39]. Under strictly anaerobic conditions, intestinal microbiota convert lignans to enterolignan metabolites enterodiol (END) and enterolactone (EL). Serum levels and urinary excretion of lignan metabolites are used as bioavailability markers for lignans intake[39, 41]. The conversion of lignans to END and EL is initiated by removal of the sugar moiety from the glycosides by bacterial β-glucosidases, followed by several metabolic steps which include reduction, demethylation, dehydroxylation and oxidation[20,36,42-44].

Secoisosolariciresinol diglucosidase (SDG), the predominant lignan in food, is metabolized in the gut to SECO, then converted to END and finally to EL, the so-called enterolignans. *Ruminococcus productus, Eubecterium limosum., Clostridium scindes,* and *Eggerthella lenta* are the common bacteria that convert SECO to END[41, 45, 46]. The metabolism of MATA to ENL occurs directly in the gut by *Peptostreptococcus productus*[41], and the bacteria *E.lenta* is involved in converting PINO to LARI and LARI to SECO[41]. Enterolignans can be directly excreted from the colon via feces or absorbed into the bloodstream[47,48] conjugated into glucuronides, sulfoglucoronides, monosulfates, and disulfates in the epithelium[49] or in the liver[50]. Some of the enterolignans may be re-excreted in the intestinal tract through bile, then leave the body through feces or are reabsorbed into the bloodstream and thus enter the enterohepatic circulation[51] or are excreted via the urine as glucuronides and disulfates[49,52].

Variations in the composition and activity of the colonic gut microbiota, intestinal transit time, colon redox state, types of lignans present in the diet, processing the lignan containing foods undergo; grinding (milling, crushing), and cooking, and the uses of antibiotics are all factors affecting the extent of lignans conversion into ENL by gut microbiota[15, 39, 41] and ultimately the health effect associated with lignan intake[45,53].

Dietary interventions of lignans

We retrieved 15 studies on lignans and body weight, seven human studies, and eight animal studies, summarized in table 2.

Two observational studies were found. In a human cross-sectional survey from National Health and Nutrition Examination Survey in the U.S., with 6,806 individuals studied from 2003 to 2008, enterolignans and creatinine were measured from a spot urine sample. High-enterodiol concentration was associated with 18% and 42% lower probability of being overweight and obese, respectively, and 48% lower high-risk waist circumference among adults. Moreover, high-enterolactone concentration was associated with a 24% and 64% lower probability of being overweight and obese, respectively[9]. A study involving 3,438 young Spaniards between 2 and 24 years old investigated the prevalence of obesity with lignan intake. It found a strong association between dietary lignan intake and the prevalence of obesity for boys, with an odds ratio of 0.34 (CI: 95%, 0.17–0.70) for comparison of the highest versus lowest quartile of lignan intake[54].

A 10-year prospective cohort study on weight change of U.S. women was performed to examine the association with baseline urinary excretion of enterolactone (EL) and enterodiol (END). The study observed that in multivariable adjustment models, women in the highest quartile of EL and END had an average BMI of 24.6 (CI:95%, 23.9 - 25.3) and 25.5 (CI:95%, 24.8 – 26.2), respectively, compared to women in lowest quartile had an average of BMI 27.5 (CI:95%, 26.8 - 28.2) and 26.6 (CI:95%, 25.9 – 27.3), respectively. Moreover, the study investigated the prospective annual weight change rate during 10-years follow up period. Women in highest quartile of END gained 0.27 kg/year less (CI:95%, -0.41, -0.12) compared to the lowest quartile. No significant weight gain change was observed in the EL excretion ($P = 0.12$). The data suggested that higher urinary enterolignans excretion was significantly associated with lower baseline BMI and modestly slower weight gain during a 10-year follow up period[55].

In a randomized crossover trial, 60 overweight or obese women consumed 30 g/day of milled lignan flaxseed for three months. The placebo group received 30g/day of milled rice. The study reported a significantly higher reduction in waist circumference (WC) and waist-to-hip ratio (WHR) by 5 cm and 0.3 cm, respectively, compared to the placebo group[56]. In another randomized trial, Barre et al. (2012) found significant waist circumference gain in 24 participants by 0.6 cm in treated compared to 2.1 cm in placebo participants. They were given 600 mg of total SDG daily for three months[57]. The study showed that the consumption of SDG would help in waist

circumference management. A third randomized controlled clinical trial of 17 subjects comparing the effect of flaxseed fiber drink and flaxseed bread for seven days showed that fecal fat and energy excretion in feces considerably increased by 50% and 23% with flaxseed drink compared to control. On the other hand, no significant difference was found comparing the effect of flaxseed drink and bread[58].

On the other hand, a single-blind randomized study showed no significant reduction in body weight and body composition in a study of 75 adolescents between 13.7 and 21 years that consumed 28 g/d of brown flaxseed, golden flaxseed, or the equivalent amount of wheat bran for 11 weeks. The low compliance to the food intake protocol may be one of the reasons why no physiological effect was observed. The adolescents consumed about half the daily amount provided, which may not have been adequate to exert the health benefits of flaxseed. In addition, no study has been done to test the beneficial effects of flaxseed on younger individuals[59].

Eight different animal studies were included in the final review. Two researches were done on the effect of SDG on weight reduction in mice on high-fat and high-fructose diets (HFFD). Supplementation with 40 mg/kg of body weight/day SDG for 12 weeks, showed significantly lower body weight gain (35.8%) compared to the control group[60]. Similarly, a study on high fat-fed obese mice given SDG (50 mg/kg of body weight/day) for eight weeks showed a significant decrease in body weight by 6.1% compared to the control group[61]. Moreover, a study reported that 60 days intake of 3 mg/kg body weight of total extract of HMRLignan™, a purified dietary supplemental form of 7-Hydroxymatairesinol from *Picea abies* extract, significantly lowered both body weight and fat mass by 11% in mice on a high-fat diet[62].

A study on mice investigated whether flaxseed intake could attenuate weight gain by modulation of obesity gene expression. Both diets with 6% (w/w) defatted flaxseed and 4% (w/w) flaxseed oil for eight weeks on high-fat diet mice significantly lower body weight gain by 16.5% in defatted flaxseed and 31.7% in flaxseed oil compared with a control group [10]. Another experimental study by Biasiotto et al. (2014) investigated the effect of sesame and flaxseeds flour on metabolic parameters on high-fat diet mice. A decrease in body fat in mice was shown when consuming 20% of sesame or flaxseed flour[63]. In addition, a high-fat fructose diet mice study on the intake of 35% of flaxseed powder for 12 weeks showed a significantly lower body weight gain by 104% compared 119% to control group[64].

Eucommia ulmoides (EU), a small native plant cultivated abundantly in China, also proved to have beneficial health effects of reducing weight in mice. Twenty-eight different types of lignans have been isolated from the bark, seeds, and leaves[65,66].

Fujikawa et al. also found that treatment with EU leaves with high lignan content significantly lowered body weight by 12-23% when EU leaf extract and 14-22% when EU green leaf powder were consumed by rats on a high-fat diet compared to the control group. In addition, the result showed that the treatment with both extract and powder enhanced metabolic function in organs, including diminishing the adenosine triphosphate (ATP) production in white adipose tissue, accelerating β-oxidation in the liver, and the use of ketone bodies or glucose in skeletal muscle[67].

Furthermore, a metabolic study of a lignan-rich diet containing *Picea abies* extract on obese mice for 60 days, showed a significantly lower body weight of 13% and fat mass of 18%. This extract is rich in 7-hydroxymatairesinol, as it is found at high concentrations in the heartwood of branches and knots of the Norway spruce trees (*Picea abies*)[62]. Table 2 shows the summary of studies evaluating the association of lignans with weight reduction.

Table 2 Summary of studies evaluating the association of lignans on body weight or related variables

Model	Number of individuals (total)	Methods	Results	References
Human- cross-sectional study	6,806 subjects, U.S.	Enterolignans were measured from a spot urine sample. Analyses adjusted for age, sex, race, total energy intake, and frequency of intake of grain, vegetable, fruit, meat, and dairy	Significant reduction of 18% and 42% of being overweight and obese, resp. in high compared to low-END concentration, 24% and 64% of being overweight and obese, resp. in high compared to low-EL concentration and 38% lower high-risk waist circumference in high compared to low-END group	9
Human- cross-sectional study	3,438 boys and girls, Spain	Dietary intake was assessed using 24-h diet recalls; lignan dietary intake was calculated using a lignan composition database. Analyses adjusted for age, income, population size and Spanish region, total energy intake, dietary fiber intake, and adherence to recommended physical activity	Strong association between dietary lignan intake and prevalent obesity was found for boys, odds ratio highest versus lowest quartile of lignan intake 0.34 (95% CI 0.17-0.70), but not for girls	54

Human- cohort study	783 women, U.S.	10 year prospective weight change study. Urinary excretion levels of enterolactone and enterodiol were measured as exposure	Highest quartile of EL and END had an average BMI of 24.6 (CI:95%, 23.9 - 25.3) and 25.5 (CI:95%, 24.8 – 26.2), respectively compared to women in lowest quartile had an average of BMI 27.5 (CI:95%, 26.8 - 28.2) and 26.6 (CI:95%, 25.9 – 27.3), respectively. Highest quartile of END gained 0.27 kg/year less (CI:95%, -0.41, -0.12) compared to the lowest quartile	55
Human- double-blind randomized crossover study	60 women with overweight or obesity, Iran	Double-blind, randomized crossover of 3 months with 30g/day of milled flaxseed vs. 30g/day of milled rice as control treatment	Significantly higher reduction in waist circumference by 5 cm and waist-to-hip ratio by 0.3 cm compared to control treatment	56
Human- double-blind, randomized crossover placebo-controlled study	16 participants with obese, Canada	Double-blind, randomized crossover placebo-controlled trial of 3 months with 600 mg total SDG/day vs. identical looking capsules as placebo. Results adjusted for age, gender, and order of treatment	Significantly smaller increase in waist circumference by 0.6 cm in treated compared to 2.1 cm in placebo group	57

Human-double blind randomized crossover study	17 participants, Denmark	Double-blind, randomized crossover trial of flaxseed fiber drink (3/day), a diet with flaxseed fiber bread (3/day), all at 7.5 g/10MJ, and a low-fiber control diet, each for 7d	Fecal fat and energy excretion increased by 50 and 23% respectively with flax seed drink compared to control.	58
Human- single-blind randomized parallel study	Seventy-five overweight adolescents, Brazil	Single-blind randomized trail, of 28 g/d of brown flaxseed, golden flaxseed, or equivalent amount of wheat bran (control) for 11 weeks	No significant reduction was observed for all variables measured: lipid profile, glycemia, blood pressure, body weight, and body composition	59
Mice		SDG (40 mg/kg of body weight/day) for 12 weeks on high-fat and high-fructose diets (HFFD)	Significantly reduced the body weight gain by 35.8% in HFFD + SDG compared with HFFD group despite no difference in food intake	60
Mice		SDG (50 mg/kg of body weight/day) for 8 weeks on high fat diet-fed obese mice and obese db/db mice	Significantly lower body weight by 6.1% in db/db mice despite no difference in food intake	61

Mice	3mg/kg body weight of total extract of HMRLignan™ for 60 days on high-fat diet	Significantly lower body weight by 11% and fat mass by 11%, liver steatosis reduced by 62%	62
Mice	6% (w/w) defatted flaxseed (DF) and 4% (w/w) flaxseed oil (FO) for 8 weeks vs 10% (w/w) whole flaxseed on high-fat diet	Significantly lower body weight gain by 16.5% in DF and 31.7% in FO compared with control group	10
Mice	20% sesame and 20% flaxseed flour for 84 days on high-fat diet	Significantly lower total body fat in both sesame and flaxseed flour fed mice by 20%	63
Rats	Lignan enriched (35%) flaxseed powder (0.02% w/w) for 12 weeks on control diet of high fat fructose diet	Significantly lower body weight gain compared to high fat diet (104% vs 119%)	64
Rats	3% and 9% of *Eucommia ulmoides* (EU) leaf extracts (ELE) and green leaf powder (EGLP) for 3 months on high-fat diet	Significantly lower body weight by 12-23% in ELE and by 14-22% in EGLP compared with control group	67

Mice	10mg/kg body weight of total extract of *Picea abies* for 60 days on high-fat diet	Significantly lower body weight by 13% and fat mass by 18%; liver steatosis decreased by 65%	62

Mechanism of action on weight reduction of lignans

Studies on lignans have tried to explain the mechanisms responsible for weight reduction and lipid metabolism[68,69]. Lignans may suppress adipose tissue growth, inhibit differentiation of preadipocytes, stimulate lipolysis, and induce apoptosis of existing adipocytes, thus reducing adipose tissue mass in the cell studies[70,71].

PPARγ has been identified as one of the key transcription factors primarily expressed in adipose tissue, promotes adipocyte differentiation, and activates the transcription of genes that regulate fatty acid metabolism in the liver[72-76]. Enterolignans act as PPARγ agonists and regulate the expression of adipogenesis[77] by effectively inhibiting the SREBPs receptor that blocks adipogenesis[78]. PPARs and SREBPs promote the expression of lipid metabolism enzymes such as adipocyte binding protein, lipoprotein lipase (LPL), and fatty acid synthetase during adipogenesis (FAS)[79]. Through these mechanisms enterolignans regulate the process of lipogenesis and lipolysis, the two primary metabolic events in adipose tissue functioning to maintain lipid homeostasis. Lipolysis is the hydrolysis mobilizing triglycerides, while lipogenesis is the process of synthesis and storage of esterified fatty acids. Both these metabolic pathways are regulated by the integration of endocrine and neural mechanisms, which is critical for progression to the final stages of adipocyte differentiation[75] and should cooperate in order to diminish fat stores, thereby combating obesity[63, 70, 80-82].

ISOFLAVONES

Chemistry and biochemistry of isoflavones

Isoflavones are secondary plant metabolites produced by the leguminous plant[83]. Isoflavones are flavonoids with a basic structure that consists of two benzene rings (A and B) linked by a heterocyclic pyrone C ring[84,85]. The benzenoid B ring position of isoflavones is in the 3-position of the pyrone C ring, which is different from the 2-position of other flavonoids[84,86]. Isoflavones are considered nonsteroidal estrogens due to their structural similarities to estrogens[87]. Approximately 98% of isoflavones are present as glucosides, which are less estrogenic than their respective aglycones. Aglycone of isoflavones is the biologically active form [88]. The main isoflavones aglycons are genistein, daidzein, and glycitein, each of which has three possible glycoside forms: β-D-glycoside, 6''-O-malonyl-glycoside, and a 6''-O-acetyl-glycoside, respectively. However, they are primarily present in foods in their β-D-glycoside form as genistin, daidzin, glycitin[89].

Occurrence in food, dietary intake, and effect of processing of isoflavones

Isoflavones are found primarily in members of the *Leguminosae* family and occur abundantly in legumes. More than 300 plants contain isoflavones, particularly seeds and roots[90]. Soy, lentils, beans, and chickpeas are some of the richest food sources of isoflavones. The US Food & Drug Administration reported that soybean and soybean products are the richest food sources of isoflavones[91,92]. Table 3 summarizes the main isoflavones content in different food sources. The foods were selected based on the main isoflavones' data availability and are reported in descending order of abundance.

Table 3 Concentration of main isoflavones (mg/100g wet weight) in different food sources

Food source	Total isoflavones*	Genistein	Daidzein	References
Fermented soybean hull	240	98	140	93
Soy flour (textured)	173	90	68	94, 95
Soy flour, defatted	150	88	65	94, 95
Instant beverage, soy, powder, not reconstituted	110	62	40	94, 96
Natto	83	38	33	94, 97
Tempeh fried	73	40	33	94
Miso soup mix	70	40	30	94
Tempeh	60	36	22	94, 96, 98
Soybeans, green, raw (includes edamame)	49	23	20	94, 98-100
Soy fiber	44	22	19	94, 101
Miso	41	23	16	94, 96, 97
Soybeans, mature seeds, sprouted, raw	35	19	13	94, 102
Tofu	34	16	16	94, 98, 103
Roasted soybeans	26	14	09	94
Red, clover	21	10	11	104
Soybeans, green, cooked, boiled, drained, without salt (includes edamame)	18	7	7	94, 105
Mayonnaise, made with tofu	17	11	6	106
Black bean, raw	14	6	8	105
Soybeans, mature seeds, sprouted, cooked, steamed	13	7	5	94
Black bean, sauce	10	4	6	94, 102
Soy milk drink	8	5	3	94, 107

*Total isoflavones: genistein, daidzein and glycitin

Soybean products containing high levels of isoflavones, such as tofu, natto, soymilk, and fermented foods such as doenjang, soy sauce, tempeh, and miso, are widely consumed in Asia[108], where the average intake of isoflavones is reported to range between 8 mg/day and 50 mg/day[109].

Food processing may alter the concentration of isoflavones in foods. A study reported that cooking soybeans increased genistein content by 60 to 80%. Moreover, soybean fermentation (tempeh) produced the most significant increase in isoflavones content by 77 to 93%[110]. Germination proved to be one of the processing approaches to enhance the isoflavone content. A study reported an increase of isoflavones on sprouted soybean up to 100% from 5 to 7 days of sprouting[111].

Another finding showed that high temperature and pressure reduced the total isoflavones content in corn-soy mixtures[112]. This finding was in agreement with a previous study by Mahungu (1999), who reported a decrease in the overall isoflavones concentration of soy protein isolates and corn mixtures with three different temperatures of extrusion (110°C, 130°C, and 150°C)[113]. Others have reported that thermal treatments reduce isoflavone content in food[114].

Bioavailability of isoflavones and microbial metabolism in the gut

Isoflavones glycosides are not absorbed intact across the enterocyte in the gut due to their higher hydrophilicity and molecular weights[115]. Isoflavones glycosides are converted to genistein and daidzein via intestinal membrane-bound enzymes such as lactase-phlorizin hydrolase (LPH) or intestinal microbiota enzyme β-glucosidase[83,84,116]. The absorption of the resulting aglycons typically ranges between 10 and 50% of the isoflavones present in the food[117]. Once absorbed, aglycones undergo phase II metabolism in the epithelial cells of the small intestine epithelium[118-120], and the enterohepatic circulation transports them to the liver, where the conjugation is completed. Aglycones derivatives from the liver are distributed through the body by the bloodstream or secreted to the intestinal lumen by bile[118].

In the large intestine, genistein is metabolized to dihydrogenistein, which can be further metabolized to p-ethyl phenol. In contrast, daidzein is metabolized to dihydrodaizen and further metabolized to equol and *O*-demethyangolensin (*O*-DMA)[85,121,122]. These steps will significantly alter the bioavailability and bioactivity of isoflavones[115]. Equol is absorbed more efficiently than daidzein through the colon epithelium[123] and remains in the plasma relatively longer than genistein and daidzein[124].

Individuals who can metabolize isoflavones into equol are "equol producers". Approximately 30-50% of the total population has bacteria that can metabolize equol in the Asian population, whereas only approximately 20% of people living in Western countries are equol producers. Bacterial composition in the intestinal tract, pH, and redox potential in the small and large intestine play a role in the formation of equol[125]. Some strains of intestinal microbes are capable of converting daidzein to equol, which are *Eggerthella lenta*, *Enterococcus faecium*, *Adlercreutzia equolifaciens*, *Slackia equolifaciens*, *Lactobacilus mucosae*, *Bifidobacterium* spp., *Slackia isoflavoniconvertens*, *Bacteroides ovatus* spp., *Streptococcus intermedius* spp., and *Ruminococcus productus* spp., among the others[126-134]. In addition, the diet influences the microbiota diversity and its metabolism of isoflavones.

Dietary intervention of isoflavones

We retrieved 13 studies on isoflavones and body weight, three human studies, eight animal studies, and two *in-vitro* studies, summarized in table 4.

One systematic review and meta-analysis was included among the final papers included. It reported on 24 studies were soy protein was consumed between 1-3m vs. 4-24m, with an intake ranging <40mg/day and >40mg/day. The lower doses (<40 mg/d of) soy protein intake significantly decreased the waist circumference in women of older ages (MD:-0.31cm; 95% CI: -0.57, -0.05; P = 0.02). On the other hand, there were no significant effects on BMI and fat mass. In 17 studies, women consumed isoflavones between 2-6m vs. 10-12m, with an intake ranging between <100 mg/day and >100mg/day. The high isoflavones intake decreased BMI (MD: -0.26; 95% CI: -0.55, 0.04; P = 0.085). In addition, the result showed that lower doses (P = 0.02) and shorter intervention lengths (P = 0.053) were more effective in weight reduction. No significant difference was found in waist circumference or fat mass[135]. All in all, the aforementioned findings showed that although soy is the major source of isoflavones, soy and isoflavones may have different impacts on weight status.

An epidemiological study performed by Roccisano and Henneberg (2012) observed a relationship between soy consumption and the prevalence of obesity using data from WHO and FAO in 167 countries. The result showed that soy consumption significantly reduced approximately 10% to 21% of obesity. Unfermented soy products are an excellent source of anti-obesity nutrients[136]. A double-blind, randomized crossover trial involving 45 obese participants was carried out and showed that gene expression involved in fatty acid oxidation in skeletal muscle was significantly reduced

after consuming 50 mg/day of isoflavone capsule (genistein). In addition, no significant differences was observed in BMI and waist circumference[11].

Eight different animal studies were included in the final review. Four animal studies were conducted on the consumption of isoflavones and soybean extracts. An animal study using a soybean embryo extract showed that two weeks of treatment of high-fat diet mice resulted in a significant reduction in body fat composition by 100%[137]. Rockwood et al. also investigated whether the intake of genistein in soybean affects weight gain by studying diabetic obese mice for 4 weeks with a 600 mg genistein/kg diet. The genistein diet significantly reduced body weight by 12% in females and 9% in male mice compared to a standard rodent diet [138]. Similarly, the intake of 2.5% of soluble soybean polysaccharides (SSPS), 0.5% of insoluble genistein, and their mixture in a normal diet mice for 12 weeks was reported to reduce their fat weight by 4.5%, 13.4%, and 17.9%, respectively[139]. Another study in which three different dosages of soy isoflavones extract (low, medium, high) were given for 13 weeks to obese rats. The results found a significant reduction in body weight gain by 21.8% in low, 23.1% in medium, and 25.6% in high dosage of extracts compared to the control group[140].

Three animal studies were conducted on the consumption of isoflavones and soybean products. A group of high-fat diet rats was given 10% (w/w) of 4 different fermented soybeans named gochujang for 8 weeks. The result showed a decrease in weight ranging from 9.5% to 22%. This experimental animal study can potentially reduce fat accumulation and thus obesity[141]. Furthermore, a study of mice fed a high-fat diet with a high soy-containing diet of fermented soy-powder milk (FSPM) with *Lactobacillus Plantarum* P1201 showed a significantly reduced body weight (10.7%) and liver white adipose tissue (32.8%) compared to the control group[142]. A study by Kwak et al. suggested that the daily intake of 16.2% steamed and 20% fermented soybean for 8 weeks in high-fat diet rats could prevent diet-induced visceral fat accumulation in mice. The study showed that weight declined significantly by 1.9% when consuming steamed and 2.8% when consuming fermented soybean. Fermented soybean also significantly reduced total visceral fat weight (35.5%), epididymal adipocyte size (26.0% and perirenal fat weight (46.1%) compared with high-fat diet group[143].

Despite the fact that soybean-based products are the most common source of isoflavones, one animal study investigated whether the root of *Pueraria montata* extract containing isoflavones has anti-obesity effects. Two different dose groups of the extract with 0.8 g/kg body weight and 0.4 g/kg body weight per day for two weeks were employed on high-fat diet rats. They found a significant reduction in body weight gain by 7.5% in 0.4 g/kg and 5.0% in 0.8 g/kg of extract compared to the control group. In

addition, the results showed that the extract induced brown adipocyte activity and triggered the formation of brown-like cells[12].

Two *in-vitro* studies were included in this review. A study of soybean embryo extract significantly reduced lipid content in adipocytes by 20% in 80 μg/ml and 30% in 160 μg/ml, respectively[137]. A 3T3-L1 cell study examined the influences of genistin and genistein on adipogenesis and lipogenesis. In a dose-dependent manner, 50 and 100 M genistin and genistein significantly inhibited lipid accumulation by 21.7% and 69.2%, respectively[144].

Table 4 Summary of studies evaluating the association of isoflavones on body weight or related variables

Model	Number of individuals (total)	Methods	Results	References
Human- systematic review and meta-analysis of randomized controlled trials	24 trials on soy protein and 17 trials on isoflavones	1-3m vs 4-24m intervention for soy protein (<40mg vs. >40mg/day). 2-6m vs 10-12m intervention for isoflavones intake (<100 mg vs. >100mg/day)	Soy protein tended to decrease waist circumference in women of older ages at lower doses <40 mg/d (MD:-0.31cm; 95% CI: -0.57, -0.05; P = 0.02). No effect on BMI and fat mass. Isoflavones tended to decrease BMI (MD: -0.26; 95% CI: -0.55, 0.04; P = 0.085). Lower doses (<100 mg/d, P = 0.02) and shorter intervention lengths (2-6 mo.; P = 0.053) were more effective. No effect on waist circumference or fat mass	135
Ecological study on WHO and FAO data	Data from 167 countries	Data on the prevalence of obesity, soy consumption, caloric intake and GDP per country.	Soy consumption correlates significantly with levels of obesity, contributing 10% - 21% to variation, adjusting for caloric intake and GDP	136
Human- double blind randomized crossover study	45 participants with insulin resistance and	Double-blind, randomized crossover of 3 months with 50 mg/day of isoflavone	Significant reduction in gene expression for fatty acid oxidation in skeletal muscle; increase in circulating metabolites of β-oxidation and ω-	11

	BMI ≥ 30 and ≤ 40 kg/m²	capsule (genistein) versus placebo.	oxidation, acyl-carnitines and ketone bodies. No significant differences was observed on BMI and waist circumference	
Mice		400 mg/kg body weight of soybean embryo extract/ 2 weeks on a high fat diet	Significant reduced body weight gain by 100%	137
Mice		600 mg genistein/kg in diet for 4 weeks in ob/ob mice on normal diet	Significant reduction in body weight by 12% in females and 9% in males; significant lower serum triglyceride levels; higher hepatic % fat content in females but not in males	138
Mice		2.5% of soluble soybean polysaccharides (SSPS), 0.5% of insoluble genistein, and their mixture/ 12 weeks on normal diet	Significant reduction in body weight gain in SSPS (4.5%); insoluble genistein (13.4%); mixture (17.9%) compared to control group	139
Rats		Soy isoflavones Extracts (low, medium, high)/ 13 weeks in obese rats	Significant reduction in body weight gain by 21.8% in low, 23.1% in medium, and 25.6% in high dosage of extracts compared to control group	140
Rats		10% (w/w) of 4 different fermented soybean/ 8 weeks on high-fat diet	Significantly reduced weight by 9.5%-22%	141

Mice	1.2 g/ body weight of fermented soy-powder milk (FSPM) with *Lactobacillus plantarum* P1201/ 12 weeks on a high fat diet	Significant reduction in body weight by 10.7% and liver white adipose tissue (32.8%) compared to the control group	142
Rats	16.2% steamed and 20% fermented soybean / 8 weeks on high fat diet	Significant reduction in body weight gain by 1.9% in steamed and 2.8% in fermented soybean. Significant reduction in total visceral fat weight (35.5%), epididymal adipocyte size (26.0% and perirenal fat weight (46.1%) compared with high-fat diet group	143
Mice	0.8 g/kg and 0.4 g/kg of body weight *Pueraria montata* extract /2 weeks on a high fat diet	Significant reduction in body weight gain by 7.5% in 0.4 g/kg and 5.0% in 0.8 g/kg of extract compared to control group	12
In-vitro	80 and 160 µg/ml of soybean embryo extract	Significantly reduced lipid content in adipocytes by 20% in 80 µg/ml and 30% in 160 µg/ml	137
In-vitro	50 and 100 µM genistin and genistein 3T3-L1 adipocytes	Significantly inhibited lipid accumulation in 3T3-L1 adipocytes by 21.7% and 69.2%	144

Mechanism of action on weight reduction of isoflavones

The mechanisms underlying the anti-obesity effect of isoflavones is not fully elucidated yet. Several studies point at a role in adipogenesis and lipid metabolism, although also some studies suggest that modulation of energy expenditure via the central nervous system plays a role[145]. Specific binding affinity to estrogen receptors (ER)s enables isoflavones to elicit both estrogenic and antiestrogenic effects depending on the tissue, as well as on isoflavone and endogenous estradiol levels[108]. The interaction of aglycones with nuclear transcription factors (PPAR) plays an important role in adipogenesis[146, 147], which improves lipid levels by activating and modulating the PPARs and sterol regulatory element-binding proteins (SREBP)s[148-150].

Moreover, isoflavones would increase the energy expenditure[145] by the increment of AMPK enzymes (AMP-activated protein kinase) and acetyl-CoA signaling cascades, which are responsible for the inhibition of adipogenesis[151,152]. AMPK can switch from anabolic to catabolic pathways by suppressing the expression of genes involved in adipogenesis, such as PPARγ[153].

Isoflavones also have been shown to have bioactivity that regulates lipid generation and thermogenesis through improved lipogenesis[148-150]. It suppresses cell proliferation and induces cell cycle arrest, subsequently decreasing lipid accumulation in the early stages of adipogenesis[154]. Another study showed that daidzein and genistein induced uncoupling protein 1 (UCP-1) expression in brown pre-adipocytes to promote cell proliferation, differentiation, and activation[12], which depleted energy depots[155].

CONCLUSION

This review summarizes the occurrence of lignans and isoflavones in plant-based foods, their bioavailability and microbial metabolism in the gut, and their mechanism of action on weight reduction. Flaxseed is the richest source of lignans, while soybean and soybean products are the richest food sources of isoflavones. In addition, processing affects the type and amount of lignans and isoflavones of final products.

The variability in intestinal microbiota activity may play an important role in further metabolism; thus, the transformation might be essential for these functions to be manifested. Since intestinal bacteria are essential for lignans and isoflavones bioavailability and are associated with health effects, nutritional strategies that promote the production of active metabolites via the microbiota appear very appealing. However,

to date, there is no evidence for a diet-driven optimization of microbial conversion of lignans and isoflavones.

Emerging evidence and reviewed data from human and animal studies support the recently increased interest in lignans and isoflavones to combat obesity. The beneficial effects of dietary lignans and isoflavones are observed in healthy and experimental animals, healthy humans, and obese subjects. The mechanism of action of these compounds on weight reduction is quite similar as they affect the expression of nuclear transcription factors, namely PPARs and SREBPs, which are the central modulators of lipid metabolism. Nevertheless, the full mechanism on how these compounds affect body weight and obesity is unclear, and this topic needs further investigation.

In addition, studies on lignans and isoflavones, and their association with microbial metabolites are crucial for understanding their role and effect on weight reduction. Similarly, little is known on the role of inter-individual differences in bioavailability and microbial conversion to achieve the potential effect on weight reduction. In summary, lignans and isoflavones may be an alternative dietary resource, with great potential in preventing and treating obesity possibly through a personalized diet approach.

DECLARATIONS

Acknowledgments

This review was supported by the Division of Human Nutrition and Health, Wageningen University & Research, Wageningen, the Netherlands, and Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

Funding

Open access funding provided by Wageningen University & Research, Wageningen, the Netherlands, within APC Discount

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval - Not applicable

Consent to participate - Not applicable

Consent for publication - Not applicable

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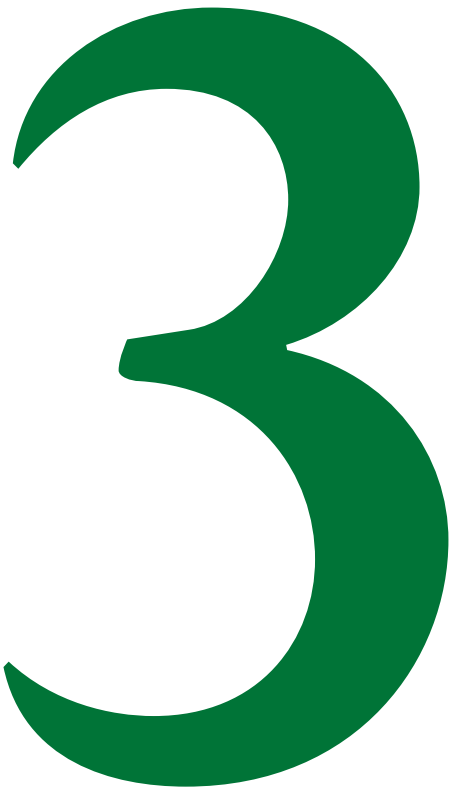

CHAPTER 3

Influence of Different Processing Method on Lignan Content of Selected Malaysian Plant-Based Foods

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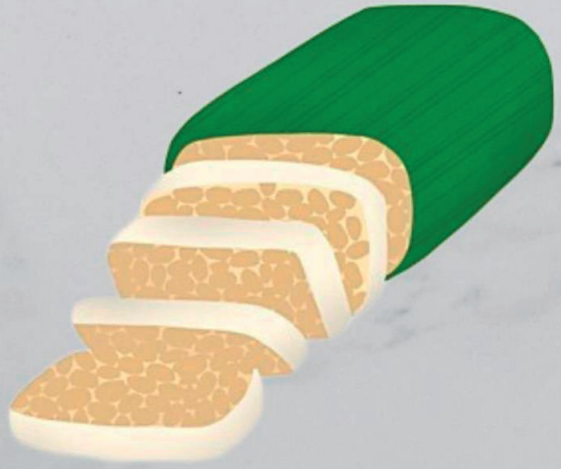
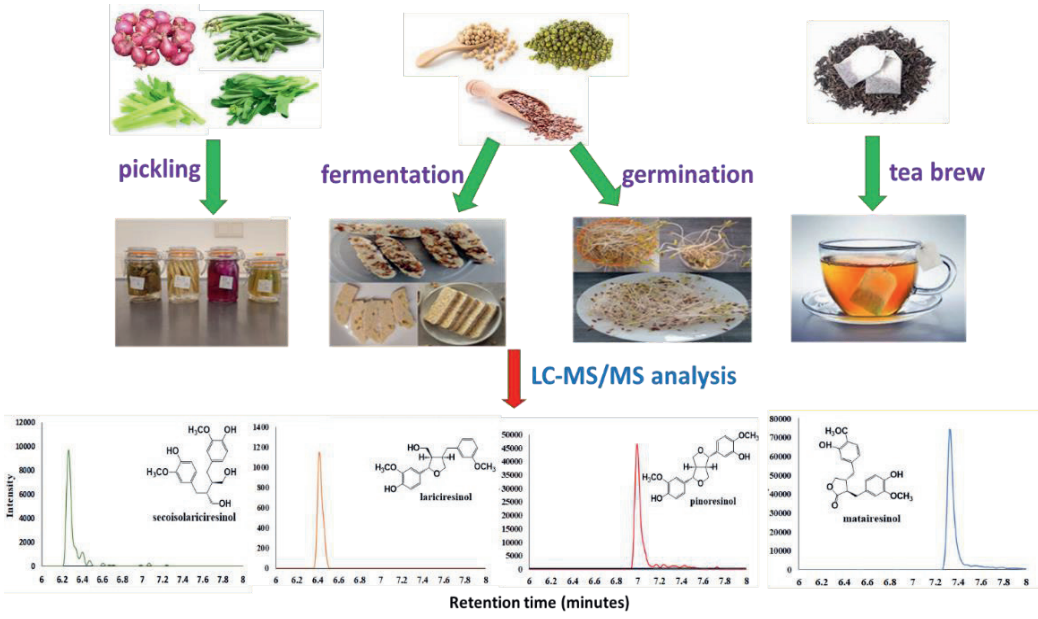
Published in Food Chemistry
(DOI: https://doi.org/10.1016/j.foodchem.2022.134607)




pickling
fermentation
germination
tea brew
LC-MS/MS analysis
secoisolariciresinol
lariciresinol
pinoresinol
matairesinol
Intensity
Retention time (minutes)

ABSTRACT

This research assessed the influence of pickling, fermentation, germination, and tea brewing on lignan content of a variety of food highly consumed in Malaysia. Lignans have been measured by a validated LC-MS/MS method. Secoisolariciresinol (SECO) was the most abundant compound in fermented and germinated samples. Pickling significantly decreased larisiresinol content by approximately 86 %. Fermentation increased lignan content in a mixture of flaxseed and mung beans (799.9 $\pm$ 67.4 mg/100g DW) compared to the unfermented counterpart (501.4 $\pm$ 134.6 mg/100g DW), whereas the fermentation of soybeans and mung beans did not significantly affect the SECO content. Germination increased lignan content, which reached its peak on day 6 of germination for all the tested matrixes. In tea brew, lignans concentration increased with brewing time reaching its highest concentration at 10 minutes of brewing. The results of this study expand the knowledge on the effect of processing on lignan content in food.

Keywords: LC-MS/MS, lignans, pickling, fermentation, germination, brewing, plant foods

INTRODUCTION

Lignans are a class of diphenolic compounds derived from combining two phenylpropanoid C6-C3 units at the β and β' carbon atoms[1]. Secoisolariciresinol (SECO) is the most abundant lignan, whereas matairesinol (MATA), pinoresinol (PINO), and lariciresinol (LARI) occur as minor components[2]. Oilseeds, nuts, cereals, vegetables (*Brassica* species), fruits, and beverages (tea, coffee, beer, and wine) are the major sources of lignans in the European diet[3], whereas leafy vegetables and beverages (tea and coffee) are the main contributors to lignan daily intake in Malaysia[4]. Some epidemiological studies have suggested that lignans reduce the risk of cardiovascular diseases and other chronic diseases (e.g., breast cancer), as well as reduce body weight and fat accumulation[5].

In Malaysia, The Malaysian Non-Communicable Disease Surveillance highlighted the rapid rise of obesity due to, among other factors, the low intake of fruits and vegetables. This vital challenge must be tackled because it will aid in preventing undesirable health conditions in society. In Malaysia, there is a research gap on the factors affecting the consumption of fruits and vegetables[6]. These dietary changes lead to a high prevalence of obesity. According to the Malaysian Adults Nutrition Survey (MANS) 2014, the consumption of "processed foods" to which salt and condiments are added, had increased, and it appears among the top ten most consumed foods[7]. The intake of these food groups is positively associated with lignan intake, thus may lower the risk of obesity.

Food processing is known to affect the content of lignans in foods[8] and may influence bioavailability and antioxidants of several compounds[9]. Bioprocessing refers to a group of sustainable technologies for extracting, purifying, and producing food and food ingredients through enzymes and/or microorganisms[10]. Fermentation is one of the bioprocessing methods traditionally applied to extend food shelf-life. 'Tempeh' is a traditional process of fermentation of soybeans, which provides many health benefits, including enhancing digestibility. Tempeh is a precooked fungal fermented bean, or cereal, bound together by the mycelium of a living mould (mostly *Rhizopus* spp.)[11].

Pickles are made by naturally fermenting fruits and vegetables. Pickling is the process of preserving edible products by immersing them in brine containing vinegar (acetic acid) and salt, followed by a heat treatment. When vegetables are brined at salt concentrations of up to about 8 % (w/v) and allowed to ferment naturally, the brine solution supports fermentation by a sequence of different types of microorganisms[12].

Germination is the physiological process through which seeds produce an embryonic root and stem within a specified time frame[13]. It noticeably modifies the seed microstructure, digestibility, and profiles of active compounds[14].

Tea is the most popular beverage globally. Malaysians drink an average of 160 mL of tea per day, making it the second most popular beverage after plain water[7] and the highest contributor to lignan intake in the Dutch population[15]. Tea consumption is linked to positive effects on human health, with polyphenols being the responsible constituents[16].

To properly tackle the challenge of growing obesity in the Malaysian population, a dietary approach based on diets rich in lignans may be a suitable strategy. For it to be successful, an accurate database of lignan content in the food items most consumed by Malaysian is necessary and, at the moment, lacking. In addition, new bioprocessing techniques are gaining popularity worldwide and are commonly used in the Malaysian diet. Studying the effect of those techniques on lignan content may help optimizing the conditions for the highest lignan intake. Thus, the current work aimed to determine the effect of pickling, fermentation, germination on the lignan content of various foods the Malaysian population mostly consumes. In addition, the tea brewing process has been investigated, given its high contribution to lignan intake in the diet. For this purpose, the lignans were extracted and hydrolyzed, and the extracts were analyzed with LC-MS/MS for lignan content and composition.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade acetonitrile (HPLC grade), methanol, acetone, sodium acetate, sodium hydroxide, glacial acetic acid, formic acid, analytical standards of matairesinol, secoisolariciresinol, lariciresinol, pinoresinol, secoisolariciresinol diglucoside and glycocholic acid (glycine-1 ^{13}C, 13C-GA) were purchased from Sigma-Aldrich Chemie NV (Zwijndrecht, Netherlands). For the enzymatic hydrolysis, β-glucuronidase with an activity of ≥ 300,000 units/g solid and a sulfatase activity of ≥ 10,000 units/g solid type H-1 from *Helix pomatia* were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). The *Rhizopus oryzae* fungus for fermentation of flaxseed, mung beans, and soybeans was obtained from Startercultures.eu/starterculturen.nl.

Sample collection

The selection of plant-based foods was based on data from The National Health Morbidity Survey, Malaysia Adult Nutrition Survey (MANS) conducted in 2014[7]. The most consumed plant-based foods (leafy green vegetables, green vegetables, legumes, other types of vegetables, and beverages) with unknown lignan content and composition were selected. Fresh shallot, celery, green Chinese mustard, long bean were purchased at three different locations (fresh products). In addition, for fresh products, a minimum of 100 g or 3 units were sampled at each location: an outlet of a nationwide supermarket chain, a local grocery, and an open-air street market. Flaxseed, mung beans, and soybeans were purchased at the local market. The commercial Malaysian black tea sample used was from the brand "Boh Tea". Flaxseed has high lignan content (3 mg/g)[15], and therefore, it was selected to evaluate lignan extractability.

Pickling, germination, fermentation, and brewing were applied to specific subsets of the samples, as indicated in table S1.

Sample treatments

Celery, green Chinese mustard, and long bean were cut into cubic pieces of 2 cm whereas shallot was sliced into slices of 2 mm of thickness. Then, the samples were freeze-dried using the freeze-dryer (Christ, Alpha 1-4 LD Plus, Salmenkipp, the Netherlands). The final moisture content of freeze-dried samples were between 1-4 %. Subsequently, the samples were stored at -20 ˚C (Liebherr, Comfort, the Netherlands) until further analysis.

Preparation of pickled samples

Shallot, celery, green Chinese mustard, and long bean samples were selected for pickling using a traditional domestic fermentation. Salt (25 g) was dissolved in water (200 mL) and vinegar (200 mL) with a final concentration of 1:16, w/v and mixed (74 ˚C, 15 min). Fresh samples were rinsed with water, cut, and placed in a clear jar (Fido 250 mL clamp jar, Bormioli Rocco, IKEA, Sweeden) with the cooled pickling solution. Then, the samples were stored indoors (10-20 ˚C) for natural pickling. Samples were collected after 5 weeks of pickling.

Preparation of fermented samples

Mung beans and soybeans were fermented following a procedure previously described with some modifications[17]. Samples were rinsed and macerated to water (1:3, w/v, 12h, room temperature (RT)). Following maceration, the samples were drained, dehulled, and split in half. The dehulled samples were placed in boiling water (1:2, w/v, 30 min, 100 ˚C). After boiling, the samples were drained, cooled down (<36 ˚C), and transferred to a container with vinegar (20 mL). Afterwards, the samples were inoculated with the fungi *Rhizopus oyrzea* at the level of 0.1 % (w/w) of boiled samples. Finally, the samples were packed in perforated polyethylene bags, and they were incubated (24-48 h, 25-30 ˚C) or until the formation of a compact white mycelium.

For the preparation of the mixture of mung beans and flaxseed, a similar procedure was followed. In brief, mung beans were rinsed and macerated in water (12h, RT). After removing the excess water, the mung beans were dehulled and split in half, drained again, and boiled in demineralized water (1:2, w/v, 30 min). Subsequently, the flaxseed was rinsed, drained, and boiled in demineralized water (1:2, w/v, 3 min). Flaxseed and mung beans were mixed (1:1, w/w) to form a white layer of compact mycelium around the beans. The sample was let to cool down (36 ˚C). The fermentation followed the same procedure as the fermentation of mung and soybeans described above.

Preparation of germinated samples

Mung beans, soybeans, and flaxseed were selected for the germination process. Firstly, the mung beans and soybeans were rinsed and were macerated in water (1:3, w/v, 12 hours, 20 ˚C). Flaxseed was soaked with water for 1 h. After removing the excess water, the seeds were placed into a container (60 x 150 mm, 20 g) and kept in the dark (RT) for germination. The sprouts were rinsed with water every 12 h to avoid microbial growth. The germination period lasted 7 days, and samples were collected every day. The collected sprouts were freeze-dried and milled using a commercial waring blender (model 34BL 99, Dynamic Corporation, USA) into a fine powder (0.5 mm sieve) and stored in the dark (RT) until further analysis.

Preparation of tea brews

Tea brew was prepared by placing one tea bag in boiling tap water (1:100 , w/v). The tea was infused for different time intervals (2, 5, 7, 10 min), and the various samples were let to cool down before 50 mL of each sample was filtered through a 1.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, USA). For each brewing period, two different tea bags were used. The samples were stored at -20 ˚C until analysis.

Lignan extraction

Fresh, pickled, fermented and germinated samples were freeze-dried, ground and stored at -20 ˚C until analysis. Lignans were extracted according to Milder et al., Norskov et al. and Penalvo et al. with slight modifications (Figure S1)[18-20]. In brief, dry samples (20 mg) were extracted in alkaline conditions (1 mL, 0.3M NaOH in methanol/water, 8/2, v/v) and were spiked with ^{13}C-GA (10 μl, 50 μg/mL). Afterwards, the samples were vortexed for 2 minutes and incubated in a vortex incubator (60 ˚C, 1 h). The samples were cooled, and the pH was adjusted to 5 with glacial acetic acid. The samples were further vortexed for 1 minute prior to centrifugation (4 ˚C, 20800 g, 15 minutes). The supernatant was transferred to a plastic tube and evaporated under a nitrogen flow at 60 ˚C. This extraction was not performed for tea samples. The supernatants from extractions and the tea samples were subjected to hydrolysis by adding 0.6 mL of β-glucuronidase/ sulfatase (2 mg/mL in 50 mM sodium acetate buffer, pH 5) to the dried samples and to brew tea (1 mL) using a vortex incubator (37 ˚C, 16 h). The hydrolysis was stopped by adding 0.5 mL of 0.4 % formic acid, and the samples were centrifuged (4 ˚C, 20800 g, 15 minutes). After centrifugation, 300 μL of acetonitrile was added to the supernatant. Finally, the samples were filtered through a Whatman 0.45 μm PTFE filter (Gelman Sciences, Ann Arbor, USA) and transferred to an amber HPLC vial for LC-MS/MS analysis.

LC-MS/MS Analysis of lignans

Sample analysis was carried with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, a LC-20ADXR solvent delivery module, DGU-20ASR degassing unit, a CTO-20AC column oven, and a FCV-20AH$_2$ valve unit. The samples (5 μL) were injected on an Acquity Premier BEH C18 Column, 1.7 μm, 2.1 x 100 mm BEH,

connected to an Acquity UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm X 5 mm (Waters Chromatography B.V., 4879 AH Etten-Leur, the Netherlands). The flow rate was set at 0.3 mL/min and the column temperature at 40 °C. The mobile phases consisted of 0.1 % formic acid (solvent A), acetonitrile with 0.1 % formic acid (solvent B) with the following elution profile (t in [min]/[%B]): (0.0/5), (0.5/5), (2.0/25), (5.0/50), (7.0/95), (8.5/95), (8.6/5) and (12.5/5). MS data was collected for 12.5 minutes. Negative ionisation mode was used for the MS analysis of all analytes except pinoresinol, which was analyzed in positive mode. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line, and heat block were set at 300 °C, 250 °C, and 400 °C, respectively. The pressure of the collision-induced dissociation gas was 4 kPa, whereas the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL/min, 3 mL/min, and 10 mL/min, respectively. The electrode voltage of Q1 pre-bias (collision cell energy entrance potential), Q2 collision cell (collision energy), Q3 pre-bias (collision cell energy exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized (Table 1) using the support software (LabSolutions, Shimadzu Corporation, Kyoto, Japan). The dwell time ranged from 3 msec to 8 msec for single reaction monitoring (SRM) of the analytes. The signal of the most abundant fragment ion was selected for quantitation.

Method validation

Calibration curves

All standards were dissolved in 100 % acetonitrile and kept at -20 °C. A working solution (50000 ng/mL) consisting of all the lignans and ^{13}C-GA in water/acetonitrile (1:1, v/v) was prepared on the day of the analysis and used for the preparation of the calibration curves. A standard curve in the range of 1000 - 10000 ng/mL (LARI), 12.5 - 10000 ng/mL (MATA), 200 - 5000 ng/mL (PINO); and 125 - 7500 ng/mL (SECO). Calibration curves were obtained by plotting the peak area against the concentration of the calibration standard and consisted 6 to 8 points depending on the analytes. Each calibration standard was injected three times, and the average value of the peak areas was used to construct the calibration curves. The standard curves showed good linearity with regression coefficients not lower than 0.997. In addition, the coefficient of variations (CV %) of calibration curves intra-day was determined.

The limit of quantification (LOQ) and the limit of detection (LOD) were calculated from the standard deviation of the intercept (S) and slope (s) of the calibration curve (LOQ = 10 X S/s and LOD = 3.3 X S/s).

Extraction efficiency

^{13}C-GA was used to evaluate the extraction efficiency of lignans by spiking the plant samples after adding the extraction solvent and the tea samples after filtration with a ^{13}C-GA solution (10 µl, 50 µg/mL). The efficiency was calculated as a concentration of ^{13}C-GA measured by LC-MS/MS compared to the estimated ^{13}C-GA concentration (357.14 ng/mL). Lignan content values were corrected according to the determined extraction efficiency percentages.

Matrix effect

The matrix effect (ME), as an indicator of the ionization suppression or enhancement, was calculated as follows: ME(%)= (B/A), where A is the peak area for each analyte in a standard solution of known concentration and B is the peak area for each analyte spiked in an extracted sample at the same concentration. Samples from various matrices were spiked with a lignan standard solution (10 ul, 2.5 µg/mL) was used to assess the matrix effect.

Statistical analysis

Data were presented as mean ± standard deviation (SD) from duplicate analyses of two independent samples. Results were statistically analyzed among samples using one-way analysis of variance (ANOVA) and Tukey HSD multiple comparison post-hoc test. *P*-values < 0.05 were regarded as statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 25 software (IBM Corp).

RESULTS AND DISCUSSION

Selection of plant food

Eight foods including flaxseed were selected for this study. Plant foods were selected that are most commonly consumed according to the data from The National Health Morbidity Survey, Malaysia Adult Nutrition Survey (MANS) conducted in 2014. The chosen foods were among the ones for which the highest intake (g/day) is reported in each of the following food groups: leafy green vegetables, green vegetables, legumes,

other types of vegetables, and beverages. Pickling, germination, fermentation, and brewing, was applied to specific subsets of the samples as indicated in Table S1.

Chromatography

A mixture of lignan standards (1250 ng/mL) was used to optimise the chromatographic conditions. Good chromatographic separation was achieved for SECO (retention time: 6.2 mins), LARI (6.4 mins), PINO (6.9 mins) and MATA (7.3 mins) (Figure S2). The short analysis time allows the screening of large sample sets. After several injections of the solution with the lignan standard mix, the analysis of blank samples showed no carry-over effects.

Mass spectrometry

The optimisation of the MRM conditions (Table 1) was performed by flow injection analysis of individual lignan standard solutions (20 μg/mL-50 μg/mL). The negative ionisation mode was the most suitable for analysing SECO, LARI and MATA in agreement with other studies [18-20]. However, PINO generated signal only when positive ionisation mode was used. LARI generated lower signal compared to the signal of the other analytes in comparable concentration. This is demonstrated by the different concentration range compared to those for PINO, MATA and SECO. Thus, the positive mode was used for PINO, which was discovered to be different from other studies.

Table 1. Compound-dependent LC-MS/MS parameters

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Q1 Pre Bias (V)	Collision Energy (CE) (V)	Q3 Pre Bias (V)
LARI	359.1000	329.1500	8	-28.0	28.0	15.0
		175.1000	8	-21.0	-21.0	24.0
		159.9000	8	-25.0	-25.0	-32.0
MATA	357.1000	83.0000	8	-18.0	28.0	14.0
		121.9500	8	28.0	35.0	26.0
PINO	358.7000	269.9000	3	-19.0	-23.0	-13.0
		226.9000	3	-11.0	-39.0	-25.0
SECO	361.0000	165.0000	5	27.0	25.0	18.0
		346.0500	5	28.0	21.0	27.0
		121.0500	5	27.0	41.0	12.0
^{13}C-GA	465.3000	75.0000	5	23.0	42.0	27.0
		402.4000	5	23.0	36.0	11.0
		382.5000	5	18.0	41.0	18.0

***** Q1 Pre Bias= collision cell energy entrance potential; CE= collision energy, Q3 Pre Bias= collision cell energy exit potential; LARI= lariciresinol; MATA= matairesinol; PINO= pinoresinol; SECO= secoisolariciresinol; ^{13}C-GA= glycocholic acid (glycine-1 ^{13}C)

Method validation

Quantitation

Calibration curves of lignan standards were used to quantify the concentrations of lignans in the samples and were constructed from calibration points for SECO, PINO, MATA and LARI (Table S2). The calibration curves consisted of 8 points in SECO, PINO and MATA, while LARI consisted 6 points. The results showed an excellent correlation between peak area and concentration of the lignan standard solutions within the used concentration range for each lignan. The LOD was 1.06 ng/mL and LOQ was 3.23 ng/mL, for LARI;, the LOD was 0.26 ng/mL and LOQ was 0.79 ng/mL for MATA; the LOD was 0.13 ng/mL and LOQ was 0.38 ng/mL for PINO; and the LOD was 0.20 ng/mL and LOQ was 0.60 ng/mL for SECO (Table S3).

The coefficient of variations (CV %) of calibration curves intra-day were range of 1.4 - 3.4 % (PINO), 1.9 - 2.6 % (MATA), 4.8 - 6.9 % (SECO) and 2.3 - 2.5 % (LARI) (Table S4).

Extraction Efficiency

The extraction efficiency of ^{13}C-GA varied vary from 70.9 to 108.2 %, and the CV values ranged from 0.3 to 19.9 % (Table S5). The ^{13}C-GA efficiency values were used to normalise the concentrations of the extracted lignans. The findings indicate that the extraction method implies that signal suppression due to matrix effects is the primary source of the deviation from the expected target of 100 %. In general, the acceptable range of extraction efficiency is 70 – 120 %[21].

Matrix effect

An overview of matrix effects (ME) in selected samples is depicted in Table S6. The ME were range of 99.1 – 125.4 % (PINO), 101.1 – 126.6 % (MATA) and 87.8 – 112.9 % (SECO). Our findings denoted a slight MS enhancement (ME>100 %). The result indicated the influence of possible ionization competition between the analyte and components in the sample matrix[22].

Influence of pickling on lignan contents

Figure 1 shows the lignan contents in fresh celery, Chinese mustard, long beans, and shallot and the lignans content after 5 weeks of storage when pickled. The level of lignans in fresh samples ranged from 0.5 to 2.0 mg/100g DW. Only LARI was detected in these samples. LARI in celery showed the most significant decrease (about 86 %) after pickling compared to the other products. Moreover, the LARI content significantly dropped by approximately 80 % in the pickled compared to the fresh Chinese mustard. No LARI as detected in shallot and long beans after pickling.

So far, little is known about the changes in the lignan content during the pickling process. One study has reported that only PINO was identified in pickled olive[23]. Nevertheless, a plethora of studies found that phenolic content decreased after pickling. Brining with salt and pickling with vinegar over three weeks led to a decline of up to 69 % in the phenolic contents of vegetables[24].

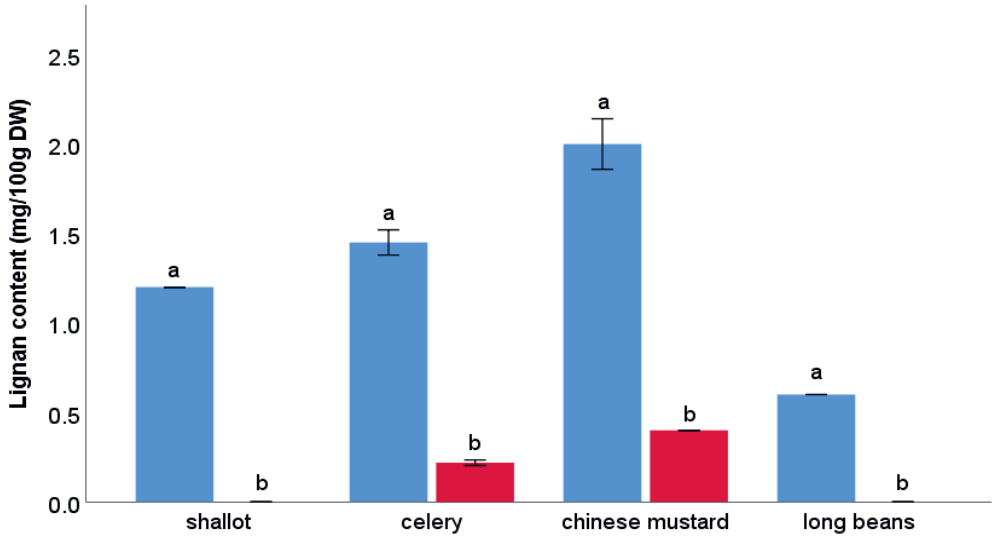


Figure 1. Lignan (LARI) content of selected foods before (blue bars) and after 5 weeks of pickling (red bars) (mean ± SD, n=2). In each sample type, different lowercase letters indicate significantly different lignan content between fresh samples and samples pickled. LARI= lariciresinol

The decline of lignan contents in our findings was in line with the reported decline in phenolics described above. Given the relatively high thermo-stability of lignans[25], its decline in the plant tissues could partially be explained by either degradation by endogenous polyphenol oxidase[26] or leaching out to the brine[27] after prolonged storage in acid and salt solution. Further investigation on lignan stability in acid and salt solution is needed to fully understand the mechanism of lignan decrease in pickled vegetables. Nevertheless, the observation that lignan content could decrease during the pickling process is important when developing products high in lignans.

Influence of fermentation on lignan contents

A mixture of flaxseed and mung beans has been used since the flaxseed seed coat's polysaccharides at the epidermal cell layer becomes viscous with water[28] and forms mucilage, a gel-like layer that forms when the seed is soaked in water. The mucilage halted the formation of a compact white mycelium; hence the fermentation was unsuccessful. Therefore, we used mung beans as a supplementary ingredient with a ratio of 1:1 to reduce the mucilage viscosity and form a white layer of compact mycelium around beans, binding them into a dense, firm cake.

The mixture of fermented flaxseed and mung beans presented by far the highest lignan content among the analyzed samples (799.9 ± 67.4 mg/100g DW, Table 2). This represents a 37 % increase compared to the unfermented mixture. Moreover, both fermented and unfermented soybeans exhibited 0.3 mg/100g DW, being the lowest lignans content of the analyzed foods.

Table 2. Lignan content (mg/100g DW) in unfermented and fermented samples (mean + SD, n=2)

Sample	MATA	SECO	PINO	total lignans
Soybeans				
unfermented		0.3 ± 0.1		0.3 ± 0.1
fermented		0.3 ± 0.0		0.3± 0.0
mung beans				
unfermented		0.4 ± 0.0		0.4 ± 0.0
fermented		0.4 ± 0.1		0.4 ± 0.1
Flaxseed + mung beans				
unfermented	0.1 ± 0.1	501.0 ± 134.4	0.4 ± 0.1	501.4 ± 134.6
fermented	0.1 ± 0.1	799.1 ± 67.3*	0.8 ± 0.1*	799.9 ± 67.4*

*indicates a significant difference in lignan content between the fermented and the unfermented sample. MATA= matairesinol; SECO= secoisolariciresinol; PINO= pinoresinol

MATA, SECO, and PINO were detected in the mixture of both unfermented and fermented flaxseed and mung beans samples. SECO represented the major contributor of total lignans. Lignan levels were not significantly different in the fermented and unfermented soybean and mung bean samples.

To the best of our knowledge, no data are available on the changes in the lignan content during fermentation using *Rhizopus oyrzae*. Nevertheless, several studies found that fermentation influenced phenolic content. A study proved the fermentation by *Rhizopus oryzae* significantly increased phenolic acids content from rice bran[29].

In grains, bioactive compounds, including lignans, are mainly located in the outer parts of the grain[30]. Prior to the fermentation of soybeans and mung beans, the hulls were removed, leaving the relatively lignan-depleted cotyledons, which may explain the low lignan content of unfermented and fermented beans.

Conversely, flaxseed was not dehulled; therefore, the lignans were higher in unfermented and fermented flaxseed. Endogenous enzymes and indigenous microbes are found concentrated in the outer layers of grains[31], and the microbes frequently express significant hydrolytic enzyme activities. Moreover, these cell wall-degrading enzymes' actions produced during fermentation may cause an increase in the release of phenolic compounds[32].

All in all, these findings demonstrate that fermentation with filamentous fungi could be considered a potential process for increasing the release of lignans, thereby contributing to the production of food products with added value.

Influence of germination on lignan contents

Figure 2 illustrates the change in lignan contents during germination of mung beans, soybeans, and flaxseed. SECO, MATA, PINO, and LARI were detected in all samples. SECO was the most abundant lignan in flaxseed. Overall, the lignan content increased during germination and reached its peak on day 6. Subsequently, the lignan content decreased at day 7 of germination. Germinated flaxseed showed the highest lignan content, which reached approximately 1500 mg/100g DW, while germinated mung beans showed the lowest lignan content (0.3 mg/100g DW). As far as we know, these are the first data on the effect of germination on soybean and mung beans. The highest level of lignans was found on day 6 for all germinated samples.

A study showed that the SECO content was increased by 4.5-fold in 8-day germinated flaxseed compared to ungerminated flaxseed[33]. Similarly, Makowska demonstrated that the total amount of lignans in triticale variety of Madilo grain increased by about 17 % – 32 % in germinated grain over 72 h[34]. Moreover, another study reported that lignan content in germinated rye grain was 80 % higher than in native grain[35].

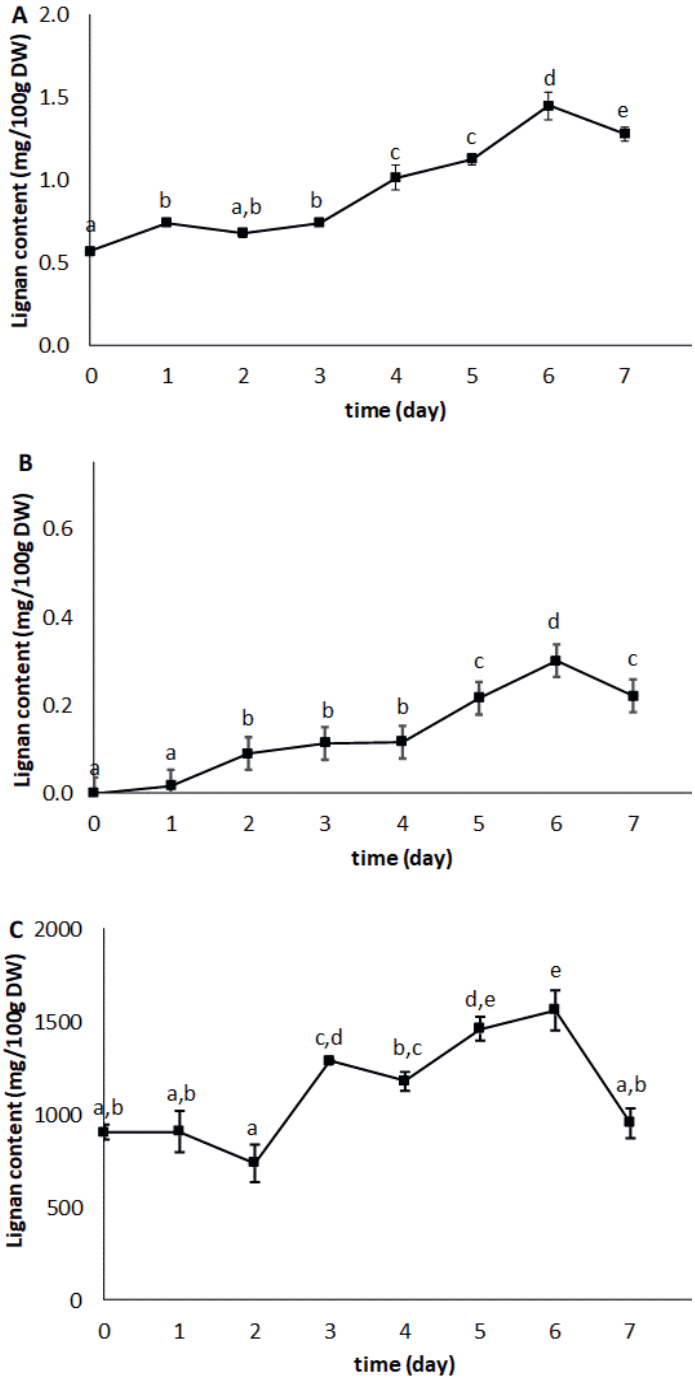


Figure 2. Lignan content during germination of A) soybean, B) mung bean, and C) flaxseed (mean ± SD, n=2). Different lowercase letters indicate a significant difference in lignan content.

The germination process is known to activate endogenous enzymes in seeds, resulting in noteworthy changes in the lignan content[36]. The biosynthesis of lignans begins with two coniferyl alcohol (CA) molecules as precursors[37]. The enzymes phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), dirigent-protein oxidase (DPO), pinoresinol-lariciresinol reductase (PLR), and glucosyl transferase (GT) are involved in the biosynthesis of lignans. PAL, and CHS enzymes are up-regulated during the germination of flaxseeds; hence it might partly explain the accumulation of lignans[33].

Influences of black tea brewing on lignan concentration

Figure 3 illustrates the content of total lignans in tea brews over four different brewing times (2, 5, 7, 10 minutes). As expected, the content of total lignans in the brew increased with prolonged brewing. The total lignan concentration was around 0.03 mg/100 mL at 2 minutes and increased more than two-fold at 10 minutes of the brewing (approximately 0.1 mg/100 mL). The lignan concentration increased 5 and 7 minutes of brewing, but no significant difference was observed between 7 and 10 minutes.

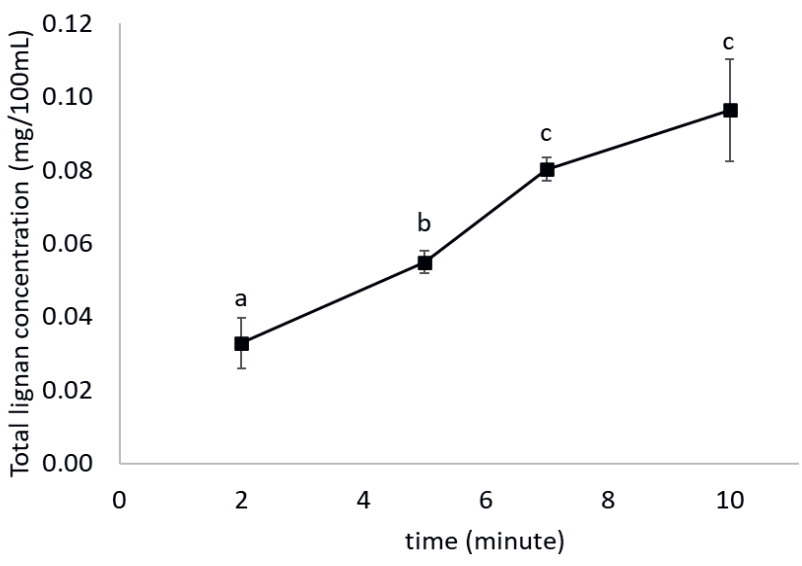


Figure 3. Total lignan concentration during the brewing time of black tea (mean ± SD, n=2 of independent brewing). Different lowercase letters indicate a significant difference in total lignan concentration.

PINO was the most abundant lignan detected in our tea sample. The presence of PINO in tea brew is in agreement with Milder et al.[19]. Moreover, a comprehensive

study of plant foods commonly consumed in the Netherlands reported that the lignan concentration in black tea brews ranged between 0.06 - 0.08 mg/100 mL after five minutes of brewing[15]. These two aforementioned studies are in agreement with our findings. Despite no report on the influence of tea brewing time on the lignan concentration could be found, a study on flavonoids and other polyphenols of commercial tea bag products (typical of those used in the UK, US, continental Europe, and the Middle East) reported that total phenolics, flavonoids, catechins, and theaflavins in brews were up to 35-55 % of the total available phenolics and that longer brewing times gave higher polyphenol concentration in tea brew[38].

In addition, it has been reported that tea infusion time and temperature both contribute to the concentration of bioactive compounds in the tea brew[39]. A study on tea brewing parameters showed that the longer the brewing time, the higher the concentration of total polyphenols[40]. The aforementioned studies corroborate our findings, revealing a continuous increase in lignan concentration with prolonged brewing. A higher concentration content might be achieved when brewing tea for longer than 10 minutes.

Water temperature used to make the infusion may affect the content in thermo-labile compounds. However, heating at 100 °C for 10 to 40 min has been reported not to change the lignan concentration, indicating that brewing temperature may have a modest effect on lignan content of brewed tea[25].

CONCLUSION

In summary, fermentation and germination increased the lignan content in food products, as did a prolonged brewing for tea. Further research into the causes of lignan accumulation in fermented foods it is necessary to establish the optimal design of fermentation conditions. The data produced widen the range of known lignan content data in foods. In addition, the selection of foods was based on data from the Malaysia Adult Nutrition Survey with unknown lignan content to investigate whether these foods could be a potential source of lignan. On top of that, this study provided insight into how different processing methods, especially bioprocessing techniques, can affect lignan content, thus providing a basis for further investigations.

Globalization is affecting Malaysia, causing a change from traditional diets and lifestyles to more Westernized eating patterns, leading to an increase in obesity and non-communicable diseases. The health of the Malaysian population is likely to deteriorate further as the transition to a more developed economy has been fast over the last decade. Therefore, the bioprocessing approaches we used may enrich foods with

lignans, increasing total lignan intake in the general population with potential beneficial effects on health. This discovery would encourage the consumption of fermented and germinated food as a better source of lignans for a healthy diet. Moreover, we could promote how to brew tea to get an optimal lignan content. These approaches are low-cost yet time-saving, and the food can be simply prepared at home.

Other processing approaches to increase lignan content of foods need to be investigated. A national strategy needs to be in place to help prevent, treat and manage obesity and non-communicable diseases in Malaysia, despite the fact that research on these areas is very much in its infancy.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Umi Kalsum Hussain Zaki: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Christos Fryganas:** Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Laura Trijsburg:** Writing – review & editing. **Edith Feskens:** Writing – review & editing. **Edoardo Capuano:** Supervision, Conceptualization, Investigation, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENTS

The study was supported by the Food Quality & Design Department, Wageningen University & Research, the Netherlands, and funded by Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

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SUPPLEMENTARY MATERIAL

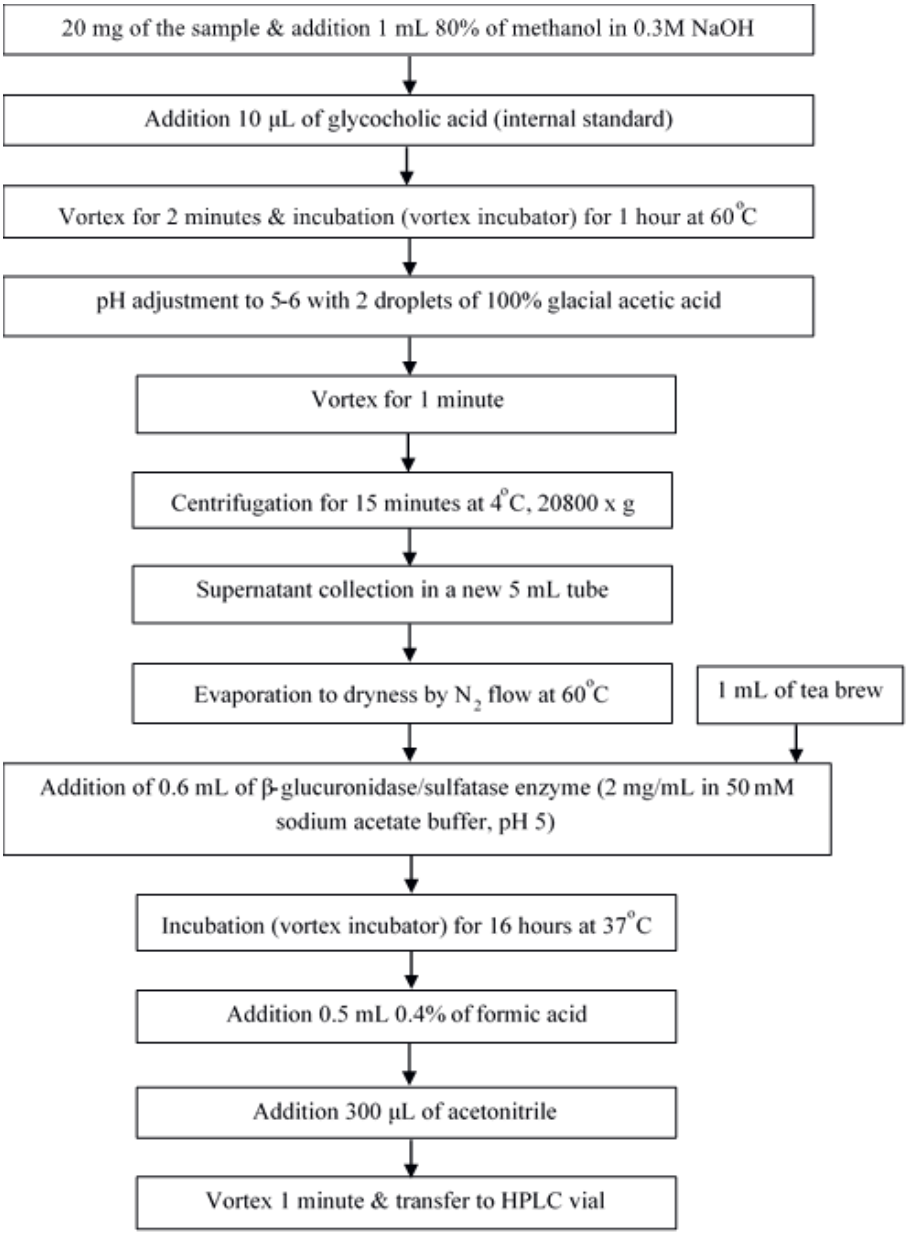

Figure S1. Flowchart of the lignan extraction from food samples

Table S1. Estimated mean intake, reason for selection, and processing conditions applied to the foods selected for this study

Category	Food item	Estimated mean intake (g/day)*	Reason for selection	Processing method applied in this study
Leafy green vegetables	Celery (*Apium graveolens*)	66	Celery is the highest consumed leafy vegetable in Malaysia.	Pickling
Brassica vegetable	Chinese mustard (*Brassicca juncea*)	19	Chinese mustard is Malaysia's most consumed brassica vegetable and cultivated plant.	Pickling
Other types of legumes	Long beans (*Vigna unguiculata*)	11	Long beans is the most consumed food in the legume group and the second-most cultivated plant in Malaysia.	Pickling
Other vegetable groups	Shallot (*Allium cepa*)	10	A family of garlic and onion. The most vegetables consumed in Malaysia among other vegetable groups	Pickling
Other vegetable groups	Bean sprout (mung beans) (*Vigna radiata)*	7	Bean sprout is the 2nd most consumed of 'other vegetable group' after shallot in Malaysia. It was grown by sprouting mung beans.	Fermentation Germination

Legumes and product	soybean (fermented soybean) (*Glycine max)*	4	Soybean products are the most consumed among the 'legumes and product' group in Malaysia	Fermentation Germination
Seed	flaxseed		As a reference sample because it has the highest lignan contents among other foods	Fermentation Germination
Beverages	tea	160**	The most consumed beverages after plain water In Malaysia.	Brewing

* from The National Health Morbidity Survey, Malaysia Adult Nutrition Survey (MANS) 2014
** unit= mL/day

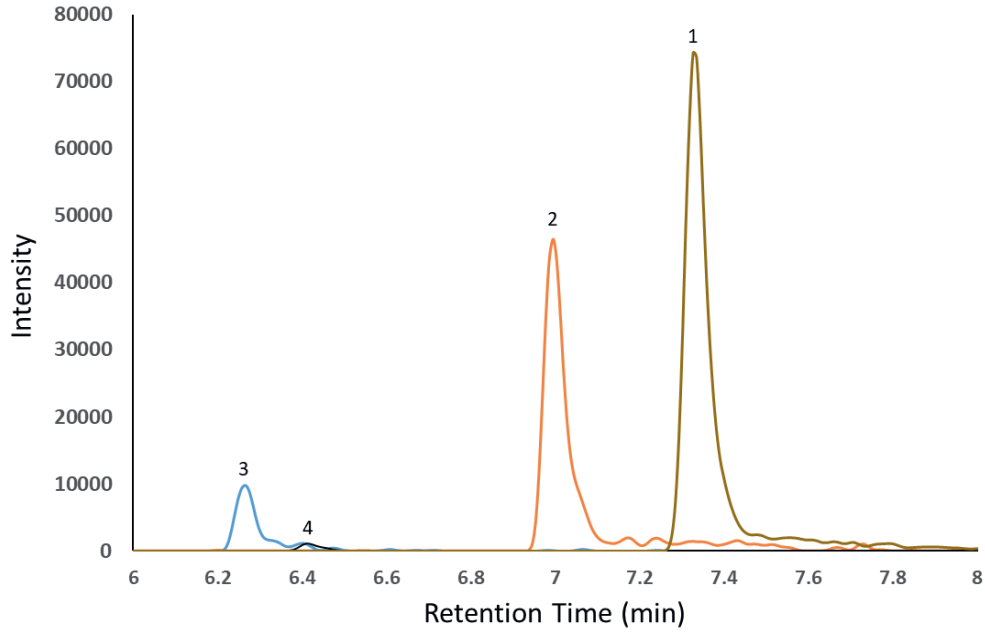

Figure S2: Typical chromatogram of lignan standard mix at 1250 ng/mL. Peaks: 1, matairesinol, RT 7.3 min; 2, pinoresinol, RT 6.9 min; 3, secoisolariciresinol, RT 6.2 min; 4, lariciresinol, RT 6.4 min.

Table S2: Information on calibration curves constructed with lignan standards in solvent

Compound	Linear range (ng/mL)	Linear regression equation	Correlation coefficient (r^2)
LARI	1000-10000	y= 1.1215x – 407.37	0.998
MATA	200-10000	y= 2000000x+ 537260	0.998
PINO	12.5 - 5000	y= 190120x + 3124.9	0.999
SECO	125-7500	y= 43390x + 27.169	0.998

y= peak area, x= concentration

Table S3: Limits of Detection (LODs) and Limits of Quantification (LOQs) for lignans

Compound	LOD (ng/mL)	LOQ (ng/mL)
LARI	1.06	3.23
MATA	0.26	0.79
PINO	0.13	0.38
SECO	0.20	0.60

Table S4: Coefficients of intra-day variations (CV %) of calibration curves

	CV (%)		
Compound	Low (200 ng/mL)	Medium (1000 ng/mL)	High (5000 ng/mL)
LARI	-	2.3	2.5
MATA	2.6	2.1	1.9
PINO	2.4	1.4	3.4
SECO	6.9	5.2	4.8

Table S5: Extraction efficiency (EE %) and coefficients of variations (CV %) of ^{13}C-GA in lignan extracts

Food sample	Extraction efficiency (EE %)	Coefficients of variations (CV %)
Pickling	95.3 – 108.2	
Fresh sample	105.7	7.6 - 18.8
Pickled shallot	81.5	15.1
Pickled celery	101.5	3.5
Pickled chinese mustard	74.9	15.0
Pickled long beans		4.0
Fermentation	73.8 – 87.8	
Unfermented soybean, mung bean, flaxseed	73.2 – 89.2	3.5 – 12.5
Fermented soybean, mung bean, flaxseed		7.2 – 15.0
Germination	70.9 – 100.2	
Mung bean	82.4 – 83.2	4.9 – 19.9
Soybean	79.4 – 80.5	0.5 – 14.9
Flaxseed		0.3 – 11.9
Tea brew	72.7 – 107.1	1.8 – 7.5

Table S6: Values of matrix effect determined for lignans

Sample	Matrix effect (ME, %)		
	SECO	PINO	MATA
Unfermented flaxseed	112.9	121.2	117.1
Fermented flaxseed	118.0	116.0	101.1
Unfermented soybean	103.7	99.1	113.1
Fermented soybean	86.0	125.4	107.6
Unfermented mung bean	111.2	110.8	126.6
Tea brew	87.8	101.2	114.2

CHAPTER 4

In-vitro gastrointestinal bioaccessibility and colonic fermentation of lignans from fresh, fermented, and germinated flaxseed

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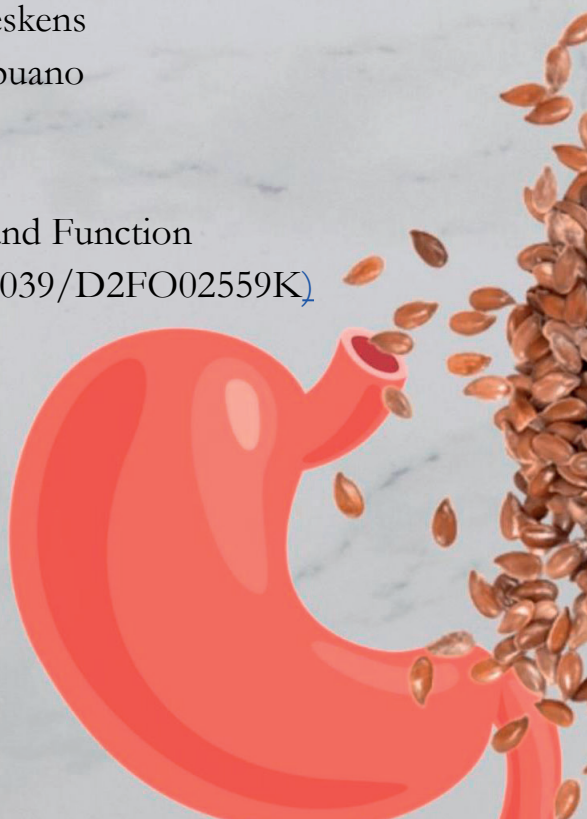
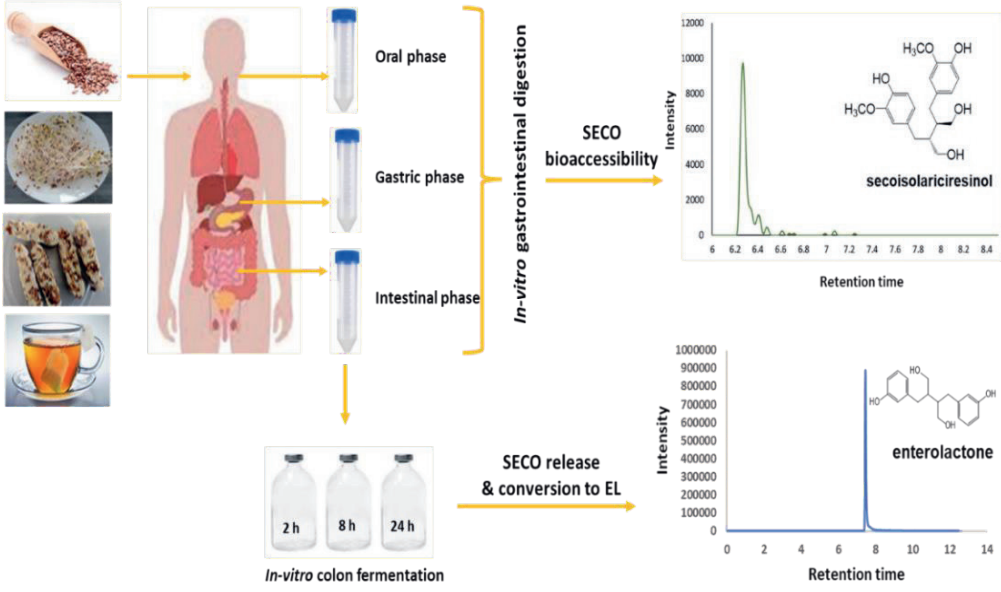
Published in Food and Function
(DOI: https://doi.org/10.1039/D2FO02559K)




Oral phase
Gastric phase
Intestinal phase
In-vitro gastrointestinal digestion
SECO bioaccessibility
Intensity
Retention time
secoisolariciresinol
2 h
8 h
24 h
In-vitro colon fermentation
SECO release & conversion to EL
enterolactone

ABSTRACT

This research assessed the influence of fermentation and germination as well as of particle size on lignan bioaccessibility from flaxseed by simulated *in-vitro* gastrointestinal digestion. *In- vitro* simulated colonic fermentation was used to study the release and conversion of lignans in enterolignans. In addition, tea was included as a representative sample to investigate the stability of lignans in the gastrointestinal tract. Only secoisolariciresinol (SECO) was detected in flaxseed samples. SECO bioaccessibility in fermented flaxseed was the highest among all the matrices but limited to ≈ 1 % ($P < 0.001$). Lignan bioaccessibility was significantly influenced by particle size too ($P < 0.001$ for both). In the colon fermented flaxseed produced the highest SECO release among all flaxseed samples, (≈ 65 %), and the highest conversion of enterolignan (≈ 1.0 %) whereas the conversion of lignans in tea brew was relatively high (≈ 15 %). Lignan conversion varies greatly among donors due to inter-individual differences in microbiota activity. Food fermentation could be a viable strategy for increasing lignan release and conversion to enterolignan.

Keywords: lignans, bioaccessibility, fermentation, germination, brewing, *in-vitro* digestion

INTRODUCTION

Lignans are phenolic compounds that belong to the class of non-flavonoids polyphenols that can exert both oestrogenic and anti-oestrogenic effects[1]. Some lignans can be metabolized into enterolignans, i.e. enterodiol and enterolactone, by the human intestinal microbiota[2] through deglycosylation, oxidation, and demethylation[3]. These metabolites have been reported to have beneficial health effects. Epidemiologic studies have shown that the intake of lignan-rich foods aid in lowering the risk of several cancers[4] and cardiovascular diseases[5], demonstrating antioxidant properties as well as estrogenic activity[6]. Differences in microbiota composition may lead to differences in absorption and excretion patterns of enterolignans[2].

It is widely known that not all of the constituents in a food matrix are fully bioavailable. The fraction of a nutrient or non-nutrient available to the human body for physiological functions and/or storage is known as bioavailability[7]. As a result, investigating the bioavailability of a food component is essential to predict its biological activity[8].

Food processing affects the lignan content of foods[9] and the levels and bioavailability of bioactive compounds[10]. Reducing particle size of plant tissue for instance, increases the fraction of broken cells thus increasing the fraction of potentially bioavailable intracellular compounds[11]. However, most lignans, as phenolic compounds in general, are covalently or physically bound to plant cell walls and thus have low bioavailbility[12]. As a result, enzymatic treatments, and fermentation processes targeting bran structure have been investigated to improve the bioavailability of lignans[13]. In addition, germination, and fermentation, are also reported to affect lignan content[14,15], but how these processing methods affect the bioavailability of lignans is unknown.

Since animal and human studies are time-consuming and expensive and may raise ethical concerns, *in-vitro* approaches that predict the behaviour of phytochemicals during GI digestion have been developed. This approach enables the screening of a relatively large number of samples and/or conditions to study the separate and combined effects of each phase of digestion on the release and availability of phytochemicals, which would be impossible with *in-vivo* approaches[8]. Bioaccessibility is the fraction of a potentially available compound released from its matrix in the gastrointestinal (GI) tract and thus becomes available for intestinal absoption[16]. Furthermore, it has been demonstrated that bioaccessibility as determined using *in-vitro* models can be well associated with bioavailability determined in human studies and animal models[17].

To date, there are relatively few reports on the effect of various processing techniques on on the bioaccessibility of lignans from food. Thus, the present study investigated lignan bioaccessibility from fresh, fermented, germinated flaxseed, and the effect of particle size using simulated *in-vitro* digestion models. In addition, tea was used to study the stability of lignans under *in-vitro* simulated gastrointestinal conditions. Moreover, the release of lignans and their conversion in enterolignans was investigated through *in-vitro* colonic fermentation with human fecal inoculums.

MATERIAL AND METHODS

Chemicals and reagents

Porcine pepsin (P6887), porcine pancreatin (P1750, 4× USP), and porcine bile salt preparation (B8631) were purchased from Sigma-Aldrich (Merck KGaA, Germany). KCl, KH_2PO_4, NaCl, $MgCl_2 \cdot (H_2O)_6$, and $CaCl_2 \cdot (H_2O)_2$, and pure ethanol were purchased from VWR International B.V. (Netherlands). KH_2PO_4, NaCl, $(NH_4)_2CO_3$, $NaHCO_3$, NaOH, HCl, and Tween 80 were purchased from Sigma-Aldrich Chemie B.V. (Netherlands), as well as the yeast extract, peptone, mucine, and L-cysteine HCl.

Acetonitrile, methanol, sodium acetate, sodium hydroxide, glacial acetic acid, formic acid, pure standards of pinoresinol (PINO), matairesinol (MATA), secoisolariciresinol (SECO), enterolactone (EL), enterodiol (END) and glycocholic acid (glycine-1 ^{13}C, 13C-GA) were purchased from Sigma-Aldrich Chemie NV (Zwijndrecht, Netherlands). For the enzymatic hydrolysis, β-glucuronidase with an activity of ≥ 300,000 units/g solid and a sulfatase activity of ≥10,000 units/g solid type H-1 from *Helix pomatia* were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). The *Rhizopus oryzae* fungus for fermentation of flaxseed, mung beans, and soybeans was obtained from Startercultures.eu/starterculturen.nl. All of the chemicals used are of analytical and chromatographic grade.

Preparation of samples

Preparation of fermented flaxseed

Flaxseed was fermented following a procedure previously described with some modifications[18]. Flaxseed produces mucilage; a gel-like layer formed when the seed is soaked in water. Water causes the polysaccharide in the seed coat's epidermal cell layer to become viscous[19]. The mucilage halted the formation of a compact white mycelium. Therefore, mung beans were used as a supplementary ingredient with a ratio of 1:1 to

form a white layer of compact mycelium around the beans and bind them into a dense, firm cake. Mung beans were rinsed and soaked in water for 12h, then were dehulled, split in half, and boiled in demineralized water (1:2, w/v) for 30 minutes. Subsequently, the flaxseed was rinsed, drained, and boiled for 3 minutes. Then, flaxseed and mung beans were mixed (1:1, w/w) to surround the beans with a white layer of compact mycelium. The sample was let to cool down to below 36 ˚C and transferred to a container with vinegar (20 mL). Next, the samples were inoculated with the fungi *Rhizopus oryzea* at the level of 0.1 % (w/w) of boiled samples. Finally, the samples were placed in perforated polyethylene bags and incubated for 24 - 48 hours at 25 - 30 °C, or until a compact white mycelium formed.

Preparation of germinated flaxseed

Flaxseed was soaked with water for 1 h. The seeds were placed in a container (60 x 150 mm), 20 g/cup, and kept in the dark and at room temperature (RT) for germination. The sprouts were rinsed with water every 12 hours to prevent microbial growth. The germination process took 6 days.

Preparation of tea brews

To make the tea brew, one tea bag was placed in boiling tap water (100 mL/g tea leaves). After infusing the tea for 10 minutes, the various samples were allowed to cool before 50 mL of each sample was filtered through a 1.2 m Acrodisc filter (Gelman Sciences, Ann Arbor, USA). The samples were kept at -20 °C until analysis.

Sample milling

The collected fresh, fermented, and germinated flaxseed were milled to obtain a coarse (> 2.5 mm mesh/sieve) and fine (< 0.5 mm mesh/sieve) fraction, freeze-dried and stored at -20 ˚C until further analysis.

Simulated *in-vitro* gastrointestinal digestion

All samples were digested using a static *in-vitro* digestion system consisting of a simulated oral, gastric, and intestinal phase, with modifications[20,21]. The compositions (%, w/w) of the simulated salivary fluid (SSF), simulated gastric fluid (SGF, pH 3.0 ± 0.05), and simulated intestinal fluid (SIF, pH 7.0 ± 0.05) were as reported[20].

For the oral phase, flaxseed samples (5 g) or tea brew (5 mL) were mixed with SSF electrolyte stock solution (3.5 mL, 37 ˚C), $CaCl_2$ (0.025 mL, 0.3 M), and Milli-Q water (1.475 mL) (Veolia water, Veolia Water Solutions, and Technologies Netherlands B.V.). The final volume was 10 mL.

Subsequently, an oral bolus (10 mL) was mixed with SGF (7.5 mL, 37 ˚C) to start the gastric phase. The pH was adjusted to 3 with HCl (0.2 mL, 1 M). Then, $CaCl_2$ (0.005 mL, 0.3 M) was added. Finally, the Milli-Q water was added to obtain the final 20 mL (approximately 0.698 mL). Porcine pepsin stock solutions (1.6 mL, 25 000 units/mL, 37 ˚C) (2000 units/mL in final chyme) was added in SGF. Then, the chyme was shaken for 2 h (37 ˚C). At the end of the gastric digestion step, NaOH (0.15 mL, 1 M) was added to reach pH 7.0, inhibiting the gastric enzyme activity.

Thereafter, NaOH (0.15 mL, 1 M) was added to the 20 mL of gastric chyme to reach pH 7.0. Subsequently, SIF (11 mL, 37 ˚C), fresh bile stock solution (2.5 mL, to reach a final concentration of 10 mM in the intestinal chyme), $CaCl_2$ (0.04 mL, 0.3 M) were added. Then, Milli-Q water (1.31 mL) water was added. Finally, a pancreatin solution (5 mL, 1600 U/mL of amylase activity) was added to reach a final volume of 40 mL. At the end of the intestinal step, samples were incubated for 2 hours (37 ˚C) on a rotating device.

In parallel, control samples with no digestive enzymes added were tested to see if these enzymes affect lignan bioaccessibility. Milli-Q water was filled to replace the enzyme solutions. All gastric and intestinal digests were centrifuged (4 ˚C, 20000 g, 10 minutes) to stop the enzymatic reaction. Supernatants (5 mL) were collected and filtered through a 0.22 µm membrane filter (Phenomenex, Netherlands) for the lignan analysis. Then, the remaining pre-digested samples were freeze-dried for further use in *in-vitro* colonic fermentation.

The bioaccessibility of lignans was defined as the content of lignans that were detected in the supernatants after digestion, divided by the total lignans initially present in the samples before digestion.

$$\text{Bioaccessibility (\%)} = \frac{\text{Lignans in the supernatant after digestion, µg}}{\text{Lignans in the samples before digestion, µg}} \text{ X } 100$$

In-vitro colonic fermentation

Fresh fecal samples were donated by three healthy adults (one Malay and two Dutch, aged 25 − 40 years old, with a body mass index (BMI) of 18.5−23.9), who declared no smoking and no antibiotic consumption for 6 months before the beginning of the study. The fecal samples were prepared according to protocols previously reported, with modifications[22, 23]. Briefly, feces (20.0 g) of feces were dissolved in anaerobic phosphate buffer (100 mL) before homogenization in a stomacher bag. The phosphate buffer contained 8.8 g/L K_2HPO_4, 6.8 g/L of KH_2PO_4, and 0.1 g/L of sodium thioglucolate. The resulting fecal suspension was filtered and considered as the fecal microbiota supernatant. The colon medium, consisting of 5.22 g/L K_2HPO_4, 16.32 g/L KH_2PO_4, 2.0 g/L $NaHCO_3$, 2.0 g/L yeast extract, 2.0 g/L peptone, 1.0 g/L mucine, 0.5 g/L L-cysteine HCl, and 2.0 mL/L tween 80. Both phosphate buffer and colon medium were flushed with nitrogen (30 minutes) before autoclaving.

Fecal supernatant (7 mL) was added to a penicillin bottle containing a buffered colon medium (43 mL). Then, pre-digested fresh, fermented, or germinated flaxseed (1 g) or tea sample (10 mL) was added, and sterile water was added to make up the mixture of fecal supernatant and the colon medium of 70 mL. The fermentation started with continuous shaking (60 rpm, 37 ˚C) under anaerobic conditions. In parallel, one control without the fecal supernatant was included to determine the release of lignans from the food matrix. All incubations were duplicated, and supernatants were collected in time intervals (2, 8, and 24 h). All supernatants were centrifuged immediately when sampled and stored at -20 ˚C until further use. For the *in-vitro* colonic fermentation, only the pre-digested samples with fine particle size were used. Two replicates were performed of two independent samples.

The release of SECO was defined as the content of SECO detected in the supernatants after fermentation, divided by the total SECO present in the pre-digested samples after *in-vitro* gastrointestinal digestion. Then, the conversion yield was defined as the content of enterolignans that were detected in the supernatants after colonic fermentation, divided by the total lignans initially present in the pre-digested samples after *in-vitro* gastrointestinal digestion.

$$\text{Release of SECO (\%)} = \frac{\text{SECO in the supernatant after colonic fermentation, } \mu g}{\text{SECO in pre-digested supernatant after gastrointestinal digestion, } \mu g} \text{ X } 100$$

$$\text{Conversion yield (\%)} = \frac{\text{Enterolignans in the supernatant after colonic fermentation, μg}}{\text{Lignans in pre-digested supernatant after gastrointestinal digestion, μg}} \text{ X } 100$$

Lignan extraction

Lignans were extracted using alkaline hydrolysis as suggested by Peñalvo et al., Milder et al., and Nørskov et al. with slight modifications[24-26]. In brief, 20 mg of coarse and fine fresh, fermented, and germinated flaxseed were extracted in alkaline conditions (1 mL, 0.3M NaOH in methanol/water, 8/2, v/v) at 60 ˚C for 1 h. Samples were centrifuged, and the supernatants were transferred to plastic tubes and evaporated under N_2 flow at 60 ˚C. The extracts from pre-digestion and *in-vitro* fermentation treatments were subjected to hydrolysis by adding 0.6 mL of β-glucuronidase/ sulfatase (2 mg/mL in 50 mM sodium acetate buffer, pH 5) and to brew tea (1 mL, without alkaline extraction) using a vortex incubator (37 ˚C,16 h). The hydrolysis was stopped by adding formic acid (0.5 mL, 0.4 %), and the extracts were centrifuged. Subsequently, 300 μL of acetonitrile was added, and the samples were transferred to an HPLC vial for LC-MS/MS analysis.

LC-MS/MS analysis of lignans

A Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) was used, coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). 5 μL of samples were injected into an Acquity Premier BEH C18 Column, 1.7 μm, 2.1 x 100 mm BEH (Waters Chromatography B.V., 4879 AH Etten-Leur, the Netherlands). The flow rate was set at 0.3 mL/min and the column temperature at 40 °C. The mobile phases consisted of 0.1 % formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B) with the following elution profile (t in [min]/[%B]): (0.0/15), (9.0/75), (11.0/75), (13.0/15), (18.0/15). MS data was collected for 18 minutes.

All analytes were analyzed in positive ionisation mode. The turbo ion-spray ionization voltage was 4.0 kV. The electrospray ionization probe, desolvation line, and heat block were all set to 300, 250, and 400 degrees Celsius, respectively. The collision-induced dissociation gas had a pressure of 4 kPa, and the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL/min, 3 mL/min, and 10 mL/min, respectively. The electrode voltage of Q1 pre-bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre-bias (collision cell energy exit

potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized using the support software (Shimadzu Corporation, Kyoto, Japan). The dwell time ranged from 11 msec to 16 msec for the analytes for single reaction monitoring (SRM). The most abundant fragment ions for SECO, 13C-GA, PINO, MATA, EL, ENT were 363.2->137.05 (collision energy:-24V), 467.20->413.3 (collision energy:-18V), 359.2->341.15 (collision energy:-9V), 359.2->137.10 (collision energy:-27V), 299.2->133.0 (collision energy:-16V), 303.0->107.0 (collision energy:-25V) respectively, and therefore were selected for quantitation. The representative chromatograms of fresh flaxseed, intestinal phase of the simulated *in-vitro* digestion, 2h and 24h of *in-vitro* simulated colonic fermentation of fresh flaxseed were provided in the Figure S1.

Method validation

Calibration curves

All standards were dissolved in 100 % acetonitrile and kept at −20 ˚C. A working solution (50 μg/mL) consisting of all the lignans and ^{13}C-GA in water/acetonitrile (1:1, v/v) was prepared on the day of the analysis and used for the preparation of the calibration curves (12.5 - 2500 ng/mL).

Matrix effect

The matrix effect (ME) was calculated as an indicator of ionization suppression or enhancement. The known amounts of lignan standard solution (10 ul, 2.5 mg/L) were spiked to tea and digested supernatants (method without extraction), pre-digested tea, fresh, germinated, and fermented flaxseed (total method). For pre-digested fresh, germinated, and fermented flaxseed, the standards were added immediately after the extraction solvent; and, for tea and digested supernatants, immediately before the addition of β-glucuronidase/ sulfatase was added. SECO, PINO, MATA, and enterolactone recoveries were relatively high (111- 134 %). The results showed that the extraction method effectively extracts the analytes from all samples.

Statistical analysis

Data of bioaccessibility are presented as mean ± standard deviation (SD) from duplicate analyses of two independent digestions. Data of release of SECO during *in-vitro* colonic fermentation, and conversion into enterolignans are averages from 3 donors. Each *in-*

vitro fermentation was performed in duplicate. One-way analysis of variance (ANOVA) was used to assess the significant differences in the bioaccessibility of fresh, fermented, and germinated flaxseed and tea brew among different digestion phases. An additional ANOVA was used to test the differences in bioaccessibility values obtained with or without the inclusion of digestive enzymes in the simulated *in-vitro* digestion. Two-way ANOVA followed by Tukey HSD multiple comparison tests was used to test the effect of type of processing (fresh, fermented and germinated flaxseed), particle size (fine and coarse) and their interaction on lignan bioaccessibility. Pearson's correlation was used to identify the correlation between the amount of SECO released and its conversion to EL by correlating each donor's data at all fermentation times. Then, a repeated measure ANOVA followed by the Tukey HSD multiple comparison post-hoc tests was applied to test the difference in SECO release and conversion yield into enterolignans during *in-vitro* colonic fermentation of fresh, germinated and fermented flaxseed and tea brew among 3 donors. An additional repeated measure ANOVA followed by the Tukey HSD multiple comparison post-hoc tests was used to test the differences in SECO release and conversion yield into enterolignans of fresh, germinated, and fermented flaxseed and tea brew within three time points. P-values < 0.05 were regarded as statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 25 software (IBM Corp).

RESULTS AND DISCUSSION

Effect of processing and particle size on lignan bioaccessibility during simulated *in-vitro* gastro-intestinal digestion of flaxseed matrixes

In the present research, the effect of fermentation and germination as well as particle size reduction on lignans bioaccessibility were assessed using simulated *in-vitro* gastrointestinal digestion. In addition to the flaxseed matrixes, tea brew was included as a representative sample to study the stability of lignans in the gastrointestinal environment and their interactions with the digestive fluids. SECO was detected in fresh, germinated, and fermented flaxseed. Only PINO and MATA were found in tea brew (Table 1). The SECO content was the highest in germinated flaxseed, with values of 15.6 $\pm$ 1.1 mg/g dry weight (DW) and 14.6 $\pm$ 0.6 mg/g DW in fine and coarse particles, respectively. Fermented flaxseed showed the second highest content of SECO with values of 11.1 $\pm$ 2.1 mg/g DW and 9.8 $\pm$ 0.6 mg/g DW in fine and coarse particles, respectively. The fresh flaxseed has the lowest SECO content with values of 3.0 $\pm$ 0.5 mg/g DW and 2.1 $\pm$ 0.4 mg/g DW in fine and coarse particles, respectively.

Table 1. Bioaccessibility (%) of lignans during simulated *in-vitro* gastrointestinal digestion of fine and coarse particles of fresh, germinated, and fermented flaxseed and tea brew (mean ± SD, n= 2)

Fine*			Coarse		
Oral	Gastric	Intestinal	Oral	Gastric	Intestinal
Flaxseed (SECO)[a]					
0.67 ± 0.07^{A}	0.72 ± 0.05^{A}	0.87 ± 0.09^{A}	0.61 ± 0.12^{A}	0.69 ± 0.08^{A}	0.67 ± 0.02^{A}
Germinated flaxseed (SECO)[b]					
0.47 ± 0.10^{A}	0.52 ± 0.05^{A}	0.55 ± 0.04^{A}	0.37 ± 0.05^{A}	0.50 ± 0.04^{A}	0.53 ± 0.04^{A}
Fermented flaxseed (SECO)[ac]					
0.70 ± 0.06^{A}	0.72 ± 0.01^{A}	1.03 ± 0.04^{B}	0.63 ± 0.06^{A}	0.69 ± 0.08^{A}	1.00 ± 0.22^{B}

Oral	Gastric	Intestinal
Tea brew (PINO and MATA)		
88.5 ± 8.42^{A}	67.0 ± 3.51^{B}	24.4 ± 4.15^{C}

Different lowercase within samples (fresh, germinated, and fermented flaxseed), uppercase within the digestion phase, and * within particle size indicate a significant difference in bioaccessibility ($P < 0.05$). SECO= secoisolariciresinol; PINO= pinoresinol; MATA= matairesinol.

As a general observation, bioaccessibility of lignans from the flaxseed food matrixes very low (1 % and less). Our findings are in accordance with a study on flaxseed that showed no lignans were released during *in-vitro* simulated gastrointestinal digestion[27].

Both type of processing and particle size significantly affected the bioaccessibility of SECO ($P < 0.001$ for both). No interaction between particle size and samples was observed ($P = 0.142$). The bioaccessibility was the highest in fermented flaxseed, reaching around 1.03 %, while it was 0.87 % and 0.67 % in fine and coarse particle of fresh flaxseed, respectively. Germinated flaxseed presented the lowest bioaccessibility of SECO (0.5 %) for both particle sizes.

Fresh flaxseed showed a significant difference in SECO bioaccessibility between the digestion phases in fine and coarse particles, $P = 0.047$ and $P = 0.002$, respectively. Similarly, the fermented flaxseed showed a significant difference in SECO bioaccessibility among digestion phases in fine and coarse particles, $P = 0.019$ and $P = 0.024$, respectively. While, in the germinated flaxseed, coarse size showed a significant

difference in SECO bioaccessibility between the digestion phases (P = 0.017), but not for fine size (P = 0.066).

In the tea brew, the lignan content was 330.5 ± 29.7 ng/mL. In tea brew, the bioaccessibility of lignans was about 88 % after the oral phase and decreased to approximately 70 % after the gastric phase (P = 0.04), and further decreased to around 25 % after the intestinal phase.

In addition, the addition of digestion enzymes did not significantly affect the bioaccessibility of SECO in flaxseed samples for fine (P = 0.640) and coarse sizes (P = 0.261). Similarly to the flaxseed samples, digestion enzymes did not significantly affect the bioaccessibility of MATA and PINO in tea brew (P = 0.062).

Our finding is in line with previous research that showed that milling and crushing of flaxseed, i.e. decrease of particle size, enhance bioavailability[28]. To the best of our knowledge, no data are available on the bioaccessibility of lignans during fermentation using *Rhizopus oyrzae*. Fungi of the genus *Rhizopus* have been traditionally used for food fermentation, specifically of tempeh in South-East Asia including Malaysia, since centuries. *Oryzae* is considered GRAS by FDA but not by EFSA, which mostly raises an issue related to mycotoxins presence[29]. The chance that the fungus may survive the passage through the gastrointestinal tract, as well as colonize and thrive in the large intestine ecology is negligible. Nevertheless, a study on the fermented raw pumpkin oil cake proved an effective release of bound phenolics which may indicate an increase in bioaccessibility[30]. Likewise, a study on fermented rice bran with *Rhizopus oryzae* reported an increased phenolic content compared to the unfermented samples[31] by releasing insoluble bound of phenolic compounds by the activity of exogenous enzymes[32].

To the best of our knowledge, there is no published data on the bioaccessibility of lignans in germinated food. However, one study has shown that the content and bioavailability of phenolic compounds increased in germinated compared to non-germinated grains[33]. This would be due to the activity of endogenous enzymes releasing the bound phenolic compounds, which increased bioaccessibility[34].

The particle size of foods affects the bioaccessibility of phytochemicals. A study showed that the bioaccessibility of the antioxidant and total phenolic content of carob flour improved with a reduction of the particle size[35]. Similarly, another study proved that the reduction in particle size in wheat bran increased ferulic acid bioaccessibility[36]. Grinding breaks down plant cells, increasing yield and bioaccessibility[37].

All in all, our findings indicate that the SECO bioaccessibility from the food matrix may be altered according to the way foods are processed.

The results show here for the first time that lignans are unstable under simulated gastrointestinal conditions by using tea brew. Other studies have reported that polyphenols may be unstable under simulated in vitro gastrointestinal digestion, with only 20% remaining intact at the end of the intestinal phase[38]. Degradation, epimerization, hydrolysis, and oxidation of polyphenols are significant transformations during digestion, particularly after the transition from the gastric to the intestinal environment[39]. Furthermore, a study speculated that the presence of bile salt in the intestinal phase would reduce the campesterol, β-sitosterol, and sitosterol in beverages due to reduce solubility of sterols[40] which may have impacted the bioaccessibility of lignans as well. However, the observation that lignans could be degraded during gastric and intestinal digestion is relevant when designing strategies to optimize the health effect of lignans.

The net amount of parent lignans were calculated that would be available to our body when the simultaneous effect of fermentation and germination on content and bioaccessibility is considered. The amount of SECO available for absorption was the highest in fine and coarse fermented flaxseed, with values of 113 μg/g DW and 98 μg/g DW, respectively. Germinated flaxseed showed the second higher of SECO content in the intestinal phase with values of 85.8 μg/g DW and 77.4 μg/g DW in fine and coarse size, respectively. Then, the fresh flaxseed has the lowest SECO content in the intestinal phase, with values of 26.1 μg/g DW and 14.1 μg/g DW in fine and coarse, respectively.

From the findings mentioned above, it was concluded that fermentation and germination could increase the amount of circulating lignans. During fermentation, the endogenous and bacterial enzymes could modify the grain constituents, thus affecting the absorption and bioavailability of bioactive compounds[41]. While, during germination, the soaking process activated the enzymes to break down the active compounds[42]. Fermentation and germination are relatively simple technologies yet widely used to increase the nutrient density in bioactive compounds by releasing the bound fraction in the food matrix, thereby increasing the bioaccessibility[43].

Lignans Released from the Food Matrix During *In-vitro* Colonic Fermentation

Next, the release of lignans in the *in-vitro* colonic fermentation phase were further investigated. Only pre-digested fine particles was used since this particle size showed a higher bioaccessibility than the coarse size. The release of SECO from fine particles of fresh, germinated, and fermented flaxseed during *in-vitro* colonic fermentation is shown in Figure 1. A control was added, where the flaxseed samples were dissolved in the fermentation medium without fecal inoculum.

Notably, the SECO can be already detected in the supernatant after 2 hours of fermentation. A significant increase was found in SECO released in fermented flaxseed from 2 to 24 hours of fermentation. The SECO released was significantly increased from 8 to 24 h in flaxseed and germinated flaxseed. After 24 hours of *in-vitro* fermentation, fermented flaxseed produced the highest SECO release among all samples, approximately 65 %, showing that the metabolic activity of the fungus *Rhizopus oryzae* may help the release of lignans in the colon. The SECO released was comparable between fresh and germinated flaxseed (approximately 10%). However, a significantly lower percentage of SECO was released in the control (approximately 2.5 %). This clearly demonstrates that microbiota activity in the large intestine aids in releasing SECO from the plant matrix.

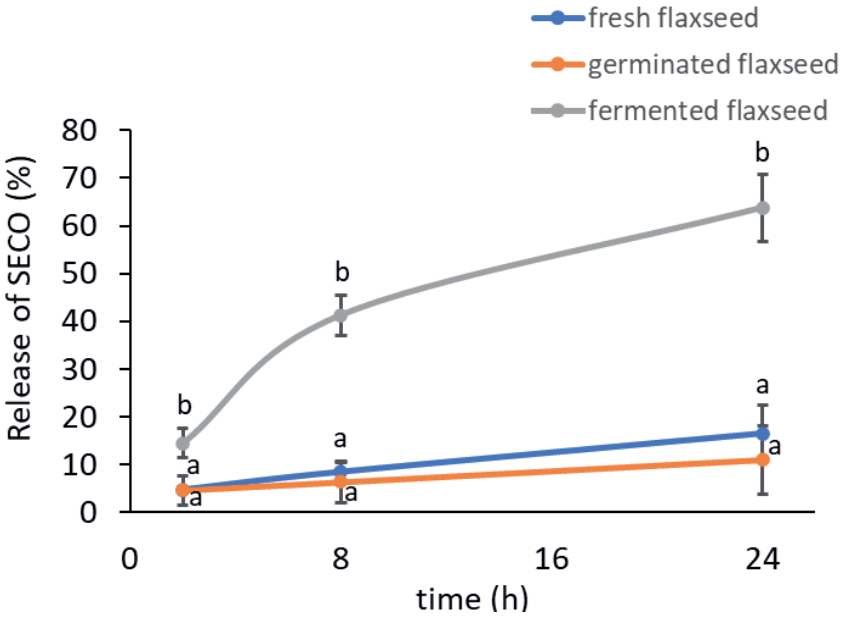


Figure 1. Release of SECO (% of the pre-digested samples) in the supernatant during *in-vitro* colon fermentation of fine particles of fresh, germinated, and fermented flaxseed. Data are from 3 donors (mean ± SD, n= 3). Different lowercase letter within samples indicates a significant difference in the % SECO released during three time points (2, 8, 24 h) in the supernatant ($P < 0.05$). SECO= secoisolariciresinol.

Lignans Conversion to Enterolignan During *In-vitro* Colonic Fermentation

In the present study, the conversion of lignans to enterolignans were also investigated. Only enterolactone (EL) was detected in the samples after the colonic fermentation, probably because of the rapid dehydrogenation of END into EL[44]. SECO conversion to EL during *in-vitro* colon fermentation of fresh, germinated, and fermented flaxseed is presented in Figure 2. The conversion of EL was significantly higher in fermented flaxseed at all times, ranging from 0.5 % to 1.0 %. *In-vitro* colonic fermentation of fresh and germinated flaxseed produced a lower conversion to EL of approximately 0.1 % after 24 hours of fermentation, with no significant difference between the samples.

The fermentation time did not significantly affect the conversion of SECO in EL in fresh ($P = 0.881$) and germinated flaxseed ($P = 0.801$). While the production of EL was significantly increased from 8 to 24 hours of fermentation in the fermented flaxseed ($P = 0.024$). As expected, no EL was detected in the control samples, because microbiota is necessary for the conversion of SECO to EL.

Furthermore, the correlation between SECO release and conversion to EL in fresh, germinated and fermented flaxseed at 24h of fermentation were evaluated. SECO release in all flaxseed samples was significantly positively correlated with EL conversion at 24h of fermentation ($r = 0.999$, $P < 0.029$). It was concluded that the more lignans released, the higher the conversion in EL because there is more available substrate.

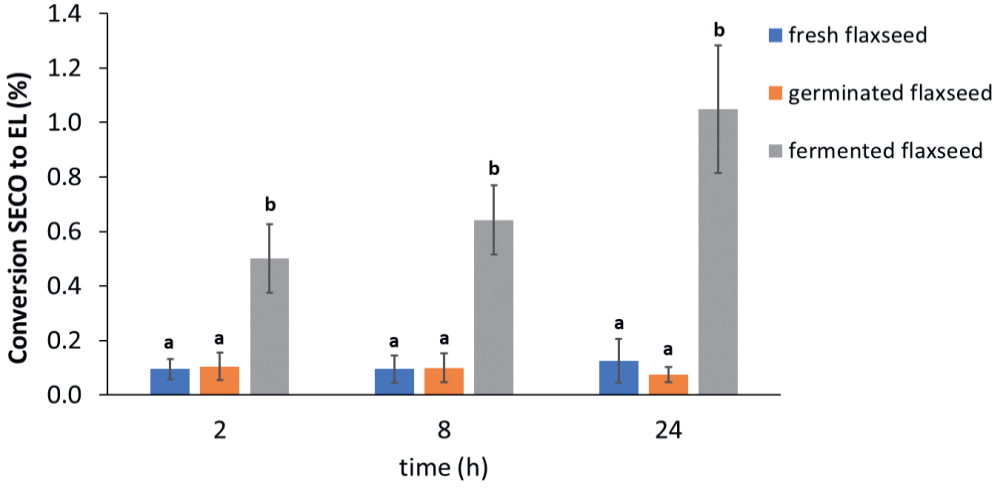


Figure 2. Lignan (SECO) conversion to EL during *in-vitro* colon fermentation of fresh, germinated, and fermented flaxseed. Data are from 3 donors (mean ± SD, n= 3). Different lowercase letter within samples indicates a significant difference in the SECO conversion during three time points (2, 8, 24 h) in the supernatant ($P < 0.05$). SECO= secoisolariciresinol; EL= enterolactone.

The net amount of EL available to our body when the simultaneous effect of fermentation and germination on content and conversion of SECO are considered were calculated. The EL content produced after colonic fermentation was by far the highest in fermented flaxseed, with a value of 116.6 μg/g DW, followed by the germinated flaxseed with a value of 10.9 μg/g DW. The EL production in the flaxseed had the lowest value of 4.0 μg/g DW. The fermented flaxseed had approximately a 29-fold difference compared to fresh flaxseed. In addition, the germination of flaxseed would substantially increase EL production with a nearly 3-fold difference compared to the fresh flaxseed. According to the aforementioned results, it was concluded that food fermentation and germination could significantly enhance EL production from flaxseed.

The inter-individual differences were looked further and reported the data for the single donors in Figure 3. Donor 3 had the highest EL conversion rate of all the samples, around 1.5 % in fermented flaxseed. Nearly a 5-fold difference in EL production was observed in fresh and fermented flaxseed between the highest and lowest producers.

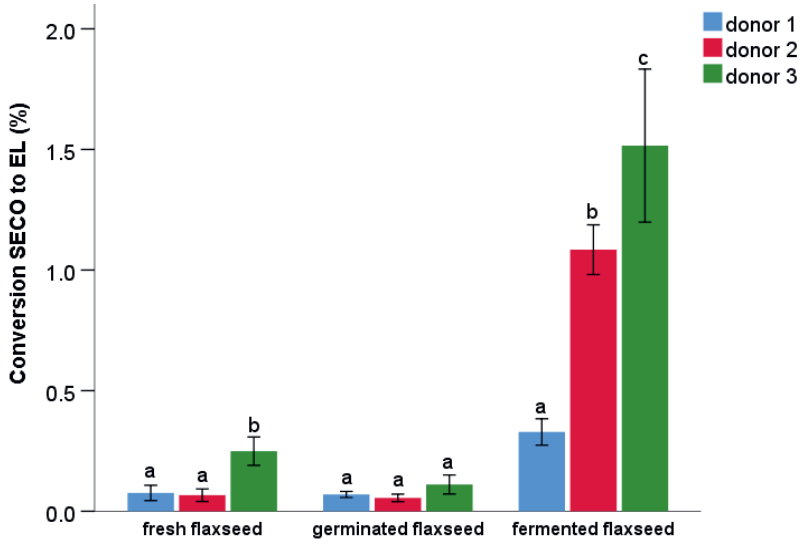


Figure 3. Lignan (SECO) conversion to EL at 24 hours in *in-vitro* colon fermentation of fresh, germinated flaxseed, and fermented flaxseed. Data are from two replications (mean ± SD, n= 2). Different lowercase letters indicate a significant difference in SECO conversion to EL among the three donors in each sample. SECO= secoisolariciresinol; EL= enterolactone.

The gut microbiota pathway for the formation of enterolignans begins with demethylation and dehydroxylation of SECO, followed by oxidation of ED to EL[45]. Factors such as diet, genetics[46], gender[28], and age[47] affect the conversion of lignans to EL by modulating microbiota[48]. In the present research, the variability observed in the

lignan conversion among donors is high, clearly due to differences in the individual microbiota. It was suggested that microbiota dehydrogenation of lignans to generate EL is a crucial step in the colon that could explain major variation in EL production[49]. The ability of gut microbiota to metabolize lignans to EL is determined by the presence of a metabolite production gradient that gives rise to "high producers" and "low producers" of metabolites[50]. To date, only a few microbiota species have been identified as enabling the production of EL. In particular, EL production has been related to the abundance of *Ruminococcus*[51], *Bacteroides, and Eggerthella* species[52]. Thus, microbiota differences are a factor that influences the outcome of the health effects of lignans[44]. Furthermore, a study hypothesized that if high lignan products are consumed for an extended period of time, the microbiota will most likely adapt to metabolizing lignans, increasing the plasma concentration of the resulting enterolignans[27].

Only PINO and MATA were detected in the tea brew in colonic fermentation. The conversion of lignans (PINO and MATA) in tea brew was relatively high, approximately 15 % (Figure 4A), which was much higher than the conversion rate observed from flaxseed matrixes. The most likely explanation for this difference in conversion yield may be the different level of lignans to which microbiota has been exposed during the fermentation experiments. This amount would be the highest when germinated flaxseed was fermented with a value of 15.1 mg lignans (in 1 g of fermented material), followed by fermented flaxseed, with a value of 11.1 mg. The lowest lignan content after the colonic fermentation was fresh flaxseed, with a value of 3.0 mg. Compared to the flaxseed matrixes, the amount of lignans in 10 mL of tea brew used in the fermentation was several orders of magnitude lower, i.e. 0.003 mg. We can speculate that the high lignan concentration may have saturated the capacity of the microbiota for the lignan conversion to enterolignans. Furthermore, the presence of fibre and its fermentation in the flaxseed may have delayed microbial metabolism of lignan as suggested elsewhere[27]. Another possible explanation for the higher conversion of lignans in tea is that MATA can be directly converted to EL, whereas SECO needs to be converted to MATA or END prior to EL production[53].

The influence of inter-individual differences on the lignans' conversion to EL were further examined (Figure 4B). Donor 3 significantly showed again the highest production of EL (approximately 25 %) compared to others.

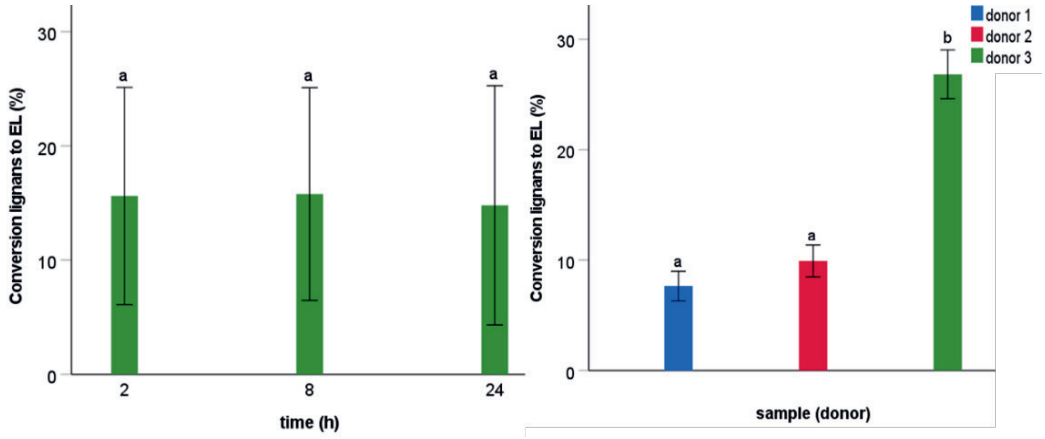


Figure 4. Lignans (PINO and MATA) conversion to EL during *in-vitro* colon fermentation of tea brew. A) Data are from 3 donors (mean ± SD, n= 3) at different fermentation time; B) Data are from two replications of tea brew (mean ± SD, n= 2) of 3 donors. Different lowercase letters indicate a significant difference ($P < 0.05$) in lignans conversion to EL. EL= enterolactone.

CONCLUSION

In the current study, for the first time, a systematic investigation of lignans bioaccessibility during gastrointestinal digestion, their release, and conversion to EL in colonic fermentation from differently processed flaxseed were reported. Our findings highlight that only a limited fraction of lignans are released in the small intestine and that processing and particle size reduction have a limited effect on the bioaccessibility of lignans. Fermentation, however might represent a potential strategy for enhancing lignan release and conversion to EL in the large intestine. Further research into further particle size reduction can be conducted to assess the impact on lignan bioaccessibility and conversion to EL.

AUTHOR CONTRIBUTIONS

Umi Kalsum Hussain Zaki: Conceptualization, Investigation, Formal analysis, Writing – original draft. Christos Fryganas: Conceptualization, Investigation, Formal analysis, Writing – review & editing. Laura Trijsburg: Writing – review & editing. Edith Feskens: Writing – review & editing. Edoardo Capuano: Supervision, Conceptualization, Investigation, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENTS

The study was supported by the Food Quality & Design Department, Wageningen University & Research, the Netherlands, and funded by Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

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SUPPLEMENTARY MATERIAL

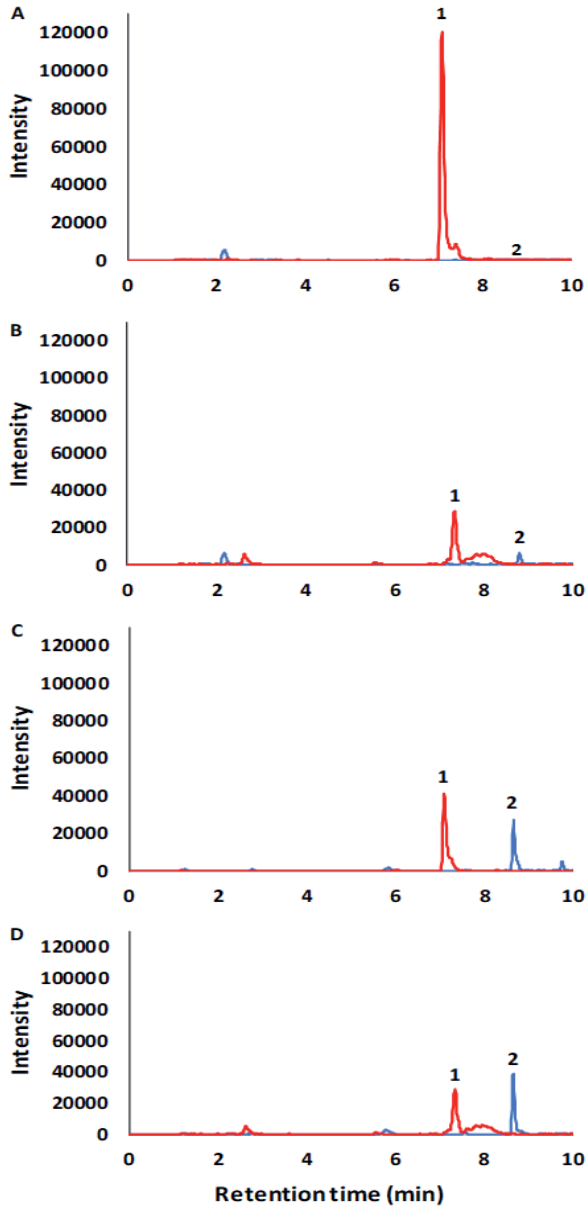

Figure S1: Representative chromatograms of A) fresh flaxseed, B) fresh flaxseed at the end of the intestinal phase of the simulated in vitro digestion, C) fresh flaxseed after 2h of *in-vitro* simulated colonic fermentation, and D) fresh flaxseed after 24h of *in-vitro* simulated colonic fermentation. In all the panels peak 1 represents secoisolariciresinol and peak 2 represents enterolactone.

5

CHAPTER 5

Association between lignan intake from different types of plant-based foods and obesity indicators in the Netherlands

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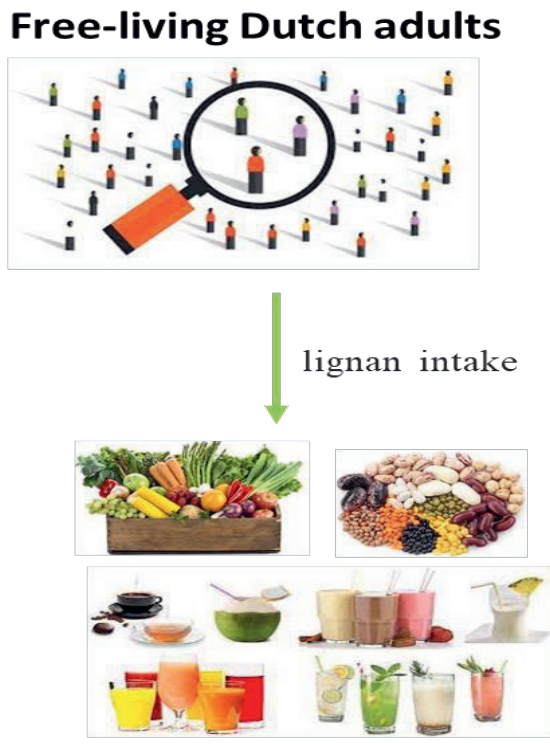
(Under review of Plant Food for Human Nutrition)


Free-living Dutch adults
lignan intake
↑ lignan intake,
↓ waist circumference

ABSTRACT

This study aimed to assess whether lignan intake is associated with lower body mass index (BMI) and waist circumference in a Dutch population. Data on lignan content of foods was extracted from a lignan database of Dutch plant-based foods and complemented with three other references to estimate lignan intake in 1012 Dutch men and women participating in the NQ-plus prospective cohort study. The association between lignan intake and BMI and waist circumference was assessed using linear regression analysis. Three multivariate models were used to investigate the association of BMI and waist circumference with total lignan intake. Model 1 included adjustments for lifestyle variables, in model 2 adjustments were made for energy intake and nutrients, and in model 3 these adjustment were combined. The median lignan intake was 3226 μg/day (mean 4763 μg/day, SD 5386 μg/day). Lignan intake was strongly skewed towards higher values, ranging from 40-50500 μg/day. The major sources of lignans were bread, which contributed 52%, nuts, seeds, and snacks (21%), and vegetables (12%) to the lignan intake. After adjustments lignan intake was associated with a lower waist circumference (-0.93 cm, 95% CI: -0.88; -1.00). No association between BMI and lignan intake was observed. A higher lignan intake is significantly associated with a smaller waist circumference in Dutch adults. To what extent a high lignan intake can reduce waist circumference in obese patients or can prevent onset of abdominal obesity remains to be tested in future intervention studies.

Keywords: lignan intake, body mass index, waist circumference, plant-based food, NQ-Plus data, Netherlands

INTRODUCTION

The global obesity epidemic has tripled rapidly in various populations and all age groups[1]. Obesity increases the risk of chronic diseases such as type 2 diabetes, cardiovascular diseases, certain cancers, dyslipidemia, poor mental health, and osteoarthritis[2]. Furthermore, obesity is one of the main factors for respiratory tract infections and functional capacity, impacting pulmonary function due to specific inflammation and immunological conditions. In addition, novel findings indicate that visceral obesity and characteristics of impaired metabolic health such as hyperglycemia, hypertension, and subclinical inflammation are strong and independently associated with a high risk of morbidity and mortality from severe SARS-CoV-2 infection/COVID-19[3].

Lignans are plant-derived diphenolic compounds that belong to the phytoestrogen class and are structurally similar to the estrogen hormone 17-estradiol[4]. They occur in edible plants[5] and are abundantly found in flax and sesame seeds[6]. Oilseeds, nuts, cereals, vegetables (*Brassica* species), fruits, and beverages (tea, coffee, beer, and wine) are the major sources of lignans in the European diet[7]. Secoisolariciresinol (SECO) is the most abundant lignan, whereas matairesinol (MATA), pinoresinol (PINO), and lariciresinol (LARI) occur as minor components[8]. Intestinal bacteria can convert some plant lignans to enterolignan metabolites: enterodiol (END) and enterolactone (EL), under strictly anaerobic conditions. These metabolites are inversely related to weight loss[9]. Studies on lignans have tried to explain the mechanisms responsible for weight reduction and their relation to lipid metabolism[10].

A study involving 3438 young Spaniards between 2–24 years old investigating the prevalence of obesity-related to lignan intake found a strong association between dietary lignan intake and prevalent obesity for boys, with an odds ratio of 0.34 (95 % CI, 0.17–0.70) in the highest versus lowest quartile of lignan intake[11]. Similarly, a 10-year prospective cohort study on weight change of U.S. women observed that the annual weight change rate among women in the highest quartile of total lignans intake was 0.20 kg less (95 % CI: −0.36, −0.04) than that among women in the lowest quartile[12]. Previously, Milder et al. performed a study to estimate the intake of lignans in a large, representative Dutch population sample using the National Food Consumption Survey 1997-1998[13] by using the lignan database of Dutch plant foods containing 83 solid foods and 26 beverages. In brief, the study showed that the amount of lignan intake in the population had previously been underestimated[14]. The survey was conducted in 1998.

Nevertheless, there are some limitations of the aforementioned studies, which only involved young participants aged 2-24[11] or women[12], while the study from by Milder was conducted more than ten years ago and only included 109 foods and beverages[13]. Hence, to estimate current intakes, our study aimed to assess lignan intake from plant-based foods in a Dutch population aged 20-70 years using an updated database, the Nutrition Questionnaires plus (NQ-plus) study conducted between 2011 to 2013. In addition, we assessed the association between lignan intake, body mass index, and waist circumference as indicators of obesity.

MATERIAL AND METHODS

DESIGN AND STUDY POPULATION

The NQ-plus study is a prospective cohort study initiated by researchers of the division of Human Nutrition of Wageningen University. This study started in May 2011 to pinpoint the specific dietary factors responsible for obesity and adverse cardiometabolic health outcomes. 1012 men and women aged 20-70 years living in Wageningen, Ede, Renkum, and Arnhem, the Netherlands, were included[15, 16].

Baseline measurements consisted of the assessment of dietary intake by multiple telephone-based 24-h recalls (24hR), a physical examination, including measurement of anthropometrics (e.g., weight, height, blood pressure), and general and lifestyle questionnaires (education, health, and smoking habits). This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human participants were approved by the Medical Ethics Committee of Wageningen University and Research. Written informed consent was obtained from each participant[16].

Dietary and lignan assessment

Multiple, telephone-based 24-h recalls (24hR) were administered by trained dietitians using the five-step, multiple-pass method, which is a validated technique to increase accuracy[17-19]. Dates were randomly selected evenly across the year and days of the week. The foods recorded on the recalls were translated into food codes and food groups using the 2011 Dutch Food Composition Table. All participants with at least two 24hR were included in this study. The record days were equally distributed over the seven days of the week. Average daily dietary intakes were calculated by multiplying the

consumption frequency by the portion size and nutrient content in grams[16]. Out of 2082 reported food items for the 24hR, 290 foods were with known lignan content and thus selected for inclusion in this analysis. The lignan data was extracted from a lignan content database of Dutch plant based foods [6] and complemented with data from three published lignan database from United Kingdom, Canada and Japan[20-22]. Foods that contained no lignans (0.0 μg), no intake from participants and unknown lignan content or composition were excluded from the calculation. For each person, lignan intake was calculated by multiplying the consumed amount of each food with its lignan content (μg/g) for each recorded day.

Anthropometrics

Height was measured with a stadiometer (SECA, Germany) to the nearest 0.1 cm, and weight was assessed with a digital scale (SECA) to the nearest 0.1 kg. Body mass index (BMI, kg/m^2) was calculated as a person's weight in kilograms divided by the square of height in meters. Participants were categorised as normal weight (BMI < 25 kg/m^2), overweight (BMI ≥ 25 kg/m^2), and obese (BMI > 30 kg/m^2)[23]. Waist circumference was measured using a measuring tape (SECA 201) to the nearest 0.5 cm.

Demographic, lifestyle, and general health

The general and lifestyle questionnaires included demographics, education, work status, smoking status, and physical activity. Most of these questions were derived from the general questionnaires of the Lifelines study[24]. The education status was divided into three groups which were low, intermediate, and high. Participants with no education or primary or lower vocational education as highest completed education were classified as low. Meanwhile, lower secondary or intermediate vocational education participants were classified as intermediate education. Participants with a high education level completed higher secondary education, higher vocational education, or university. Smoking status was classified into three categories: non, current, and former smoker. Information about the participants' usual physical activity was obtained using the Short QUestionnaire to ASsess Health enhancing physical activity (SQUASH)[25]. The SQUASH contains questions evaluating the participants' compliance to the Dutch physical activity guidelines, specifically, a physical activity pattern including at least 30 min of moderate-intensity physical activity on ≥ 5 days/week[26].

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25 software (IBM Corp). The initial sample consisted of 7437 participants having the required information. We excluded participants who did not have complete information on all nutrients. Then, we eliminated participants who only having one 24hR record. One participant was excluded from the analyses, since she/he provided only nutrient information. Thus, the complete data on all nutrients intake variables with at least 2 days of 24hR were available for 1012 participants. The generated dataset was checked for duplicates and missing values for BMI, waist circumference, education, smoking status and physical activity. Automatically generated values replaced all the missing observations in the confounding database through multiple imputations. All the variables were entered in the imputation model, and five imputations were carried out with 10 iterations under a fully conditional specification imputation method. The missing observation represented 5.6 % out of the entire data. Pooled data was used for further analysis[27]. The study population consisted of 1012 participants: 534 men and 478 women. Participant characteristics were reported as means with standard deviation (mean $\pm$ S.D.), median (IQR), or n (%). ANOVA tests were performed for normally distributed continuous variables, Kruskal-Wallis tests were performed for skewed continuous variables, and chi-square tests were performed for categorical variables to compare the baseline characteristics over tertiles of total lignan intake. The energy intake data was checked before proceeding to further analysis. The energy intake data was in the normal range (804 – 4410 kcal), and the mean energy intake was 2105 kcal. In addition, the food values of those participants with the lowest and highest energy intake were checked, and no misreporting values were found. Thus, these participants were included in the study. The energy intake was based on participants' total dietary intake, and the value was used for the energy adjustment.

The contribution of individual foods and food groups to the mean lignan intake was calculated, and the percentage of users of each food or food group was determined. Mean lignan intakes for men and women, adjusted for age, and intakes in age categories adjusted for gender, were compared using analysis of covariance after log transformation of the individual lignan intakes. Similarly, mean lignan intakes stratified by other selected lifestyle characteristics were adjusted for age and gender.

In addition, three multiple regression models were used to investigate the association between BMI and waist circumference and total lignan intake. The waist circumference was used an outcome variable as it is the simpler, most practical, and accurate measure of abdominal obesity for practice in public health research[28, 29]. We applied the Z-score calculation to standardize the variables with a 1-SD increase in intakes of total lignan. The model with crude (unadjusted energy) and energy-adjusted

results were compared with three multiple regression models. A list of potential confounders was selected from published research on lignan intake in relation to obesity. Ethanol intake, physical activity and education are listed as potential risk factors for obesity and may be correlated with lignan intake. Smokers have greater oxidative stress than non-smokers; thus, the lignans' antioxidant effects might be more marked in smokers. In addition, dietary fiber intake may influence the lignan bioavailability, affecting intestinal transit time[30]. Model 1 included adjustments to the lifestyle variable: age, sex, education, smoking status, ethanol consumption, and physical activity with energy adjusted. In model 2, adjustments were made for macronutrients, and fiber with energy adjusted. Model 3 (full model) was the adjustment variables of models 1 and 2. The residual method was used to adjust for energy intake. P-values were considered significant if $P \leq 0.05$.

RESULTS AND DISCUSSION

LIGNAN INTAKE

Mean lignan intake of the study population was 4763 μg/day (ranging from 40 to 50500 μg/day) on the average days surveyed, and was strongly skewed towards higher values (Fig. 1). The correlation of lignan intake between the two non-consecutive days of recalls was low ($r = -0.009$, $P = 0.69$), indicating a large day-to-day variation.

There was no significant difference in total lignan intake between men and women in this population (Table 1). Our finding is in accordance with the study of Milder et al.[13], reporting no significant difference in lignan intake according to gender. In contrast, in a Finnish study total lignan intake was shown to be higher in men than in women[31]. Also, no differences according to age-category, educational level, smoking or BMI were observed for all participants (data not shown). Participants in the highest tertile of total lignan intake had a lower waist circumference than those in the lowest tertile ($P = 0.008$) (Table 1). There was no significant difference in other selected characteristics across the tertiles.

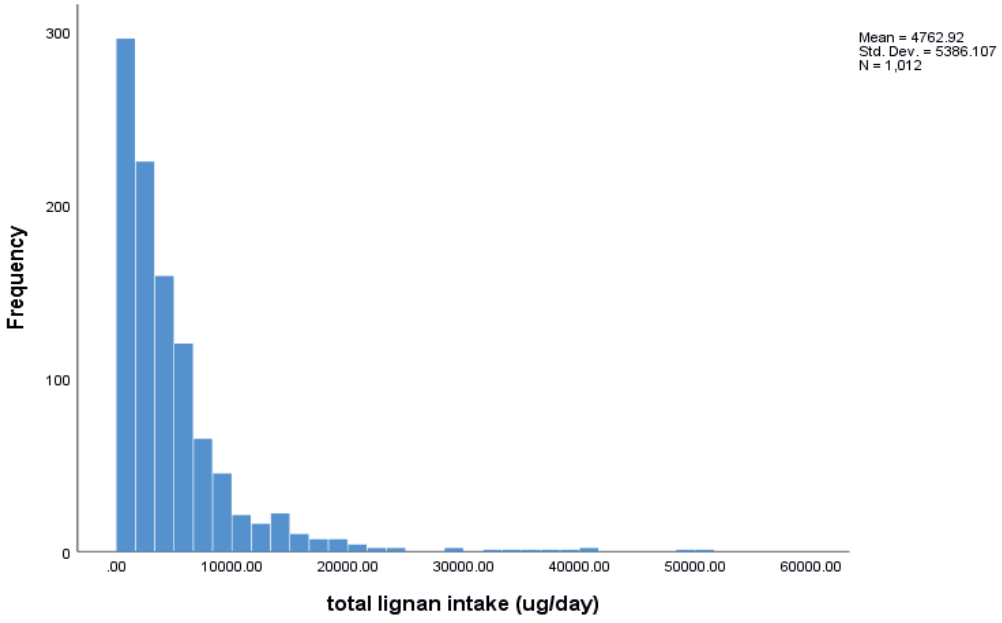


Figure 1 Distribution of total lignan intake among 1012 participants in the NQ-Plus study

Table 1 Selected baseline characteristics and nutrients by tertile of lignan intake among 1012 participants in the NQ-Plus study

Tertile	Total	T1 (low)	T2	T3 (high)	P†
n	1012	338	336	338	
Lignan intake, µg/day†	3226 (1369-5948)	996 (726-1372)	3226 (2574-3971)	7674 (5943-11351)	
• **Men**	2877 (2642 - 3126)				0.16
• **Women**	3140 (2870 - 3428)				
Sex, men (%)	534 (52.8)	194 (57.4)	159 (47.3)	181 (53.6)	0.03
Age, years†	55 (45-62)	56 (46-63)	54 (45- 61)	52 (44 – 62)	0.09
Age, years (%)					
• **19-29**	61 (6.0)	24 (7.1)	16 (4.8)	21 (6.2)	0.58
• **30-49**	290 (28.7)	88 (26.0)	105 (31.3)	97 (28.7)	
• **50-69**	651 (64.3)	223 (66.0)	213 (63.4)	215 (63.6)	
• **≥70**	10 (1.0)	3 (0.9)	2 (0.6)	5 (1.5)	
BMI, kg/m²†	25.2 (23.1-28.0)	25.5 (23.4-28.1)	25.1 (23.1-27.9)	25.3 (23.1-27.9)	0.77
***BMI category, kg/m²* (%)**					
• **<25**	461 (45.6)	148 (43.8)	162 (48.2)	151 (44.7)	0.57
• **25-30**	411 (40.6)	147 (43.5)	125 (37.2)	139 (41.1)	
• **>30**	140 (13.8)	43 (12.7)	49 (14.6)	48 (14.2)	
Waist circumference (cm)†	90.5 (82.7-99.0)	92.9 (83.4-99.8)	89.4 (81.6-98.5)	89.5 (82.5-98.5)	0.008
Smoking status, n (%)					
• **Non**	554 (54.7)	172 (50.9)	186 (55.4)	196 (58.0)	0.17
• **Current**	375 (37.1)	136 (40.2)	117 (34.8)	122 (36.1)	
• **Former**	83 (8.2)	30 (8.9)	33 (9.8)	20 (5.9)	
Total Physical activity level, minutes per week†	2212 (1373-2784)	2185 (1211-2805)	2233 (1451-2772)	2216 (1457-2785)	0.39

Education, n (%)					
• **Low**	65 (6.4)	20 (5.9)	25 (7.4)	20 (5.9)	0.36
• **Intermediate**	301 (29.7)	92 (27.2)	111 (33.0)	98 (29.0)	
• **High**	646 (63.8)	226 (66.9)	200 (59.5)	220 (65.1)	
Dietary intake					
• **Energy, kcal/day†**	2064 (1778-2354)	2093 (1797-2363)	2038 (1752-2332)	2039 (1788-2364)	0.17
• **Dietary fiber, g/day†**	22.3 (18.1-26.8)	21.9 (18.0-26.9)	22.4 (17.8-26.7)	22.6 (18.3-26.9)	0.65
• **Protein, g/day**	80.3 (69.0-92.2)	80.9 (69.8-92.9)	79.9 (68.2-94.1)	80.2 (69.3-91.6)	0.78
• **Fat, g/day†**	77.4 (64.1-94.4)	77.5 (65.1-94.0)	76.5 (63.4-95.0)	78.3 (63.9-94.5)	0.60
• **Carbohydrate, g/day†**	219.9 (184.3-261.5)	219.5 (184.2-264.7)	218.0 (185.1-256.7)	224.7 (183.8-260.4)	0.37
• **Ethanol consumption (g) †**	8.8 (0.1-18.4)	9.8 (0.0-20.4)	7.2 (0.3-16.6)	9.1 (0.1-18.4)	0.22

*To compare baseline characteristics over tertiles of total lignan intake, ANOVA tests were performed for the mean of normally distributed continuous variables, Kruskal-Wallis tests were performed for a median of skewed continuous variables, chi-square tests were performed for categorical variables. ¥Mean ± S.D. (all such values), †Median, IQR in parentheses (all such values).

The primary source of lignans intake was bread, which contributed 51.6 % of the total lignan intake in this population. The contribution was mainly from multigrain bread with seeds, contributing 48.5 % (Table 2). The second primary source of lignan was the nuts, seeds, and snacks group (21 %), where flaxseed provided the highest lignan mean intake (19.6 %), although the seeds were consumed only by a small part of the population. The vegetable group was in third place of the total lignan intake, contributing 11.9 %. Boiled kale curly was the major vegetable source of lignan (3.3 %), followed by boiled broccoli, 2.8 %. Tea contributed a similar amount as boiled kale curly, and was the beverage providing the largest contribution to total lignan intake (3.3 %). Fruits contributed to 4.3 % of the lignan intake, and berries were the major lignan fruit source with a contribution of 1.0 %.

Furthermore, we examined 102 participants with more than 10,000 μg/day of lignan intake to identify which foods contribute to this high intake level. Moreover, we thoroughly checked the raw data but found no incorrect code or unusual values in the dataset. Multigrain bread/roll/bun with seeds was an important source of these 102 participants , with an average intake of 110 g/day. Also, flaxseed bread, flaxseed, tea, broccoli, sesame seeds, kale curly boiled, Brussel sprouts boiled, sesame paste tahin, contributed to the high lignan intake. The energy intake of participants with high lignan intake (> 10000 μg/day) ranged from 1076 to 4410 kcal/day. The highest intake of bread and flaxseed were 490 g/day and 18 g/day, respectively (data not shown). We compared the food group intake of participants who consumed more than 10,000 g per day to that of all participants. The high lignan group consumed more flaxseed and multigrain bread than the general population. In addition, the gender of these participants was evenly distributed (52 men, 50 women), and their ages ranged from 24 to 69.

Milder et al. studied the lignan intake and major food sources of lignans using the Dutch Food Composition Survey from 1997 to 1998[13]. She reported a mean intake of lignan of only 1241 μg/day, which was far lower than our findings. This may be partly due to the fact that they accounted for 109 foods while we included lignan intake for 290 foods. Moreover, we included multigrain bread, as this food was reported by our study population studied in more recent years (2011-2013). As the lignan composition of this food is relatively high, this may well account for the higher level of intake we observed.

We concluded that the large variation of the lignan intake in the Dutch population over the 10 years is due to the healthy diet transition to plant-based food. More food in the study contributed to high lignan content, e.g.; flaxseed and multigrain

bread. In addition, more lignan food data is available, thus increasing the accuracy of lignan intake.

Table 2 Full list of total lignan intake and % contribution of food items

Food items	total lignan intake	% contribution	Rank
bread	**5065095**	**51.6**	
• **Bread/roll/bun multigrain average with seeds**	4760328	48.5	1
• **Bread/baquette/roll brown/wholemeal average**	100804	1.0	
• **Crispbread sesame**	71250	0.7	
Fruits	**418586**	**4.3**	
• **berries & currants**	94045	1.0	9
• **Pear**	58083	0.6	
• **Avocado**	36175	0.4	
Vegetables	**1169898**	**11.9**	
• **Kale curly boiled**	322271	3.3	3
• **Broccoli boiled**	270780	2.8	5
• **Beans French/runner/mangetout boiled**	102389	1.0	8
Nuts, seeds & snacks*	**2062362**	**21.0**	
• **Flaxseed**	1921095	19.6	2
• **Sesame seeds**	55179	0.6	
Soy product	**16811**	**0.2**	
Legumes	**5080**	**0.1**	
Cereal	**34358**	**0.4**	
Potatoes	**30961**	**0.3**	
Herbs & spice	**1807**	**0.0**	

Savoury	**150896**	**1.5**	
• **Sesame paste tahin**	141902	1.4	6
Pastry, cakes, and biscuits	**99266**	**1.0**	10
Sugary product	**19045**	**0.2**	
Fat & oil	**40366**	**0.4**	
Soup	**30511**	**0.3**	
Composite food	**57786**	**0.6**	
Milk & milk product	**8510.01**	**0.1**	
Miscellaneous	**1788**	**0.0**	
Beverages	**607647**	**6.2**	
• **Tea prepared**	324193	3.3	4
• **Coffee prepared**	107062	1.1	7
• **Wine red/port wine**	62555	0.6	
• **Beer**	50304	0.5	

***** Snack is part of the group, the contribution was lower than 0.1%.

Another cross-sectional study of 301 Dutch women aged 60–75 years reported a lignan intake ranging from 650 ug/day to 2300 ug/day for the lowest and highest quartile[32]. The most common lignan classes were SECO, MATA, PINO, and LARI. In this study, only SECO and MATA were considered, while we and Milder et al. included four individual lignans (SECO, MATA, PINO and LARI). However, PINO and LARI only contribute small amounts to total lignan intake[13], thus we can compare these findings.

Other recent studies report a large range of lignan intake as well. For instance, low mean lignan intake was reported in France (200 μg/day) in a study conducted from 2006 to 2007 (HELENA study)[33]. Similarly, EPIC cohorts observed a low lignan intake in Italy (700 μg/day). Limited information was provided on the lignan content of the products consumed, such as various types of coffee and herbal tea. Also, this estimate was based on a single 24-h recall and the variation includes within-person variation[34].

Meanwhile, the Latvian population generally had a high lignan intake, with the estimated mean lignan intake being 4300 μg/day. This could be explained by a high intake of grain products (86 %), including seed breads which were most commonly consumed[35]. Additionally, the highest lignan intake (9100 μg/day) was reported in a vegetarian/vegan UK population consuming mainly plant-based foods[34].

Our reported total lignan intake was in the range of the Latvian population. Despite study variances, the existence of large multi-center studies such as the European Prospective Investigation into Cancer and Nutrition (EPIC) and the Healthy Lifestyle in Europe by Nutrition in Adolescents (HELENA) permits assessment of lignan intakes across Europe using comparable methods[33, 34, 36]. But differences in estimated lignan intake can be due to differences in foods available over the years, population composition, and the lignan food database used. We filled the data gaps in the existing older Dutch database with lignan values with data from three published lignan database from United Kingdom, Canada and Japan[20-22], hence we had few missing values.

The lignan sources in our study were similar to those found in a previous study of women in the Netherlands and Germany, in which bread, nuts, and seeds contributed the most to lignan intake[37]. We observed that flaxseed, multigrain bread, and seeded buns contributed significantly to this population's lignan intake, similar to the main lignan food sources in northern European countries, including Scandinavian and Baltic countries including Latvia[38]. Moreover, in our study, *Brassica* vegetables such as broccoli, kale curly, and Brussel sprouts were among the sources and contributed more to lignan intake than Milder's previous study. In addition, tea and coffee also contributed to total lignan intake.

Cereals were the major lignans source in most Western countries because of their regular daily consumption[39]. Furthermore, the study by Tetens concluded that cereals and grain products are important contributors to lignan intake in all Scandinavian countries[36]. Other findings showed that the total lignan intake varies among countries due to the different dietary habits[40].

ASSOCIATION OF BMI AND WAIST CIRCUMFERENCE WITH TOTAL LIGNAN INTAKE

We next investigated the association between BMI and lignan intake adjusted for lifestyle and nutrient variables (Table 3). A higher total lignan intake was significantly associated with a lower BMI in unadjusted analysis, and in model 2 ($P < 0.05$). No significant association was observed when adjusting for lifestyle confounders (model 1) and in the full model. All models significantly associated a higher total lignan intake with a lower waist circumference (Table 3). Moreover, men are significantly associated with a higher total lignan intake with a lower waist circumference in a lifestyle-adjusted and full model. The regression coefficient adjusting for all potential confounders amount to -0.93 (95 % CI: -0.88; -1.00), indicating that with a 1SD higher lignan intake waist was 0.93 SD lower.

So far, few studies have investigated the relationship between lignan intake and obesity. An observational study of 115 postmenopausal women in Canada showed that the high dietary lignan intake group had a significantly lower BMI and total body fat mass than women in the low lignan intake group [41]. One cross-sectional study indicated that higher lignan intake was associated with less abdominal fat mass[42]. Similarly, a Spanish study observed a strong association between dietary lignan intake and reduced prevalence of obesity for 2 – 24 years old Spaniards boys[11]. Overall, the aforementioned studies were in line with our findings. They support the notion that increased lignan consumption might potentially lead to less weight gain and reduced waist circumference, an indicator of abdominal obesity.

Although the exact underlying biological mechanisms are not completely clear, several studies have suggested that the enterolignans' estrogenic effects contribute to weight reduction [10]. Lignans have been shown to suppress adipose tissue growth, inhibit differentiation of preadipocytes, stimulate lipolysis, and induce apoptosis of existing adipocytes, thus reducing adipose tissue mass in the cell [43]. In addition, it has been shown that the intestinal microbiota are responsible for metabolizing lignans to enterolignans [44], and this may be one of the pathways through which they affect body weight and fat mass [45].

The present study results should be considered in light of some limitations. We did not assess the habitual intake since at least participants with 2 days of 24HR were included. Although no adjustment for within-person variation was made, the means of the lignan intake remain valid. Residual confounding due to unknown or unmeasured factors and confounding due to other dietary constituents cannot be completely excluded. In addition, lignan intake was estimated by matching daily food consumption and its lignan content obtained from a database, which may not reflect the actual amount of the compounds reaching the target organs after digestion, absorption, and metabolism, and as usual in this type of observational studies, metabolic conversion in the body was not taken into account. Research on the impact of different food matrices and synergy between various foods in determining bioavailability will be relevant for future studies.

An important strength of our study was that we used a Dutch lignan database [14] that contained lignan data of more than 90 % of our selected foods, and this database was exclusively developed to study the Dutch population. The dataset included all major plant foods in the Netherlands, habitually processed and prepared. On top of that, all values in this database were obtained using the validated LC-MS-MS lignan analysis method [46] with identical sample preparation for all food items.

Table 3 Association of BMI and waist circumference according to intake of lignans among participants in the NQ-Plus study, per 1-SD increase

	Total lignan intake B (95 % CI)	P-value	Total lignan intake, B (95 % CI)	P-value	Total lignan intake, B (95 % CI)	P-value
	Total population		Men		Women	
BMI (kg/m²)						
Crude (energy unadjusted)	-0.92 (-0.87;-0.98)	0.006	-0.95 (-0.85;1.04)	0.200	1.04 (-0.95;1.11)	0.367
Energy adjusted	-0.93 (-0.87;-0.98)	0.011	-0.95 (-0.85;1.04)	0.200	1.04 (-0.96;1.12)	0.383
Model 1	-0.95 (-0.89;1.04)	0.068	-0.94 (-0.83;1.03)	0.148	1.06 (-0.97;1.114)	0.221
Model 2	-0.91 (-0.85;-0.96)	0.001	-0.94 (-0.84;1.04)	0.236	1.10 (-0.96;1.13)	0.345
Full model	-0.96 (-0.91;1.02)	0.213	-0.93 (-0.83;1.03)	0.174	1.07 (-0.97;1.15)	0.204
Waist circumference (cm)						
Crude (energy unadjusted)	-0.90 (-0.85;-0.96)	0.001	-0.92 (-0.98;1.00)	0.063	1.00 (-0.92;1.11)	0.866
Energy adjusted	-0.89 (-0.84;-0.95)	<0.000	-0.92 (-0.98;1.00)	0.063	1.01 (-0.92;1.11)	0.790
Model 1	-0.92 (-0.86;-0.98)	0.012	-0.90 (-0.98;-1.00)	0.031	1.05 (-0.95;1.16)	0.347
Model 2	-0.88 (-0.83;-0.94)	<0.000	-0.92 (-0.98;1.00)	0.076	1.01(-0.92;1.11)	0.781
Full model	-0.93 (-0.88;-1.00)	0.042	-0.91 (-0.98;-1.00)	0.037	1.05 (-0.95;1.12)	0.314

Model 1: age, gender, education, smoking status, ethanol consumption, physical activity, energy intake

Model 2: energy intake, fat, protein, fibre, carbohydrate

Full model: model 1+ model 2

CONCLUSIONS

In conclusion, the present study results add further evidence to earlier observations of an association between a higher lignan intake and less obesity, as we observed an associated decreased waist circumference in a Dutch population. Future studies, including a prospective design and randomised controlled trials, are warranted to confirm our results and provide additional information about the optimal dietary intake of lignans required to achieve the expected positive health outcomes. Further investigation of the metabolism of lignans to enterolignans by gut microbiota in the colon will also be useful.

ACKNOWLEDGEMENTS

All authors read and approved the final version of the manuscript. This present work was supported by the Division of Human Nutrition and Health, Wageningen University & Research, Wageningen, the Netherlands and was funded by the Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

FUNDING

This present work was funded by the Malaysian Agricultural Research and Development Institute (MARDI), Selangor, Malaysia.

DATA AVAILABILITY

All data generated during this study are included in this published article and its supplementary information files.

AUTHORS' CONTRIBUTION

Umi Kalsum Hussain Zaki: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Laura Trijsburg:** Supervision, Conceptualization, Investigation, Writing – review & editing. **Edith Feskens:** Supervision, Conceptualization, Investigation, Writing – review & editing.

Ethics approval - Not applicable

Consent to participate - Not applicable

Consent for publication - Not applicable

Declaration of Competing Interest - The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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6

CHAPTER 6

General discussion

There is widespread agreement that the dynamics of modern food systems are a crucial driver of unhealthy diets and related non-communicable diseases such as obesity, which are major causes of death and disability worldwide[1]. The use of plant-based foods is an important part of the current recommendations to improve health and, at the same time, limit the diet's impact on the environment. Phytoestrogens could be one of the plant-based components that exert an anti-obesity effect[2-4]. However, to further understand the impact of phytoestrogens on health, their bioavailability, as well as the role of food processing, needs to be investigated.

The aim of this thesis was to assess the intake and bioaccessibility of phytoestrogen compounds as well as their relationship with overweight and obesity indicators. Lignans were selected as the class of phytoestrogens for further investigation. The influence of processes such as pickling, fermentation, germination, and tea brewing at different time intervals on lignan content was evaluated. Several experiments and studies were conducted. These included a systematic review of the literature to identify the class of phytoestrogens that shows the most potential evidence in relation to obesity **(Chapter 2)**, the assessment of the lignan content in foods that Malaysians commonly consume, and the effect of bioprocessing techniques **(Chapter 3)**, *in-vitro* models of gastrointestinal digestion and colonic fermentation to study the behaviour of lignans in the digestive tract **(Chapter 4)**, and for a Dutch population-based dietary intake study assessing lignans intake from the usual diet in relation to obesity indicators **(Chapter 5)**.

The main findings from each chapter will be highlighted and interpreted in the context of the broader literature. Main methodological issues will also be discussed. Finally, potential future research directions will be discussed.

MAIN FINDINGS AND INTERPRETATION

Systematic review on phytoestrogens (lignans and isoflavones)

The findings of the studies described in this thesis are schematically summarized in Figure 1. Lignans and isoflavones are two major groups of phytoestrogens whose intake is associated to body weight reduction[5-10]. However, the mechanism of action of lignans and isoflavones linked to obesity are still not completely clear. Thus, in **Chapter 2**, a systematic review on lignans and isoflavones and their association with overweight and obesity was carried out.

Lignans and isoflavones exist in the plant as glycosides, and are converted to active forms by microbiota in the intestinal environment[11-13]. The microbiota is required

for lignans and isoflavones to exert their full bioactivity, which is linked to its beneficial health effects. The effect of various processing techniques and particle size reduction of lignans or isoflavones on their bioaccessibility, release, and conversion has been further investigated in **Chapter 4** using simulated *in-vitro* digestion models.

In Chapter 2, data from human and animal studies were reviewed. Fifteen studies on lignans and thirteen studies on isoflavones, including human, animal, and *in-vitro* studies, were retrieved and reviewed on their association with body weight and obesity. The reviewed studies demonstrated significant weight loss effects in both human and animal studies. Lignans were selected for further investigation, as overall, the review showed a greater potential for on weight reduction compared to isoflavones. Thus, this chapter served as a starting point for evaluating lignan content in various foods and assessing processing effects and its bioaccessibility.

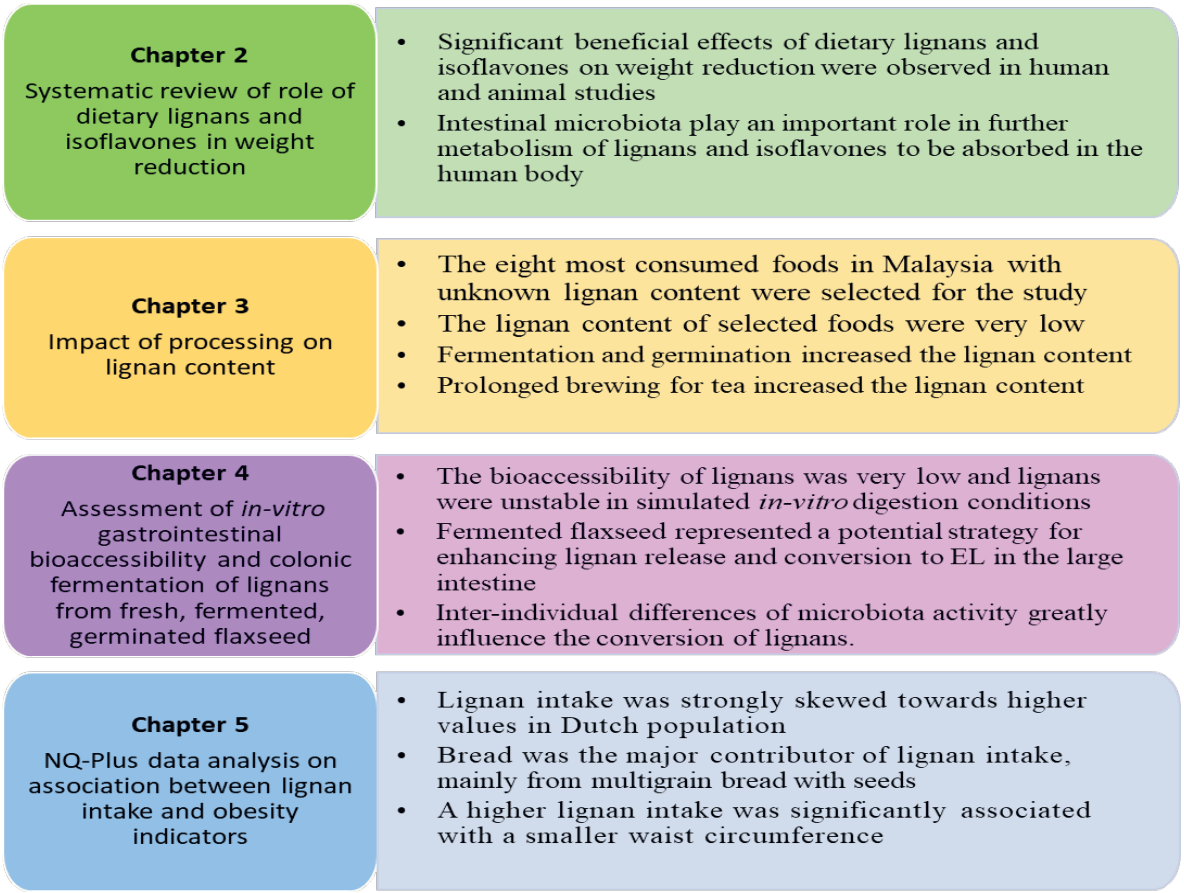

Figure 1: Overview of this thesis and its main findings

Expanding the knowledge of the lignan content of food and its effect on processing

Interestingly, we discovered a lack of data on lignan content in Malaysia's most commonly consumed foods. Plant-based foods were selected based on the National Health Morbidity Survey, Malaysia Adult Nutrition Survey (MANS), conducted in 2014[14]. The chosen foods were among the ones for which the highest intake (g/day) was reported in each food group: leafy green vegetables, green vegetables, legumes, other types of vegetables, and beverages. Pickling, germination, fermentation, and brewing were applied to specific subsets of the samples. Lignans were measured by a validated LC-MS/MS method[15-17].

Furthermore, we gathered information on the effect of processing on lignan content in the systematic review **(Chapter 3)**. We did not find available data on the effect of several bioprocessing techniques on lignan content. Bioprocessing is a green technology that uses enzymes and/or microorganisms to extract, purify, and produce food and food ingredients[18]. Fermentation, germination, and pickling are some of the traditional food bioprocessing methods that have been suggested to influence phytochemical content[19-21].

Bioprocessing is also a strategic approach for Malaysia since these methods are low-cost while saving time, and the food can be easily prepared at home. Therefore, the influence of different bioprocessing techniques on lignan content was further evaluated in **Chapter 3**.

Shallot (*Allium cepa*), celery (*Apium graveolens*), green Chinese mustard (*Brassicca juncea*), and long bean (*Vigna unguiculata*) were selected for pickling, and the samples were collected after five weeks of pickling. Pickling showed a significant decrease in the lariciresinol content by approximately 86%. To the best of our knowledge, this is the first study to explore the lignan content of pickled foods.

The decline of lignan content in pickled foods could be due either through degradation by endogenous polyphenol oxidase[21] or through leaching out of the brine[22]. Further analysis of lignan stability in acid and salt solution after prolonged storage would be useful to understand the mechanism of lignan reduction in pickled foods.

Other than bacteria, fungi are also involved in food fermentation. Meat, fish, vegetables, milk, and cereals are some of the foods that are typically fermented with fungi[23]. Tempeh is a precooked fungal fermented bean, bound together by the mycelium of a living mould (mostly *Rhizopus* spp.), which provides many health benefits, including

enhancing protein digestibility[24]. Moreover, *Rhizopus* spp can produce extracellular enzymes[25,26]. In addition, it could tolerate and grow in any adverse environment[26]. *Rhizopus oryzae* is one of the filamentous fungi used in fermentation of agricultural by-products[27].

In our study, mung beans, soybeans, and a mixture of flaxseed and mung beans were fermented with the fungi *Rhizopus oryzae* until a compact white mycelium was formed. Our findings showed that fermentation increased lignan content in a mixture of flaxseed and mung beans (799.9 $\pm$ 67.4 mg/100g DW) compared to the unfermented counterpart (501.4 $\pm$ 134.6 mg/100g DW). In contrast, the fermentation of soybeans and mung beans did not significantly affect the SECO content. As far as we know, no data on the lignan content of fermented products by *Rhizopus oryzae* fermentation is available. Nevertheless, a study proved that the phenolic acid level was remarkably higher in oat fermented with filamentous fungi than in non-fermented oats[28]. The production of cell wall-degrading enzymes by fungi[29] could explain the increase of lignan content in fermented foods.

Germinated beans, also called bean sprouts, are common vegetables, mainly used in Asian countries[30]. According to the Malaysian Adults National Survey 2014 (MANS 2014), the estimated mean intake of bean sprouts was 7.03 g/day and was listed as 4th in the 'other vegetable group'[14]. Germination is a low-cost and effective technology for improving the nutritional quality of grains or seeds[31].

In a study **(Chapter 3)**, germination increased lignan content, which reached its peak on day 6 of germination for all the tested matrixes. Our results are in accordance with a recent study that found that the lignan content of germinated flaxseed increased by approximately 6-fold on day 8 of germination[32].

Based on our data, we calculated the lignan intake of fermented soybean (tempeh) and germinated soybean (bean sprout) in Malaysia. For fermented soybean, we arrived at a value of 0.01 mg/day, and for germinated mung beans (bean sprout), this resulted in an estimate of 0.02 mg/day.

Furthermore, we investigated lignan content of tea brewing at different time intervals. In Malaysia, tea is the second most popular beverage after plain water, with an average of 160 mL consumed[14]. Tea is one of the main contributors to lignan intake in the United Kingdom[33] and the highest contributor to lignan intake in the Dutch population[34]. Hence, tea could potentially be an important lignan source in the Malaysian diet.

We used black tea since this is generally consumed in Malaysia. As shown in **Chapter 3**, lignan concentration increased with brewing time, reaching its highest concentration at 10 minutes. Our results can be used to promote the best way to drink tea while maximizing its potential health benefits. Based on our data, we calculated the lignan intake in the Malaysian population. On average, the lignan intake from tea consumed by the Malaysian population could increase to 0.05 mg/day with two minutes of brewing time. Then, the lignan intake could be increased to 0.16 mg daily when the brewing time reaches up to 10 minutes. Different types of tea may contain different levels of lignans. A study showed that black tea has two times higher lignan content than green tea[35]. Nevertheless, we expect that the effect of brewing would be the same irrespective of the type of tea investigated.

In summary, in **Chapter 3**, we provided evidence that fermentation and germination are potential processing methods to increase the lignan content in food products, as did a prolonged brewing time for tea. On the other hand, pickling decreased the lignan content in foods. Our findings widen the range of known lignan content in foods. Our data can contribute to the well-known Phenol-Explorer database, the first comprehensive database on polyphenol content in foods (http://phenol-explorer.eu). Moreover, our results fill the gap in knowledge on lignan intake in Malaysia by providing data on some frequently consumed products. Aside from that, this information is useful in educating farmers and consumers on how to increase the lignan content of the foods that they have readily available, e.g., by selling and consuming germinated and fermented products. Further study of other potential beans, seeds, or foods that can be fermented or germinated to enhance their lignan content can also be recommended for foods outside Malaysia.

The behaviour of lignans in the gastrointestinal tract and its effect on the food matrix

In conjunction with findings obtained from Chapter 3, we studied the effect of bioprocessing on bioaccessibility and gut microbiota conversion in enterolignans using flaxseed as the most abundant source of lignans (**Chapter 4)**. The idea is that processing can affect not only the content but also the bioavailability of lignans. Therefore, in this chapter, we reported a systematic investigation of lignans bioaccessibility from fresh, fermented, and germinated flaxseed, and the effect of particle size using simulated *in-vitro* gastrointestinal models with 3 digestion phases (oral, gastric, and intestinal).

Moreover, the release of lignans and their conversion in enterolignans in the large intestine was investigated via *in-vitro* colonic fermentation with human fecal

inoculums. In addition to the flaxseed matrixes, tea brew was used as a representative sample to investigate lignan stability and interactions with digestive fluids.

Bioaccessibility refers to the quantity of bioactive compound released from the food matrix in the gastrointestinal phase, which eventually becomes available for small intestine absorption[36]. Chemical structure, food matrix properties, host physiological conditions (gastric emptying and intestinal transit time), and food processing strategies impact bioaccessibility[37]. To our knowledge, there have been few studies on the effect of processing on lignan bioaccessibility.

It has been reported that reducing particle size in plant foods substantially increases the protein digestibility in yellow and black soybean pellets[38]. Another study reported that the bioaccessibility of ferulic acid increased as the particle size of wheat bran decreased[39]. Similarly, the total phenolic content and antioxidant activity significantly increased for smaller particle sizes of carob flour[40]. Thus, it is suggested that particle size reduction can improve the release of phytochemicals from the matrix and possibly enhance their bioaccessibility in the gastrointestinal phase[41, 42]. Reducing particle size increases the fraction of broken cells, thus, allowing a greater proportion of the intracellular content to be absorbed[43, 44].

Our study found that SECO was detected in fresh, germinated, and fermented flaxseed; PINO and MATA were detected in tea brew. The bioaccessibility of lignans in fresh, germinated, and fermented flaxseed reached approximately 1% or less. The particle size and processing significantly affected the bioaccessibility of SECO ($P < 0.001$). Conversely, no interaction between particle size and samples was observed ($P = 0.142$). In tea brew, the bioaccessibility of lignans was about 88% in the oral phase, before decreasing to approximately 25% in the intestinal phase. A study of tea showed a decrease in flavanols which are very prone to oxidation and disappear in the intestinal phase during *in-vitro* digestion[45]. Thus, the aforementioned study could explain the lignan decrease in our findings, possibly due to oxidation in the intestinal phase.

Further investigation was done on the released lignans and conversion rate in the *in-vitro* colonic fermentation phase. Fermented flaxseed produced by far the highest release of SECO, approximately 65%. Then, we further observed the conversion of lignans to enterolignans. Only EL was detected in all samples. A study showed that ED was rapidly converted to EL via oxidation[46]. Similarly, other findings reported that microbiota could metabolize SECO to ED and then quickly convert to EL[47]. Thus, our findings are in accordance with the studies as mentioned earlier and could explain EL detection, possibly due to rapidly conversion of ED to EL.

Next, we observed that the fermented flaxseed has the highest EL at all fermentation times, ranged between 0.5% to 1.0%. Moreover, the conversion of lignans in tea brew was relatively high, approximately 15%. A possible explanation for the higher lignan conversion in tea is that MATA can be directly converted to EL. In contrast, SECO must be converted to MATA or ED before converting to EL[48]. In addition, the low conversion of EL in flaxseed samples is probably due to soluble fibre. The soluble fiber fermentation, such as mucilage, may have delayed the microbial conversion of lignans[47].

We further calculated the amount of lignans to which microbiota had been exposed during the fermentation analyses. Germinated flaxseed gave the highest with a value of 15.1 mg lignans, followed by the fermented flaxseed, with a value of 11.1 mg lignans (in 1 g of fermented material). Fresh flaxseed had the lowest lignan content after colonic fermentation, with a value of 3.0 mg. Our results could explain that the difference in EL conversion yield could be due to the different matrix levels of lignans. The amount of lignans in 10 mL of tea brew used in the fermentation was several orders of magnitude lower (0.003 mg) compared to the flaxseed matrixes. Hence, we can speculate that the microbiota capacity may have been saturated by the high lignan concentration for the lignan conversion to EL.

Inter-individual differences are probably caused by the differences in microbiota and, based on the amount of metabolites produced, can be classified as "high producer", "moderate producer," and "low producer"[49, 50]. In the present study, the variability observed in the conversion of lignans to enterolignans is high. There was a nearly 5-fold difference in EL production between the highest and lowest producers of fresh and fermented flaxseed. Another human study also reported that the variability in lignan conversion is due to inter-individual differences in microbiota metabolism[51].

Lignan intake and its association with obesity indicators

Beyond assessing the lignans' bioaccessibility in the *in-vitro* study, we also assessed the relationship between lignans and obesity indicators by exploring how the dietary pattern of lignan food consumption was associated with obesity. In **Chapter 5,** the associations between lignan intake and obesity indicators, body mass index for general overweight or obesity, and waist circumference for abdominal obesity were examined using a real-life prospective cohort study in the Netherlands, "Nutritional Questionnaire plus" (NQplus).

The lignan intake of the study population ranged from 40-50500 μg/day, which was strongly skewed towards higher values. The median lignan intake was 3226 μg/day (mean 4763 μg/day, SD 5386 μg/day). As expected, the values are skewed due to some foods being high in lignan content. Multigrain bread with seeds contributed nearly half of the population's lignan intake. On top of that, flaxseed consumption resulted in a high lignan intake with a contribution of 19.6%. In other studies, lignan intake ranged from 200 to 9100 μg/day[52-54], and our findings were comparable. The vegetarian and vegan UK population reported the highest lignan intake since their intake is mainly derived from lignan-rich plant-based foods[53]. The Latvian population also had a high lignan intake, estimated at 4300 μg/day, as grain products accounting for 86% of it[54].

Three multiple regression models were built to examine the association between lignan intake and BMI and waist circumference. The results showed that a higher lignan intake is significantly associated with a smaller waist circumference in Dutch adults (P= 0.042 in the full model). The regression coefficient adjusted for all potential confounders amounts to -0.93 (95% CI: -0.88; -1.00), indicating that the waist circumference decreased with 0.93 cm upon a 1-standard deviation (1-SD) (1.24 μg/day) higher lignan intake. No association between BMI and lignan intake was observed. The inter-individual differences in converting lignans to enterolignans, as reported in Chapter 4, could partly explain the low associations between lignan intake and BMI. Notably, the findings in this chapter could be valuable in exploring how dietary patterns of lignan food intake impact health, particularly obesity.

Several studies have suggested that lignans reduce body weight and fat accumulation[55-57]. The mechanism responsible for the impact of lignans on weight reduction and lipid metabolism includes suppression of adipose tissue growth, inhibition of pre-adipocyte differentiation, stimulation of lipolysis, and induction of apoptosis of existing adipocytes, thus reducing adipose tissue mass[58, 59]. Interestingly, phytoestrogens, including lignans, are known to inhibit the activity of peroxisome proliferator-activated receptor-gamma (PPARγ) and activate the protein kinase (AMPK) signaling pathway, associated with fat and energy metabolism[60-63]. The molecular pathways need further elucidation.

METHODOLOGICAL CONSIDERATIONS

Extraction and lignan analysis by LC-MS/MS

We followed the validated lignan analysis method by Milder et al., Norskov et al.; and Peñalvo et al.[15-17] with slight modifications in **Chapters 3** and **4**. In our study, we achieved good chromatographic separation for SECO (retention time: 6.2 mins), LARI (6.4 mins), PINO (6.9 mins), MATA (7.3 mins), EL (7.5 mins), and ED (6.6 mins) at a short run time of 12.5 mins compared to Milder's method. Moreover, PINO generated a signal only when positive ionisation mode was used, which was discovered to be different from other studies. The PINO ionisation mode was found to be an added value since it gave the high sensitivity of PINO detection.

We also calculated the matrix effects (ME) as an indicator of ionization suppression or enhancement. The ME is defined as an unexpected suppression or enhancement of the analyte response due to co-eluting matrix constituents. ME values calculated from the peak area for each analyte spiked in an extracted sample is divided by the peak area for each analyte in a standard solution of known concentration[64]. The ME ranged from 99.1 to 126.6% in the dried samples, while the ME ranged from 111-134% in the digestive fluids. The results indicate the influence of possible ionization competition between the analyte and components present in the matrix[65].

Plant lignans occur bound to one or more sugar moieties as glycosides. Lignans are usually measured after hydrolysis to aglycones since not all glycosides are known. Enzymatic extraction with β-glucoronidase/sulfatase was performed to degrade the glycosidic bonds and release the lignan aglycones[66]. Thus, only aglycones could be measured with this method. In regards of our study, the secoisolariciresinol diglucoside (SECO glycoside) could not be detected since it had been degraded to SECO (aglycone form). In addition, we combined alkaline hydrolysis and enzymatic extraction to optimize the extraction recovery. Combining alkaline hydrolysis and enzymatic extraction improved lignan yield[15].

Bioaccessibility of lignans during gastrointestinal digestion and fermentation process

In Chapter 4, *in-vitro* methods were used to investigate the bioaccessibility of lignans. We applied a static simulated *in-vitro* gastrointestinal digestion. Food samples are digested sequentially simulated oral, gastric, and intestinal phases, with parameters such as electrolytes, enzymes, bile, dilution, pH, and digestion time based on physiological

data[67]. The static *in-vitro* digestion models are useful in forecasting *in-vivo* digestion outcomes. A study showed that *in-vivo* and *in-vitro* protocols have a comparable peptides profile; hence the *in-vitro* digestion proved to be a good approximation to the physiological of gastrointestinal digestion[68].

Nevertheless, the *in-vitro* gastrointestinal digestion method has some limitations. Firstly, the system lacks tissues such as epithelial mucosa and variable pH throughout the gastrointestinal phase. Hence it can not mimic the dynamics of the digestion process or the physiological interactions with the host[69]. Moreover, we used Greiner tubes as the gastric and intestinal phase material, which is far from the actual stomach. Soft materials such as silicone might be more suitable as an added value to make *in-vitro* gastrointestinal closer to the real one[70].

Then, *in-vitro* simulated colonic fermentation protocol for further understanding the release of lignans relates to the microbiota and conversion to EL. It has been used to study gut microbiota fermentation of different food matrixes, giving insight into their ability to degrade particular substances, which can contribute to developing personalized nutrition strategies. Furthermore, batch fermentation can help explain the metabolic routes and intermediate metabolites that appear by exposing gut microbes to specific compounds. As a result, this could provide signs on how to direct microbial metabolism toward a specific goal, thus, bringing gut microbiota modulation through diet one step closer[71]. The batch fermentation allows many samples to be studied at once in a short time, at low cost, is simple to set up, and is handy for determining metabolite profiles produced by fecal microbiota that consume diet components[72-74]. On top of that, no animal or human ethical clearance is needed.

But there are also some limitations which should be acknowledged. The host colon function is only partially simulated due to the accumulation of some microbiota waste products; hence, microbiota growth could probably be affected[71,75]. *In-vivo*, the intermediate metabolites can be absorbed and conjugated by enterocytes[76] before they are further converted; thus, the overall pathway could be different. Moreover, the effects on microbiota composition and its metabolic activity are solely due to the applied experimental treatment[77], therefore may influence the production of enterolignans. Furthermore, even when using the same medium in the analysis, concentrations in the buffering solutions, nutrients, fecal material source, and fecal slurry concentration vary across experiments[71]. Additionally, when the previous *in-vitro* gastrointestinal digestion is carried out using different protocols across analysis, it will result in different amounts of digestion components available for colonic fermentation, which could influence the results.

Despite the fact that simulated *in-vitro* gastrointestinal digestion and colon fermentation models cannot directly mimic *in-vivo* conditions, these models may be useful for investigating the effect of the food matrix bioaccessibility in order to obtain critical data to support claims of the biological relevance of compounds in the context of nutrition and human health[78].

Dietary lignan intake and its association with obesity

Self-report dietary assessment methods are well known to suffer from measurement errors, which reduce the precision of intake estimates and reduce statistical power to access potential associations. The measurement errors will often lead to underestimating diet-health outcomes[79-81]. Random errors, such as day-to-day variation in food intake, could be reduced by applying repeated measurements. Multiple 24hR recalls are an example of repeated measurements. Consequently, we calculated the estimated lignan intake in a Dutch populations in the Netherlands using repeated 24hR in **Chapter 5**. Participants with at least two 24hR were included in this study.

Average daily dietary intake was calculated by multiplying the consumption frequency by the portion size and nutrient content in grams[82]. Only foods with available lignan content was selected for inclusion in the dietary intake. We used the Dutch plant base foods database[35], and to increase the accuracy of the result, we complemented the lignan data with another three references (Gunter G.C. Kuhnle 2009, Thompson, 2006 and Penalvo, 2007)[83-85]. 90% of our 209 selected foods were extracted from the Dutch lignan database solely developed for the Dutch population study. Moreover, a validated LC-MS/MS method was used by Milder et al. to quantify the lignan in processed and prepared foods[15].

In the current thesis, the estimation of lignan food intake was achieved from an existing free-living cohort (NQ-plus study), with distinct advantages and limitations over an intervention approach. Using a free-living cohort with complex and uncontrolled diets could be more efficient than conducting an intervention study for each food of interest.

Nevertheless, the results should be considered in light of some limitations. The metabolic conversion of lignans in the body was not considered, as this is a typical observational study. It did not reflect the amount of compounds reaching the target organ after digestion, absorption, and metabolism. Furthermore, residual confounding from unknown or unmeasured factors, as well as confounding from other dietary

constituents, cannot be completely ruled out despite the confounding factors for which the regression models were adjusted.

Three multiple regression models were built to assess the association between BMI and waist circumference with total lignan intake in **Chapter 5**. A number of factors were considered in the adjustment of the models that could confound the association. Age, gender, education, smoking status, ethanol consumption, physical activity, energy intake, and intake of fat, protein, fibre, and carbohydrates were listed as potential risk factors for obesity and may be correlated with lignan intake. The model adjusted for confounding lifestyle variables was Model 1, while model 2 included energy intake and nutrient variables. In the full model, model 3, the combination of all potential confounders were used.

There was no association between BMI and lignan intake, while a higher lignan intake was significantly associated with a smaller waist circumference. Several studies are in concordance with our findings and reported that the intake of lignan has a potential of waist circumference management[3, 86]. There can be several interpretations for these findings. Our baseline characteristic findings showed no significant difference between persons with low, normal and high BMI and their lignan intake. The categories of BMI could be more specific, e.g., underweight (BMI <18.5) and obese (BMI >39.9), since these categories have different microbiota compositions that might influence the lignan conversion. BMI is also affected by fat-free mass, not only fat mass, and hence waist circumference may have been a better outcome meausure as this is related to visceral or abdominal obesity. The use of a food frequency questionnaire (FFQ) could also be a recommendation for future observational studies. The FFQ is one of the most common dietary assessment instruments that might better capture the habitual food and nutrient intake in large populations[87]. Thus, the FFQ could help extend the assessment of lignan intake associated with obesity.

The health benefits of lignan foods could be attributed to other components in the food matrix such as carbohydrates, proteins, and lipids; we did not take the bioavailability of lignans into account. Further research on the association between lignan intake and enterolignan production using serum and urine could be explored to investigate the bioavailability of lignans and the correlation between the intake and health benefits. The potential mechanism of action underlying this association might be explained by the improvements observed in gut microbiota. Hence, a more comprehensive database and consensus studies with obesity indicators in a longitudinal design are warranted to delineate further the role of lignan intake in obesity.

FUTURE RESEARCH AND CONCLUSION

This thesis adds to the current state of the literature on phytoestrogen compounds with added information on lignans and their association with obesity. Simultaneously, several knowledge gaps were revealed in enhancing the lignan content in various food processing and influencing its bioaccessibility, release and conversion to enterolignans. Moreover, the understanding of their intake in association with obesity was discovered. Potential future research directions will be presented together with their implications for public health, the food industry, and consumers.

Expending further food processing technologies, such as encapsulation, a combination of germination and fermentation, fermentation of foods with other fungi (eg; *penicillium* and *monascus*), and fermenting and germinating other seeds, beans, or foods, could be further investigated to increase lignan content and its bioavailability from selected foods. Encapsulation can improve bioaccessibility and bioavailability by coating the compounds, targeting delivery to a specific part of the digestive phase, and controlling its release[88]. The data generated expands the lignan content database for more accurately estimating the dietary intake of enterolignan precursors. Documenting the lignan content of new innovative fermented foods could open new research avenues for understanding how their consumption affects health and diseases, specifically obesity. Additionally, such information could benefit the food industry by developing enhanced functionality products. Furthermore, we can manipulate the food matrix through formulation or processing to modulate lignan conversion to EL in the large intestine.

The variation in microbiota between individuals may be useful in future personalized nutrition initiatives and tailored dietary advice. Further targeted studies are needed to better understand the gut microbiota and its interactions with host health, opening up new paths for personalized nutrition within plant-based diets. Moreover, a comprehensive understanding of the role of specific gut-derived metabolites and gut microbiota can lead to developing preventive strategies for obesity. Targeted microbiota which acts specifically to inhibit the activity of PPARs and AMPK signalling pathway on energy metabolism, could lead towards weight reduction.

Given that the NQ-Plus population is relatively healthy, investigation in a larger cohort or nested case-control study would also present a larger gradient of obesity indicators between participants, which may be helpful for observing associations between food lignan intake and obesity. On top of that, a comprehensive study needs to be implemented with an increased number of 24h recalls enhancing the correlation between the day-by-day correlation of lignan intake. Moreover, further research could

use the FFQ since it is typically designed to measure the usual food intake. The present study set up could also be repeated in Malaysia to investigate the lignan intake in Malaysia associated with obesity and other health problems.

All in all, the strategies mentioned above could only be implemented with good teamwork between government, industry, professional bodies, non-governmental organizations, communities, and individuals that could help battle obesity globally. As a scientist, we need to educate the general population in a simple yet understandable way on how to incorporate scientific knowledge into daily life. For example, on how to increase lignan intake by promoting the consumption of fermented, germinated foods and longer time of brewing. Apart from that, the present research on processing techniques could help develop low-cost and time-saving food products with beneficial properties.

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Summary

Obesity represents one of the most significant health and socioeconomic burdens to modern society. Lignan foods are commonly consumed in diets worldwide and can be an important part of an effective strategy to help prevent and manage obesity. However, precise associations between the consumption of lignan foods and obesity have not been reported extensively. Furthermore, the insight in the impact of different processing methods on bioaccessibility and conversion to gut microbial metabolites that are more bioactive is lacking. Therefore, this thesis aimed to look at the definition of phytoestrogens, particularly lignans, the lignan content of various foods, their bioaccessibility, and conversion into microbial metabolites in the human large intestine. Further, we assessed the lignans food intake in relation to obesity indicators in a Dutch population.

Firstly, to capture the existing phytoestrogen compounds in regard to obesity, a systematic review was conducted, and the result is presented in **Chapter 2**. The lignans and isoflavones studied showed significant weight loss effects in both human and animal studies. As a result, lignans were chosen for further investigation since the review revealed that they have a more significant potential influence on weight loss than isoflavones.

In **Chapter 3**, the influence of different bioprocessing techniques on lignan content was further evaluated. The plant-based foods were selected based on data from The National Health Morbidity Survey, Malaysia Adult Nutrition Survey (MANS) conducted in 2014: shallot, celery, green Chinese mustard, long bean, mung beans, soybeans, flaxseed and tea. Pickling significantly reduced the content of lariciresinol by approximately 86%. Conversely, fermentation and germination increased lignan content. Moreover, the lignan content increased with prolonged brewing in tea. Thus, fermentation, germination, as well as prolonged brewing of tea have shown to be potential techniques to improve the lignan content in foods.

The analysis of the lignan foods was further expanded in **Chapter 4**, where the effect of bioprocessing on bioaccessibility and gut microbiota conversion in enterolignans using flaxseed were studied. In addition, tea brew was used as a representative sample to investigate lignan stability and interactions with digestive fluids. The bioaccessibility of lignans was very low (1% or less), and lignans are unstable in simulated *in-vitro* digestion conditions in all fresh, germinated, and flaxseed for both fine and coarse particle sizes. The particle size and processing significantly affected the secoisolariciresinol (SECO) bioaccessibility. Fermentation, on the other hand, could be a viable strategy for increasing lignan release and conversion to enterolactone (EL) in

the large intestine. Moreover, the inter-individual microbiota influenced the conversion of lignans to EL.

Finally, in **Chapter 5**, the lignan intake association with obesity indicators (body mass index and waist circumference) were evaluated in a Dutch adult population using a real-life prospective cohort study, "Nutritional Questionnaire plus" (NQplus). Bread was the most common source of lignans, followed by nuts, seeds, snacks, and vegetables. A higher lignan intake was associated with a smaller waist circumference also when adjusted for potential confounders. Whether lignans can reduce waist circumference in obese people or prevent the onset of abdominal obesity must be evaluated in future intervention studies.

Collectively, the findings from these chapters indicate the potential for further exploring the lignan content in foods, and processing techniques to increase the bioaccessibility and conversion of lignans to enterolignans. The current research could aid in the development of low-cost and time-saving food with manifold healthy properties.

Acknowledgments

Everything happens for a reason. Trust your believer. His plans are the best!

My 4 years PhD journey has come to an end. I have not travelled in a vacuum during this journey. I am blessed to be encircled by great supporters and a positive atmosphere throughout my journey.

First and foremost, I would like to thank my thesis supervisors for paving the way for me to pursue my studies at WUR.

Prof. dr. Edith J.M. Feskens: I remember meeting with you during your Christmas break, and from that, you permitted me to pursue my PhD under your wing. No words can describe my feelings since a great professor had time to receive my call from Malaysia. Thanks for your warmth, thoughtfulness, and guidance throughout my journey. When I told you that I was not good enough to do something, you replied with a simple yet meaningful phrase, "that is why you are here." Indeed, you were right, Edith. I came far from Malaysia to the Netherlands to challenge myself and leave my comfort zone. I am comfortable with you not only about academics but also about personal and family issues.

Dr. Edoardo Capuano: I remembered you as a terrific lecturer in the Food Digestion course. I admired your lecture delivery style. Working with you and the people at Food Quality Design was an excellent opportunity. I am eternally grateful for your encouragement, mentoring, and constructive criticisms, which enabled me to progress significantly in my career.

Dr. Laura Trijsburg: I am honored to have you not only as a supervisor but as a friend. You always motivate me in any challenging situations. Whenever I need your help, I know you will be there for me. When the COVID pandemic started, I needed to do the data cleaning of NQ-Plus at home, and you guided me closely even though we were physically apart. We had a call 2-3 times a week to ensure that I performed my data analysis correctly. You continued to guide me while you were far away in Malawi. Your patience and kindness in getting things done were so adorable. Your unwavering support and backing at all times made my path one of the most mesmerizing experiences.

I would like to express my gratitude to **Christos Fryganas**, an incredible laboratory officer who helped me in the laboratory work perfectly. The lignan analysis could not have been possible without your enthusiastic work ethics. I now know how to calculate

the result from raw data and generate a nice chromatogram from LC/MS-MS. You stayed with me doing laboratory analysis until 10 pm.

I humbly would like to express my deeply indebted to my family. My Ph.D. journey won't be possible without your prayers.

My steadfast hero, **Ahmad Mursyid Ahmad Rudin**: You are my strength. You have stood by me through all my travails, my absences, my fits of pique and impatience. Thank you for granting me to pursue my study. Without your blessing, I won't be able to make it happen. Nothing can ever make up for your sacrifice. You abandoned your dream and career to ensure I 'climbed the mountain' peacefully and successfully without worry. You always remind me to check our priorities, seek blessings from parents, and be kind to them. What we have accomplished is unquestionably due to their blessings. Behind every successful woman stands a great man, and he is you.

I am immeasurably grateful to my beloved hot princes and the gorgeous princesses: **Umi Nurin Irdina, Umi Nurin Insyirah, Ahmad Najiy Ifwat, Umi Nurin Ilmanafia,** and **Ahmad Najiy Irham**. Thank you, thank you, and thank you. You are astounding and superior gifts from Allah to me. Your struggle and sacrifice to learn Dutch delighted me, and you did great in school. I am a proud mama. You helped me do house chores, ease my study and calm me when I was in trouble. Your unequivocal support and great patience at all times, for which my mere expression of thanks likewise does not suffice.

To Ma, **Hasnah,** and Abah, **Hussain Zaki**: my heartfelt thank you for your blessing and prayers for my family and me. You have been so supreme, nurtured my learning, and supported my dreams. You are my best security blanket, always available to share my joyful or challenging stretch. Abah, your wisdom words nourish my spirit.

To my in-laws: Mok, **Ramlah,** and Abah, **Ahmad Rudin**: I sincerely thank you for your unfailing prayers. Without your blessing, I might not accomplish my vision.

To my sisters: **Mariam, Zainab, Aminah,** and **Shuhada**, thank you for patiently hearing my worries and issues, listening to my secrets, and making them yours. I am blessed to have your unparalleled love, support, and prayer.

I would like to appreciate the extraordinary ladies of the secretariat of the Human Nutrition and Health Division for your being helpful and supportive; **Jeanine, Jasmijn, Gea,** and **Sjanneke**. Your swift responses to any questions are awe.

To my paranymphs: **Asrul** and **Inga**. Thank you for being there for me and helping to organize the defense and reception flawlessly. You are not only my defense paranymphs but also my entire Ph.D. journey paranymphs. You kept me calm when I was attacked by anxiety and during the hardest phase of the journey.

A Ph.D. journey is not all about research. It is a balance between work and life. This endeavor journey would not have been possible without colleagues and friends who I've crossed paths with at WUR for the friendship and bonding time: **Tesfaye, Son, Duong, Arli, Maria (Mexico), Liangzi, Taymara, Koen, Marie-Luise, Maria (Ecuador), Ursula, Katherine, Claudia, Tsitsi, Giulia, Marijke, Marion, Mahsina, Max, Ruoxuan, Anneleen, Alida, Marion, Pol, Santiago, Aafke, Perdana, Luc, Betul, Dan, Dan Kirk, Lisse, Julia, Fiametta, Ranti, Ruth,** and **Fabiola**. Thank you for the companionship and shared Ph.D. activities. Last but not least, thank you to the efficient and effective FQD technicians, **Frans, Erik, Jelle**, and **Nienke**, for your assistance made laboratory work went smoothly.

I could not have undertaken this journey without thesis defense committees: **Prof. Dr Jean-Paul Vincken, Prof. Dr. Amin Ismail, Dr. Rina Agustina,** and **Dr. Eva Corpeleijn**, who generously provided knowledge and expertise. Thank you for devoting time and invaluable patience to evaluating my thesis.

To my Malaysian family members in the Netherlands, too numerous to mention by name, thank you for being part of my relatives who shared and helped our family to stay comfortably in the Netherlands. Not to forget my extended relatives and close buddies in Malaysia, who have had unflagging support and prayer throughout my study.

Last but not least, I would like to thank my workplace institute, MARDI (Malaysian Agricultural and Development Institute), who granted me an HCCBP scholarship to pursue my study.

About the author

CURRICULUM VITAE

Umi Kalsum Hussain Zaki was born on February 20, 1980 in Tumpat, Kelantan, Malaysia. She obtained her Bachelor of Science in Food Science & Nutrition at the Universiti Kebangsaan Malaysia. Then, she continued to pursue a Master of Science in Nutrition at the same university. In 2004, Umi started her carrier as a researcher in nutrition in the Food Science & Technology Centre at Malaysian Agricultural and Research Development (MARDI).

She was involved with several nutrition committees at the national level, such as the National Coordinating Committee on Food and Nutrition and Malaysian National Health Policy. Umi received an Excellent Staff Award in 2013 and won several medals in various competitions throughout her carrier. In 2013, she was promoted to senior researcher. Umi has several publications such as a book, chapter in book, journals, newsletters, and lecture notes. In addition, she was awarded two intellectual properties (IP). Umi was involved with 17 research projects since 2004 as a project leader and collaborator. In late 2018, Umi started as a doctoral candidate at Wageningen University and Research, the Netherlands, under the supervision of Prof. Dr. Edith F.M. Feskens in the Division of Global Nutrition & Food Quality Design Department, under the supervision of Dr. Edoardo Capuano. This multi-disciplinary research project aims to investigate the lignan in various foods, its bioaccessibility and conversion, and its relationship with obesity. The result of this research is presented in this thesis. During her study, she attended several international conferences and courses; and was involved in tutoring Bachelor's and Master's students.

List of publications

Publications in peer reviewed journals

Published and accepted paper

Hussain Zaki, U.K., Fryganas, C., Trijsburg, L., Feskens, E.J.M., & Capuano, E. In-vitro gastrointestinal bioaccessibility and colonic fermentation of lignans from fresh, fermented, and germinated flaxseed, *Food Funct*, 2022. DOI https://doi.org/10.1039/D2FO02559K

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Ho, C.W., Lazim, A.M., Fazry, S., **Hussain Zaki, U.K.**, Lim SJ. Effects of fermentation time and pH on soursop (Annona muricata) vinegar production towards its chemical compositions. Sains Malaysiana. 2017 **1;46(9)**:1505-12.

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Mirfat, A. H. S., Razali, M., Salma, I., & **Hussain Zaki, U.K.** Antioxidant and nutritional values of selected under-utilised Mangifera species in Malaysia. *Indian Journal of Plant Genetic Resources*, 2015, **28.1**: 72-79.

Submitted papers (under review)

Hussain Zaki, U.K., Trijsburg, L., & Feskens, E.J.M. Association between lignan intake in different types of plant-based food and obesity indicators in the Netherlands. *Plant food for human nutrition.*

Hussain Zaki, U.K., Trijsburg. L., Kuijsten, A., Capuano, E. & Feskens, E.J.M. Beneficial role of dietary lignans and isoflavones in weight reduction: A review. *Phytochemistry reviews.*

Abstracts and presentations

Hussain Zaki, U.K., Trijsburg. L., Kuijsten, A., Capuano, E. & Feskens, E.J.M. Role of lignans in weight reduction: A review. *13th European nutrition conference*. 2019.

Hussain Zaki, U.K. Introduction to the website of Malaysian Food Composition Database (MyFCD). *Foodcomp*. 2019.

Hussain Zaki, U.K., Trijsburg, L., & Feskens, E.J.M. Association between lignans intake in different types of plant based food and obesity in the Netherlands. *Dutch nutritional science days (NSD).* 2021.

Overview of completed training activities

Name of the course/activity	Organizer	Year
Discipline specific courses and activities		
13th European Nutrition Conference (poster presentation)	The Nutrition Society	2019
FOODCOMP Course (presentation)	HNE	2019
Global One Health Symposium	HNE	2019
Nutrition 2020 Live Online	ASNutrition	2020
Nutrition and Health: Human Microbiome	WageningenX: MOOC	2020
Wageningen Food Science Symposium	FQD	2020
Dietary Biomarker Symposium: Advances, Challenges, and Future Directions in Food Biomarker Research	School of Public Health, Harvard University	2020
NF Virtual Event: Nutrition and COVID-19	British Nutrition Foundation	2020
Spring Conference 2021: Gut Microbiome and Health	The Nutrition Society	2021
The Science of Weight Loss: Dispelling Diet Myths (Introductory course)	NewcastleX: SWL101x (Online course)	2021
Nutrition 2021 Live Online	ASN	2021
6th Annual Agriculture, Nutrition and Health (ANH) Academy Week	ANH Academy	2021
Healthy & Sustainable diets: Synergies & trade-offs	VLAG	2021
Dutch Nutritional Science Days (NSD), 7th Oct 2021 (presentation)	The Dutch Academy of Food Science (NAV)	2021
General courses and activities		
VLAG PhD week	VLAG	2018
Research Data Management	WUR library	2018
PhD Workshop Carousel	WGS	2019
Essential of Scientific Writing & Presenting	Wageningen Into Languages	2019
Introduction to R	WGS	2020

PhD Competence Assessment (online training)	WGS	2021
Popular Science Writing	WSSS	2021
PhD Workshop Carousel	WGS	2021
Reviewing a Scientific Manuscript	WGS	2021

Other courses and activities

HNE-32306 Clinical nutrition research (tutor)	HNE	2019
HNE-24806 Introduction to epidemiology & public health (tutor)	HNE	2019
Proposal writing		
Paper cafe	Global nutrition	2018-2022
Chair group meeting	Global nutrition	2018-2022
Seminar, colloquia organized within a department/research group	Global nutrition	2018-2022
Committee of PhD study tour	VLAG	2021

Colophon

The research described in this thesis was financially supported by the Malaysian Agricultural Research & Development Institute (MARDI) awarded to Umi Kalsum Hussain Zaki.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Muhammad Asrullah
Printed by Digiforce | Proefscriftmaken.nl