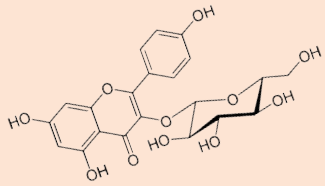
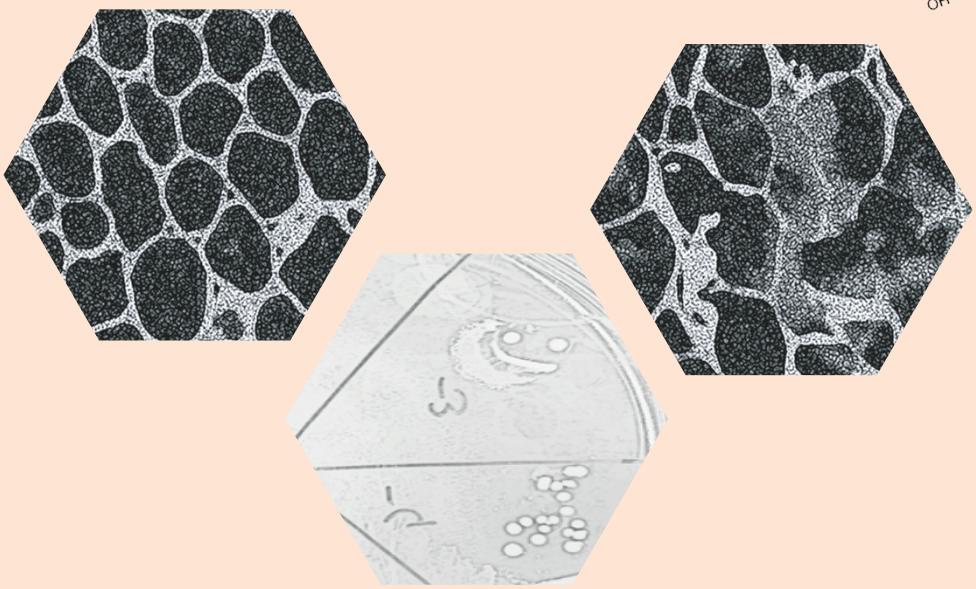
Food Matrix and Disease Prevention

Bioprocessing approaches to improve bioactivity and acceptability of pulse seeds





Elisa Di Stefano

Propositions

1. Bioprocessing can effectively be used to modulate content, profile and bioaccessibility of bioactive components in pulses. (this thesis)
2. Bioactivity of a food molecule should always be considered within its matrix and after digestion and absorption at the target site.
   (this thesis)
3. An holistic approach in food and nutrition research provides more realistic, but difficult to trace back and defend results.
4. A better exploitation of microbial metabolism scales down to a jar what is achieved by a multistage food processing plant.
5. Multidisciplinary research provides tremendous opportunities but leads to a tremendous amount of stress.
6. Where pizza is treated as a junk food, it should not be called pizza.

Propositions belonging to the thesis, entitled

Food matrix and disease prevention: bioprocessing approaches to improve bioactivity and acceptability of pulse seeds

Elisa Di Stefano

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Food matrix and disease prevention: bioprocessing approaches to improve bioactivity and acceptability of pulse seeds

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Elisa Di Stefano

Thesis

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CHAPTER 1

General Introduction and thesis outline

Introduction to the thesis

1. Metabolic syndrome epidemics and glucose regulation

Noncommunicable diseases (NCD) are responsible for 71 % of the yearly deaths globally. Cardiovascular diseases (CVD), cancers, respiratory diseases and diabetes are the most common NCD, accounting for over 80 % of the deaths.[1] The term metabolic syndrome (MetS) defines a combination of metabolic disfunctions coexisting at the same time, which significantly increase the risk of type 2 diabetes mellitus (T2DM) and CVD.[2] The International Diabetes Federation identifies MetS in the presence of central obesity together with two among (i) raised triglycerides level, (ii) reduced HDL-cholesterol, (iii) raised blood pressure, (iv) raised fasted plasma glucose.[3] Ethnicity and genetic factors, and the transition to sedentary lifestyle and an energy and sugar-rich diets are considered major contributors.[4,5] The incidence of MetS is on the rise in both developed and developing nations, affecting ~ 25 % of the adult population globally.[6] While the incidence increases with age, recent data showed a 4 – 7 % prevalence also in children and adolescents, with 1/3 young adults (aged 18 – 30) exhibiting at least one component of MetS[6,7] The factors involved in the development of MetS are complex, but visceral obesity seems to be the main trigger, and chronic inflammation and insulin resistance the major contributors to the metabolic disfunctions.[2] The syndrome progresses stepwise, and drastic lifestyle changes or therapeutical interventions can halt or even revert it.[2]

Glucose regulation and metabolic homeostasis

Glucose is a major energy source for cells and tissues, with brain and red blood cells depending exclusively on it.[8,9] An interplay of organs, hormones and physiological signals, coordinated by the central nervous system (CNS), operate in concert to maintain the constant blood glucose concentration of 90-120 mg/dl required for correct functioning of the human body.[10] The ratio between the pancreatic hormones insulin and glucagon is the main regulator of glucose homeostasis. After digestion (fasted state), the metabolism gradually transitions to a catabolic state and glucose is supplied to the circulation through breakdown of liver glycogen storage and gluconeogenesis in liver and kidneys.[11] After food consumption (postprandial state), pancreatic insulin secretion causes suppression of glucagon secretion, and a reduction in adipose tissue lipolysis, with decrease in levels of circulating non esterified fatty acids. This causes a decline in gluconeogenesis and an increase in glucose uptake and use by skeletal muscle, and dietary lipids uptake from the adipose tissue. In the liver, insulin causes a decrease in glucose synthesis, and increase in glucose uptake and storage as glycogen. After 1 hour from food consumption, about 50 % of the ingested glucose is converted to

glycogen in the liver, while the remaining 50 % is used by skeletal muscle, brain, adipose tissue and kidneys.[11]

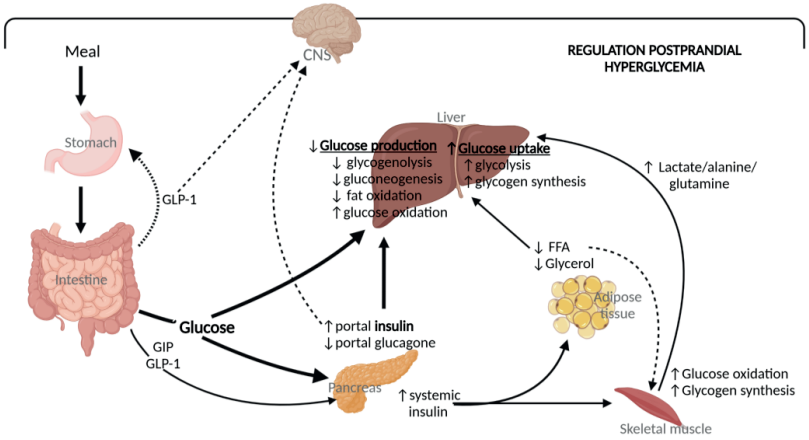

Figure 1. Postprandial glucose regulation. Figure adapted from Dimitriadis et al.[11] and Kowalski et al.[12]

1.2 The role of incretin hormones

Another important player in postprandial glucose regulation is the gut, which coordinates with the CNS the secretion of various peptides regulating gastric motility, absorption, satiety and insulin/glucagon secretion. A group of gut peptides called incretins, primarily glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are secreted within few minutes from food ingestion and contribute to insulin and glucagon regulation.[11] In presence of hyperglycemia, GIP and GLP-1 enhance glucose-induced insulin secretion from β-cells, a phenomenon known as "incretin effect", responsible for up to 60 % of the postprandial insulin release.[11,13,14] GLP-1 additionally inhibits glucagon secretion from α-cells.[14] The interest in GLP-1 arose about 25 years ago, when it was observed that its intravenous administration to T2DM patients would lead to normal postprandial insulin and glucose levels.[15] However, later studies found that GLP-1 and GIP were physiological substrates of the ubiquitous enzyme dipeptidyl peptidase-IV (DPP-IV), and that the main circulating form of GLP-1 in healthy individuals was actually the inactive cleaved form of the peptide.[16] DPP-IV is an amino-peptidase that cleaves out dipeptides from the N-terminal of its substrates. It mainly exists as integral membrane protein, expressed in the brush border of intestine and kidneys, in vascular endothelial cells, adipose tissue and other sites.[17] The active domain of the enzyme is located in the extracellular protrusion of the protein, connected to the intracellular N-terminus by a flexible section. As this can be cleaved, DPP-IV is also present as a "circulating" form (aa 49-766) in plasma and other body fluids.[18] Due to the activity of DPP-IV, the half-life of GLP-1 is of about 2 minutes and only 10 - 20 % of its active form is found in the plasma.[19,20] Moreover, the incretin effect is considerably impaired, if not completely lost, in T2DM patients.[21]

1.3 β-cells disfunction and insulin resistance in T2DM progression

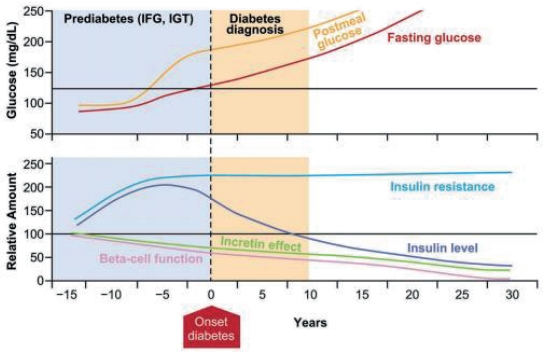

Figure 2. Onset of type 2 diabetes mellitus. IFG = impaired fasting glucose; IGT = impaired glucose tolerance. From Mazze R, et al.[22]

As previously described, postprandial glucose metabolism is a complex and tightly controlled process primarily regulated by insulin and glucagon. However, due to genetic or environmental factors, defects in postprandial insulin secretion and sensitivity can exist. Insulin resistance (IR), a condition in which insulin is unable to sufficiently stimulate glucose uptake by target tissues, and hyperinsulinemia are characteristic in people diagnosed with MetS, and are initiators of T2DM development. In fact, IR causes an increase in plasma glucose levels, triggering a compensatory response from pancreatic β-cells, which increase insulin secretion in the effort of restoring normal glucose levels. The sustained increased demand in insulin causes β-cells disfunction over time. As β-cells become unable to compensate for IR, postprandial glucose cannot be properly disposed, and glucose intolerance develops. This eventually leads to loss of β-cells number (β-cell mass) and metabolic productivity (β-cell function) and overt diabetes (Fig. 2). Differently, in type 1 diabetes mellitus, glucose intolerance is caused by autoimmune destruction of pancreatic β-cells.[23,24] IR can originate by genetic defects and lifestyle, with recent studies suggesting the possibility of a fetal metabolic programming for IR.[25,26] The involvement of physical inactivity and lipid oversupply have been well documented, with a recognized chronic inflammation caused by proinflammatory cytokines secreted by the adipose tissue being a major driver of metabolic disfunctions.[23]

1.4 Approaches for T2DM management

The management of diabetes mellitus focuses on normalizing postprandial glucose levels. In type 1 diabetes mellitus, as β-cells are destroyed, the only available treatment is the provision of insulin. In the case of T2DM, oral drugs have been developed to enhance the insulin response by targeting different mechanisms (Supplementary table 1). However, this “curative” approach causes individuals and nations substantial costs from chronic dependence on medical treatments and decreased productivity, exacerbating economic inequality in societies and across countries.[27,28] Conversely, primary interventions are considered the most powerful approach for decreasing MetS and T2DM incidence, with tremendous decrease in the economic burden, and should become a major target of public health policies worldwide.[28] Physical inactivity and unhealthy dietary patterns are the major modifiable contributors to MetS development.[29] The combination of the so-called Western diet, characterized by processed foods rich in saturated fats, salt, refined cereals and sugars, and low in fruits and vegetable, and sedentary lifestyle are considered as the main drivers of the global epidemic of MetS.[30,31] Animal studies showed that a cafeteria style diet, rich in human ultra-processed foods and sugary beverages, was effectively inducing MetS in a short period of time.[32] On the contrary, diets rich in whole grains, nuts, legumes, complex carbohydrates, fresh fruit and vegetables such as the Mediterranean and DASH (Dietary Approaches to Stop Hypertension) are inversely associated with T2DM and MetS development.[29,33,34] Bioactive constituents of these diets, such as polyphenols, unsaturated FAs, fibers and plant proteins seems to be majorly involved in the observed benefits. Legumes seeds alone, a rich source of bioactive phytochemicals, complex carbohydrates, plant-

proteins, and low in fat, have vastly been related to amelioration of glucose and lipid metabolism, with direct implications on many components of the MetS in animal models.[35,36]

Pulses: an underutilized valuable food

Belonging to the *Fabaceae* family, pulses are the legume crops harvested for their dry seed only, and not for their edible oil.[37] Pulses such as cowpea, common beans and lentils are traditionally included as staple foods in Southeast Asia, Sub-Saharan Africa and Latin America diets, being the main protein source in many developing nations.[38–40] Their connotation of "protein of the poor man" in the Asian culture, and an erroneous perception as low quality food sources hinders their consumption in richer and Western nations.[41] The global westernization of diets and economic transition further favoured animal-proteins over pulses, further decreasing their consumption.[42] Yet, increasing concern for environmental impact of animal products is driving consumers interest towards plant-proteins. Pulses have a great potential in this context, considering the high protein content (~ 20-30 % of dry weight) and their excellent macro- and micronutrient compositions (Table 2). Although the limitation in the amino acid methionine lowers their protein quality score compared to animal sources, their culinary combination with the methionine rich and lysine poor cereals often encountered in traditional foods compensates this deficiency, providing a higher protein quality profile.[43]

	Chickpea (*Cicer arietinum*)	**Faba bean (*Vicia faba*)**	**Bean (*Phaseolus vulgaris*)**	**Lentil (*Lens culinaris*)**	**Pea (*Pisum sativum*)**
Macronutrients (g / 100 g dry weight)					
Carbohydrates	50.7 – 57.3[44]	42.2-47.3[45]	51.4 – 57.2[44]	52.3 – 60.5[44]	52.5 – 52.6[44]
Total starch	41.4 – 45.0[46,47]	27.0 – 50.0 [48,49]	31.9 – 36.2[50]	43.7 – 4.79[46,51]	36.9 – 49.0 [52,53]
RS	3.4 -4.4[44,54]	17–29 % of starch[49]	2.9 – 9.1[44,55]	1.6 – 4.3[44,51]	2.1 – 6.3[44,53]
Soluble dietary fibers	0.0 - 1.8[44,47]	0.55 – 4.7[56,57]	1.5 – 3.2[44,55]	1.3 – 20.0[44,51]	1.73 – 5.5[44,56]
Insoluble dietary fibers	13.9 – 28.8[44,47]	9.1 – 15.9 [56,57]	18.4 – 20.1[44,55]	11.4 – 20.3[44,51]	10.00 – 20.7[44,56]
RFOs	7.4–7.5 (Raf: 0.4-1.2; Sta: 2.0-3.6; Ver: 0.6-4.2)[58]	3.1-15.0 (Raf:0.1 – 3.9; Sta:0.7 – 13.7; Ver:1.7 – 15.0)[57,58]	2.6-6.6 (Raf: <0.05 - 0.93; Sta: 0.5 - 4.1; Ver: 0.06 - 4.0)[58]	3.0-7.1 (Raf:0.3 - 1.0; Sta:1.7 - 3.1; Ver:0.6 - 3.1)[51,58]	5.1-8.7 (Raf:0.3 - 1.6; Sta:1.3 - 5.5; Ver:1.6 - 4.2)[58]
Lipids	5.3 – 7.3[44,47]	1.50 – 2.12 [45,48,56]	2.3 – 2.7[44]	2.0 - 2.3[44]	1.3 – 2.3[44,56]
Proteins	15.0 – 20.2[44,47]	22.7 – 36.0[48,56,57]	19.4 – 22.4[44]	20.4 – 29.2[44,51]	20.4 – 23.4(g/ 100g)[44,56]
Ash	2.5 – 3.3[44,47]	2.7 – 4.0[48,56,57]	3.5 – 4.1[44]	2.5 – 2.8[44,51]	2.8 – 3.5[44,56]
Micronutrients (mg / 100 g dry weight)					
K	718[59]	1084.2 – 1205.5[60]	1885.4 – 2217.1[61]	843.5-1002.4[51,61]	926.5 – 1135.9[61]
Ca	47.2 – 64.4[61]	92.8 – 103.0[60]	7.1 – 12.3[62]	26.8 – 76.2[51,61]	59.6 – 106.9[63]
Cu	0.7 – 0.9[61]	0.75 – 0.91[60]	0.9 - 1.2[61]	0.7 - 1.13[51,61]	0.5 - 0.6[61]
Fe	4.9 – 7.3[47,61]	1.8 – 21.3[57]	5.8 - 7.3[61]	6.25 - 10.0[51,61]	4.5 -5.8[49,61]
Mg	152.5 - 190.2[61]	129.2 – 147.8[60]	184.5 - 238.3[61]	93.6-109.0g[51,61]	109.8 – 127.9[61]
Mn	2.1 - 2.6[61]	1.30 – 1.59[60]	1.1 - 1.5[61]	1.00-1.50[51,61]	0.9 – 1.6[61]
P	288.7 - 299.2[47]	433.9 - 605.5[60]	400 - 442[64]	308.0-394.0[51]	270.3 – 950.5[63]

Zn	2.1 - 2.8[61]	0.9 – 5.2[57]	2.5 - 3.3[61]	2.63 - 5.0[51,61]	2.8 – 6.3[49,61]

Table 1. Nutritional and antinutritional content of common pulse seeds.

2.1 Pulses and glucose regulation

Pulse consumption has long been associated with improved health outcome. Clinical trials showed a marked amelioration in blood lipid profile, blood pressure, inflammation biomarkers and anthropometric measurements (body eight, waist circumference) upon daily consumption of pulses.[36,65] Dietary fibers, particularly oligosaccharides and resistant starch, were advocated as major source of bioactivities, as their characteristic bulkiness hinders energy intake and extends the transit time in the stomach, stimulating the secretion of appetite regulating hormones and therefore promoting satiety and decrease in body weight.[65,66] Moreover, fibers' digestion by commensal bacteria leads to increased propionate production, which was shown to inhibit hepatic cholesterol synthesis in animal models, and butyrate production, promoting glycogenesis and consequently amelioration of body weight and glucose control.[67–69] The mineral profile also participates in the beneficial activity on CVD and MetS, as the low sodium and high potassium levels are associated with decrease in blood pressure.[70] Furthermore, i*n vitro* studies analysing individual components of pulses started compiling in recent years, highlighting the potential role of other bioactive components. Particularly, pulse-derived phenolic acids, flavonoids and bioactive peptides showed a certain ability to inhibit major enzymes involved in glucose regulation and body weight management such as α-amylase, α-glucosidase, DPP-IV, pancreatic lipase, GLUT1 transporter (Supplementary table 2). Phenolic compounds have also been suggested to form complexes with proteins and fibers during digestion. This was initially contributing to the classification of phenolic as anti-nutritional compounds, however recent investigations suggested that phenolic compounds reaching the colon could be bio-transformed or metabolized by commensal bacteria to biologically active molecules, in turn reabsorbed by the colonic epithelium.[71,72] Terpenoids such as triterpene saponins have also been reported to improve glucose control via inhibition of glucose regulating enzymes, stimulation of glycogenesis and decrease glycogenolysis.[73,74] Fig. 3 shows some of the hypothesised sites and way of action of pulse bioactive components on glucose regulation in humans. While most of these studies are still at the beginning and clinical assessments in humans are still scarce, they open a new way of re-thinking about foods as a functional components of diets. Current limitation such as the implication of food matrix, preparation and cooking techniques, digestion, absorption, *in vivo* functionality must be addressed in order strengthen these findings and design effective functional foods.

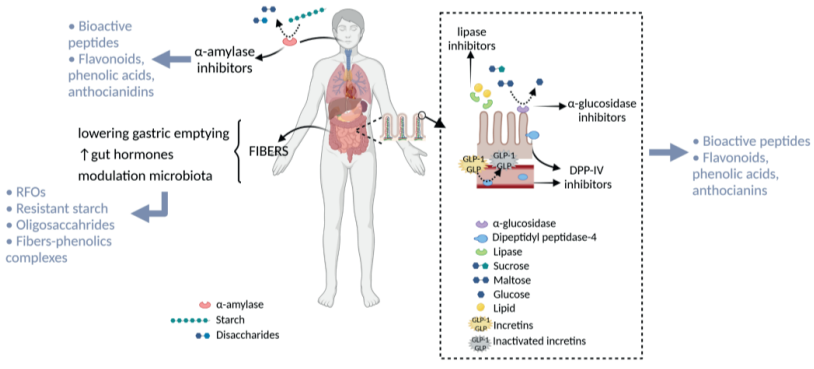

Figure 3. Sites of action of pulse-derived bioactive glucose regulating components (grey text).

2.2 Limitations to pulse exploitation in human nutrition

2.2.1 Plant cell wall and bioaccessibility

In plant physiology, seeds have the function to provide energy and protection for the growing plant. Accordingly, while being a rich source of macronutrients, they also contain secondary metabolites for protection by external stresses and hazards.[75] The legume seed structure is shown in Fig. 2. The nutritive tissue is stored in the endosperm, while the seed coat (testa), the first defensive barrier against extrinsic factors, contains protective compounds such as phenolics and antimicrobial peptides on the inside (parenchyma) and thick-walled lignified cells on the outside (palisade region). Besides the testa, in plant tissue each cell is surrounded by a thick cell wall.[75] Legume seed cell wall is composed of cellulose as fibrillar phase, and a range of non-cellulosic polysaccharides (pectin, xyloglucans, heteroxylans, (galacto) glucomannans) in the matrix phase, with the relative composition changing for each legume (Fig. 4 C).[75,76] As human digestive enzymes cannot easily break down the fibrous shield of the plant cell wall, most of nutrients remain encapsulated within this structure during digestion, with pores size dictating their absorption rate.[77,78] Processing of the plant material prior ingestion is crucial for improving its digestibility. Extrusion and microwave cooking showed to improve digestibility of plant proteins.[79] On the contrary, thermal treatment promoted solubilization and loosening of the polysaccharides in the cell wall (*e.g.*, pectin) and dissociation of cells from each other's, but with preservation of cell wall integrity even when followed by gastrointestinal digestion.[80,81] The entrapment within the cell wall and the densely packed macronutrient matrix was shown to limit protein denaturation upon heating, starch digestibility and iron absorption in beans.[80,81] Higher protein denaturation was instead observed upon heat treatment of bean flour.[81] However, great variability in response to the processing exists among plant sources and more research is needed to elucidate individual digestibility properties of the various plant food sources to improve digestibility of legume seeds and advance their inclusion in diets.

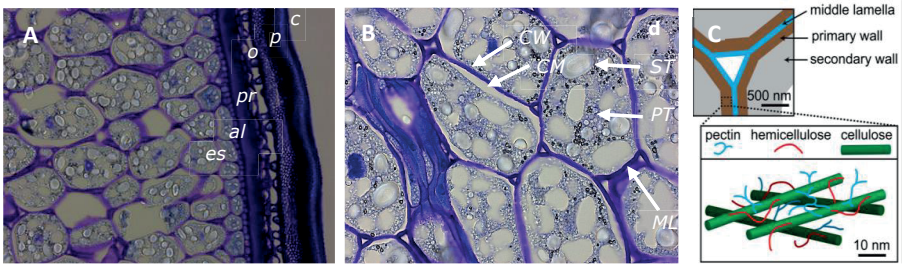

Figure 4. Yellow pea visualised by Toluidine blue O. A: seed coat (testa), brightfield, 20x. Bt: cotyledon, brightfield, 40x. c: cuticle; p: palisade cells; o: osteosclereids; pr: parenchyma; al: aleurone; es: endosperm; CW: cell wall; CM: cell membrane; ML: middle lamella; ST: starch; PT: protein. C: cell wall structure.

2.2.2 Acceptability and off-flavours

While consumers are aware of the health benefits of legumes and appreciate the ethical and environmental benefits, this doesn't reflect in an increased inclusion in daily diets.[82] Limited knowledge on preparation techniques, time, inconvenience, gastrointestinal discomfort and family acceptance are major factor limiting their inclusion in Western diets.[82–85] Moreover, characteristic green, grassy, beany aroma attributes of legumes are often perceived as off-flavours. Aldehydes and alcohols such as hexanal, nonanal, 1-hexanol, 1-nonanol, (E)-2-hexenal, (E,Z)- 2,6-nonadienal and (Z)-2-hexen-1-ol are formed upon oxidation of polyunsaturated fatty acids in pulses and are responsible for the characteristic grassy, beany flavour in yellow pea and green lentils.[86] Saponins and phenolic compounds can contribute to the bitter and astringent off-flavors.[87] Degradation of amino acids and formation of Maillard reaction products during processing and heat treatment can further lead to the formation of aromatic aldehydes and compounds contributing to off-flavours.[88]

2.2.3 Flatulence factors

Consumption of legumes is also often associated with gastrointestinal discomfort.[85] Flatus formation is due to presence of large amounts of indigestible oligosaccharides in plant seeds, which are fermented by commensal bacteria with gas formation. Fructans and raffinose-family oligosaccharides (RFOs) are the most abundant water-soluble carbohydrates in plants, where they function as reserve carbohydrates and stresses protectants.[89,90] Sharing similar physiological activities, they usually do not occur in the same plant. RFOs are characteristic in pulse seeds. After the initial formation of sucrose from photosynthesis, RFOs are formed by a stepwise addition of galactose residues via and α-1,6-glycosidic linkage.[90] Raffinose (Suc-$[Gal]_1$), stachyose (Suc-$[Gal]_2$) and verbascose (Suc-$[Gal]_3$) are the most abundant RFOs in pulse seeds. When required by the plant, RFOs are metabolized by the endogenous α-galactosidase. However, this enzyme is not produced by humans and monogastric animals, which are therefore unable to digest RFOs. Undigested RFOs reach the colon and are metabolized by commensal bacteria with accumulation of CO_2, hydrogen and methane, which constitute ¾ of the flatus.[91–93] As RFOs are water-soluble, soaking can decrease the content of RFOs by about 20 to 40 %.[94,95] Cooking was shown to additionally decrease RFOs content in some, but not others, legumes.[94] However, a considerable amount of RFOs persist in the seeds even after these pre-treatments, and flatulence is still experienced by consumers. Approaches targeting a more substantial reduction in RFOs could therefore be an important step in increasing the choice of pulse foods by consumers.

2.3 Bioprocessing as a strategy to improve digestibility and acceptability of pulses

Bioprocessing describes processes using living sources (e.g., microorganisms, plant or animal cells) or their components (e.g., enzymes, chloroplasts) to produce value added products.[96] Applications include, for instance, production of chemicals, improving aroma and converting indigestible biomasses to energy sources.[97,98] Recent studies explored the use of bioprocessing for formation of bioactive compounds in food products and by-products, such as the release of bioactive peptides by enzymatic hydrolysis of proteins.[99] Germination and fermentation were also suggested to potentially improve pulse seeds digestibility and bioactivity.[100,101] In fact, germination defines the transition from seed dormancy to maturity and requires energy and nutrients that are made available by breaking down phenolic-protein complexes, storage proteins and polysaccharides.[101,102]

Fermentation is one of the oldest bioprocessing and preservation technology in human history, with signs of its application dating back to the 7000 B.C.[103] The process can be performed in a submerged state (SMF), where the microorganisms operate in a liquid environment (e.g., yoghurt), or in a solid-state (SSF), with a water content of 12-70 % and microorganisms typically degrading the substrate from its surface (e.g., bread).[104105] According to the biological niche in which they evolve, microorganisms can use a variety of metabolic pathways for metabolizing substrates and generate energy.[103]

Besides holding unexplored potential due to their biochemical effects on the substrate, germination and fermentation recently gained interest from consumers seeking more “natural”, health-promoting and sustainable food products such as probiotics and functional foods.[106–108] This offer even greater momentum for the exploration of bioprocessing in healthy and functional food development, with vast opportunities particularly in the plant-based domain.[108,109]

3. Food digestion and absorption and the role of food matrix

Routinely, the nutritional value of a food product is determined by estimating the proximate amounts of its macro- and micronutrients, and the caloric content by multiplying the macronutrients by defined factors (proteins: 4 kcal/g, lipids: 9 kcal/g, carbohydrates: 4kcal/g, alcohol: 7 kcal/g) based on average digestibility (Atwater system).[110] While this is an old system, it is still the legally required method for food products labelling in Europe and USA.[111,112] However, new understandings about the implications of food matrix on digestibility of nutrients are questioning this approach.[113] The so called “food matrix effect” refers to the observation that food components show different properties and bioactivities when they are in their free form (e.g. a phenolic compound) or when they are part of a food

microstructure.[114] The novel concepts of bioaccessibility, the "fraction of an ingested compound (nutrient, bioactive) which is released or liberated from the food matrix in the GI tract", and bioavailability, the "fraction of a given compound or its metabolites that reach the systemic circulation" were also recently introduced to address this important issue.[114]

3.1 The digestion process

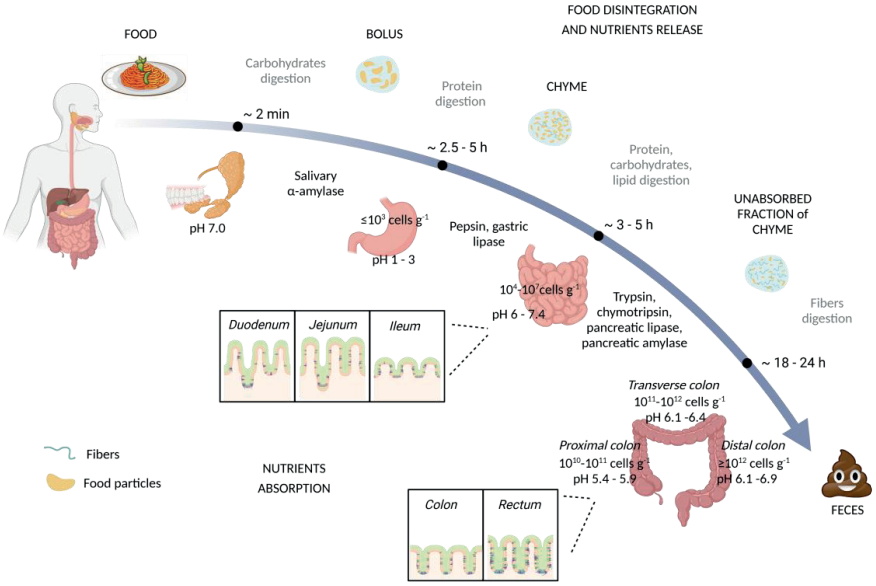

Figure 5. Overview of the digestion process and absorption sites.

The digestive process can be dissected into four major stages: oral, gastric, intestinal, colonic. After ingestion, mastication provides the first fragmentation of food. The saliva moistens the food, favouring the entrance and action of salivary α-amylase, which initiates carbohydrates digestion. After the oral phase, the bolus (= mixture of food particles and saliva) is swallowed through the oesophagus to the stomach. The entrance of food triggers mucin secretion for lubrification and emulsification of the bolus. In the stomach, food disintegration continues via physical and biochemical actions. The muscular contractions (peristalsis and mixing) allow uniform mixing and mechanical disintegration of food particles. At the same time, hydrochloric acid (HCl), gastric lipase, intrinsic factor and pepsinogen are secreted by the stomach. Starch digestion from α-amylase continues until when hydrochloric acid propagates in the food matrix and inactivates it. In the acidic environment, pepsinogen is converted to pepsin, an endopeptidase with optimal activity at pH ~ 2 that digests proteins into peptides.[115] About 0.7 L of gastric juices are secreted for each meal, containing not only enzymes, HCl, intrinsic factor and mucin, but also electrolytes (NA^+, H^+, K^+, CL^-) and hormones.[116] Major GI hormones are cholecystokinin (CCK), gastrin, secretin, motilin and gastric inhibitory polypeptide (GIP). Gastrin is secreted by the stomach and stimulates gastric motility and acid secretion. Secretin, CCK and GIP are secreted by the duodenum and inhibit stomach motility. GIP also triggers insulin secretion by the pancreas.[117] The muscular contractions occur ~ 3 times/min and moves the bolus from the upper to the lower region (pyloric sphincter) of the stomach, where they intensify and ensure further breakdown of the remaining large material in the chyme to 1-2 mm particles. This is then emptied in the small intestine, which consists of duodenum (~ 25-30 cm), jejunum (~ 2.5 m) and ileum (~ 3.5 m). The entry of chyme stimulates secretion of bicarbonate to neutralise the gastric juice and supply the

suitable pH for the activity of intestinal enzymes in the duodenum. At the same time, pancreatic juice and bile are released. Bile plays an important role in lipids digestion and absorption, enabling emulsification and break down of large fat globules into smaller ones, facilitating digestion by lipases. Bile also enables solubilization and absorption of fat soluble nutrients by the intestinal mucosa.[119] The pancreatic juice contains various enzymes such as the proteases trypsin and chymotrypsin, pancreatic lipase, pancreatic amylase.[118] Pancreatic lipase hydrolyses triglycerides to fatty acids and monoglyceride, further hydrolysed by phospholipase. Pancreatic proteases include endopeptidases and exopeptidases. The activity of the gastric pepsin first, and the intestinal proteases after lead to the release of oligopeptides and free amino acids. The oligopeptides are further hydrolysed by brush border enzymes on the apical side of the enterocytes. Pancreatic amylase complete the digestion of starch and glycogen remaining after the activity of salivary amylase. Amylases release disaccharides which are further digested to absorbable monosaccharides by brush border enzymes.[120] It takes about 3 to 5 hours for the chyme to move though the small intestine, allowing for nutrient absorption. The mucosa has a characteristic folding into microvilli, increasing the absorptive surface area to ~ 4500 m^2.[118] Most of the absorption takes place in the duodenum.[118] The unabsorbed components of the chyme then moves to the large intestine, which consists of caecum, appendix, colon, rectum and anus. The colon is the most studied section in the context of food digestion, due to the high concentration of commensal microbes in this site. The colon itself consists of three major sections: proximal (10^{10}-10^{11} cells/g), with bacteria specialized in carbohydrates digestion and large production of short chain fatty acids (SCFA), transverse (10^{11}-10^{12} cells/g), and distal (≥ 10^{12} cells/g), primarily dedicated to protein fermentation with limited production of SCFA. After 18-24 hours, the unutilized material is excreted from the body via defecation.[116]

3.2 Food matrix implications

Before ingestion, the composition and rheology of the food already defines the microstructure/network and the nature and strength of interactions among macro and micronutrients. For instance, matrices with higher buffering capacity will lead to an increased pH in the stomach, preserving the activity of salivary α-amylase for longer time and therefore affecting the rate of carbohydrates digestion , and reducing the activity of gastric acids. Dairy products and protein-based foods are example of food with good buffering capacity, causing a gastric pH of 3-4.[121,122] Food structure and processing also regulates food disintegration, enzyme diffusion and subsequent bioaccessibility of nutrients. For instance, Hiolle et al. noticed that lipolysis was significantly higher in a biscuit structure compared to a custard which was prepared with the same ingredients. Opposite behaviour was observed for luteolin bioaccessibility, while other macro and micronutrients tested were not affected.[123] Oil droplet size and strength of the gel matrix in which they are dispersed were described as main player in the digestion rate of lipids.[124] Pineda-Vadillo reported the effect of food structure on anthocyanins profile and recovery during digestion. While the incorporation of anthocyanins to a food matrix overall protected the compounds from intestinal degradation when compared to a water solution, differences in recovery rate could be observed according to the structure of the food. Solid foods (pancake and omelette) led to a better recovery of anthocyanins compared to liquid foods (custard, milkshake). Particularly, glucosylated and acetyl-glucosylated anthocyanins showed the highest recovery rates (75-110 %).[125] Protein gel porosity positively associated with digestion rate, with more porous gels such as the one from pea protein being hydrolysed more rapidly than the less porous soy protein one.[126] Food composition and rheological structure also affects stomach motility. Generally, (semi-)solid foods have are more satiating than liquid foods of equivalent macronutrient and caloric content, mainly because of the higher gastric retention time which in turn stimulates secretion of GI hormones.[127,128] Moreover, foods rich in lipids have a longer transit time in the stomach, but continuous consumption of a high-fat diet causes adaptations of the GI processing with acceleration of gastric emptying time and possible decrease of the satiating effect of the meal.[129]

4. *In vitro* and *ex-vivo* models of human digestion, fermentation and absorption

Human clinical trials remain the golden standard for testing food and nutraceutical products. However, due to high cost and ethical constraints, they can only be performed as validation of the findings at a final stage of product development. Moreover, high interindividual variability exists in the human digestive process, making it challenging to draw conclusions from intervention studies. *In vitro* or *ex vivo* models are therefore a valuable resource for food and nutrition research, providing cheaper, faster and more standardized alternatives to human interventions. A wide range of models have been developed in recent years, accommodating different research questions (Table 3). *In vitro* digestion systems can be either static or dynamic, single or multi-compartmental.[116,118] Intestinal absorption and brush border activity is commonly investigated with cell culture models such as Caco-2 or HT-29, which differentiate in small intestine phenotype but lack the 3D and cellular complexity.[130] Tissue biopsies represent a valuable tool in this context, which can be used as they are in the Ussing chamber, or for generating intestinal organoids (enteroids).[131] The simulation of the activity of the microbiota in the colon can span from simplified batch fermentation models to highly complex continuous fermentation systems.[132131] A new direction in the field of *in vitro* models of human digestion is the use of microfluidic chips which miniaturize the GI organs and connect them into a continuous system on chip. The motility is also retained and the microbiota can be included.[133] Commercial gut-on-a-chip systems are still under development and therefore their validity and applicability for food and nutrition studies is yet to be tested.

Name of the model	Application in food research	Modelled system	Advantages	Limitations
Gastrointestinal digestion models				
INFOGEST (static)[134]	Digestion kinetics and bioaccessibility of food	Oral, gastric and intestinal stages	Simple and affordable set-up Digestive fluids and enzyme activity closely resemble *in vivo* composition Allow for screening of large volume of samples Standardization of the model allows for inter-laboratory comparisons	No brush order enzymes and no intestinal absorption Lack physiological relevance (peristalsis and mixing) Design doesn't match human anatomy
TIM (dynamic) [116,118]	Digestion kinetics and bioaccessibility of food, feed, pharmaceutical products. Prediction of glycaemic response, protein quality, lipid digestion	Multi-compartmental system: stomach + small intestine (TIM-1) + large intestine (TIM-2)	Validated protocols for wide range of meals in young, adults, elderly and animals. *In vivo* validation Dynamic adjustment of pH and gastric secretions Contractile and peristaltic movement Removal of digestion products (dialysis of water-soluble fraction and filtering of lipophilic fraction prior transfer to small intestine)	Costly and complex set-up No intestinal absorption No simulation of satiety stimulus Design doesn't match human anatomy
DGM (dynamic) [116,118]	Bioaccessibility of food components, probiotic survival, drugs delivery systems	Stomach (fundus, body, antrum) + static duodenum	Dynamic adjustment of pH and gastric secretions Contractions of the body (3/min) Antrum has high shear and mixing Can hold a full meal (800 mL)	Vertical design of body and antrum doesn't match human anatomy No simulation of satiety stimulus Costly and complex set-up Parameters need to be validated for new test meals No intestinal absorption
DIDGI (dynamic) [116,118]	Digestion of human milk and dairy products, food microbes survival	Stomach and small intestine (multi-	Dynamic adjustment of pH and gastric secretions Good *in vivo* correlation for infant formula and dairy	No satiety signals controlling digestion velocity No intestinal absorption

		compartment system)	products studies (proteolysis kinetics)	No mechanical simulation (contractions, peristalsis) Vertical design of body and antrum doesn't match human anatomy
Cellular models				
Caco-2 cell line[131]	Intestinal absorption of nutrients/ transport studies	Small intestine enterocytes (polarized apical brush border)	Spontaneously differentiate into a polarized monolayer of cells with characteristic features of mature absorptive enterocytes found in the small intestine Express most of the brush border enzymes (receptors, transporters, metabolizing enzymes) Validated against *in vivo* data from pharmacology and transport studies	Only enterocytes, other cell types missing No mucus layer (because no goblet cells) No flow Over/ under expression of certain proteins compared to *in vivo*
HT29 cell line[131]	Study of anticancer food compounds digestion and metabolites production; food digestion and bioavailability; study reaction to bacterial infections; models for study cell differentiation	Small intestine enterocytes (polarized apical brush border); can be used in co-culture with Caco-2	Formation of monolayer with tight junctions, apical brush border and some of the enzymes Mucin-producers	Longer differentiation time (~30 days) compared to Caco-2 Lower enzyme activities compared to Caco-2 cells Impaired glucose metabolism Limited number of enterocytic brush border enzymes expressed, not best model for transport studies Over/ under expression of certain proteins compared to *in vivo*
Human gut fermentation models				
Batch fermentation (faecal inoculum)	Metabolism dietary substrates	Colonic fermentation	Cheap and easy set up Allow to screen high volume of samples Suitable for monitoring SCFA production	No pH control Fermentation can be carried out for short period of time due to accumulation of toxic compounds, depletion substrate, pH drop
TIM-2[116,118]	Effect of food/drugs on activity and composition of microbiota and identification of metabolic products	Proximal colon, usually coupled to TIM-1	Peristaltic movements Water and metabolite absorption Presence of dialysis membrane avoid accumulation metabolites Possible to run up to 10 units in parallel at the same time	No epithelial or immune cells No feedback mechanisms Systems developed from data from healthy individuals; use in disease studies needs to be validated
SHIME[116,118]	Effect of food/drugs on activity and composition of microbiota and identification of metabolic products	Multi-compartment: stomach + small intestine + large intestine (ascending colon, transverse colon, descending colon)	Vessels connected by peristaltic pumps Differentiate microbial communities of AC, TC, DC M-SHIME: addition of mucine Preservation of metabolic phenotype from faecal inoculum; allows inter-individual studies	No physiological parameters (peristalsis and contractions) No dialysis between small intestine and colon Design doesn't match human anatomy Limited biological replicates High cost
PolyFermS[132]	Effect of food/drugs/bacteria	Proximal colon	Preservation of metabolic phenotype from faecal	Limited biological replicates

	on activity and composition of microbiota and identification of metabolic products		inoculum; allows inter-individual studies Stable cultivation of complex intestinal microbiota Allow for direct comparison of various experimental conditions on multiple sub-reactors fed with the exact same microbiota Use of beads for inoculum allow to retain microbial diversity over time Validated for microbiota of elderly, child and various animals	Complex setup and labour intense operation High cost Design doesn't match human anatomy No physiological parameters (peristalsis and contractions)
In vitro intestinal tissue models				
Intestinal Crypt Organoids (enteroids)[131]	Nutrients and drugs intestinal absorption and metabolism	Various GI sections (stomach, ileum, colon)	Variety of GI cells (e.g. Paneth cells, goblet cells, absorptive and hormone secretive epithelial cells) provide excellent physiological model Isolated from human/animal tissue, allow for personalized nutrition/medicine Long-term culturing Possibility to develop organoids of the various GI sections (stomach, ileum, colon)	Inwards orientation (apical side enclosed inside organoid) make it difficult to use for transport studies Variability in size and structure among organoids Lack interaction with other systems (enteric nervous, vascular, lymphatic, immune) Costly and training required
Ussing Chamber[131]	Transport of digested foods, toxicology studies, secretion of satiety hormones	Intestinal epithelium	Use intestinal tissue, so morphological and physiological features retained	Limited availability of human tissue often require to switch to animal tissue Limited viability of intestinal tissue (~120 min) allow testing of limited parameters Low throughput (few segments of epithelial tissue at the time)

Table 2. Examples of the different types of in vivo and ex-vivo models used for food and nutrition research

5. Aim of the thesis and outline

Pulses are an excellent source of nutrients and bioactives, however the low digestibility and entrapment of bioactives within cell wall restricts their absorption by humans. DPP-IV and α-glucosidase inhibitors have been observed in a wide variety of plant-derived isolates such as purified protein hydrolysates and phenolic extracts. However, little attention has been placed on the interaction of these bioactive components within the seed matrix, and the related bioaccessibility. Moreover, such bioactivities could also be derived by bioprocessing of the whole pulse seed, improving the "naturalness" and sustainability of the food product. Importantly, to promote inclusion of pulse products in daily diets, flavor and intestinal discomfort also have to be addressed. Off-flavors attributes such as "beany, green, earthy" are commonly associated with pulse consumption. Formation of flatus, primarily associated with the high content of raffinose family oligosaccharides (RFOs), is a second major problem. Accordingly, the aim of this thesis was to explore the use of bioprocessing for improving bioaccessibility of nutrients and bioactive components in pulses, and to assess the implications of the bioprocess on the acceptability and colonic fermentation of pulse products. Aroma compounds and flatulence factors were used to assess the acceptability.

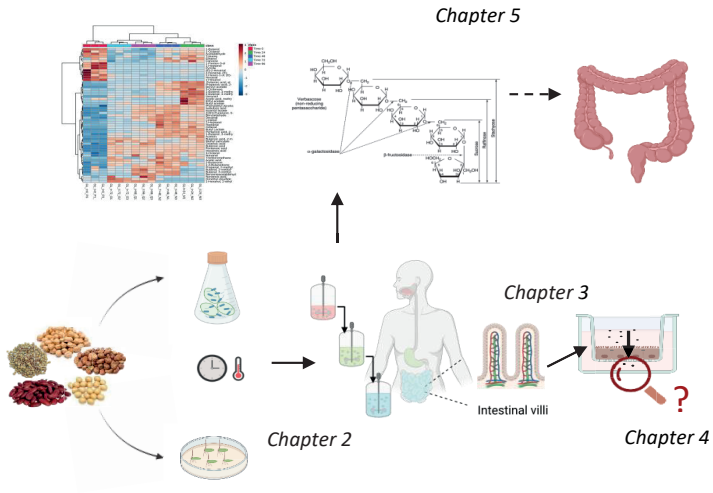

Figure 6. Graphical visualization of thesis chapters.

In **Chapter 2**, germination and solid-state fermentation with *L. plantarum* were applied on five common pulse seeds (chickpea, faba bean, kidney bean, green lentil, yellow pea). Their implications on seed microstructure, bioaccessibility of proteins and phenolic compounds, and *in vitro* DPP-IV and α-glucosidase inhibition were investigated.

Following up with a bioprocess that showed promising results, in **Chapter 3** the bioactivity of microbially fermented green lentil flour was assessed in a polarized Caco-2 cell model. Dipeptidyl peptidase-IV activity was measured in the cell extract (*in situ* activity) and in the basolateral fluids containing the bioactive molecules transported across the intestinal monolayer (circulating DPP-IV). The transport of glucose across the monolayer was further assessed, to test whether the bioactivity extended to the glucose transporters.

In **Chapter 4**, the implications of the microbial fermentation on the bioaccessibility and intestinal absorption of peptides from green lentil was investigated.

Chapter 5 focuses on the influence of the bioprocess on the acceptability of green lentil and yellow peas flour. The volatiles present and/or formed during microbial fermentation, particularly focusing on the compounds associated to "beany, grassy" off-flavors were profiled. The major raffinose family oligosaccharides (RFOs) responsible of flatus formation upon pulse consumption were also quantified. Aiming at decrease the RFOs while retaining the beneficial prebiotic effect of pulses, the *in vitro* digested fermented flours were further fermented by commensal bacteria isolated from human faeces in a continuous *ex vivo* fermentation model of the proximal colon.The profiles of bacteria and short chain fatty acids were analysed.

Ultimately, in **Chapter 6**, the major findings of the thesis are summarized and discussed. The methodological approach and limitations are also considered, and future research directions are proposed.

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Supplementary Material

Class of drugs	Commercialized product	Activity
Biguanides	Metformin	Interfere with multiple mechanisms in various tissues, resulting in increased glucose usage in the gut, increased insulin signaling and decreased glucagon activity in the liver, enhanced insulin-dependent glucose uptake in muscles and raised circulating GLP-1 levels. [135]
Insulins	Various types of human, analogs, biosimilars or combinations	Act in a glucose-dependent manner on barely all glycemic targets, however the administration is very burdensome, as it needs to be injected before meals with dosage adjustments based on diet, activity and weight.[136]
Incretin-based therapies: GLP-1receptor agonists (incretin mimetics)	Exenatide, liraglutide, lixisenatide, dulaglutide and albiglutide.	Peptides resistant to DPP-IV degradation but able to activate the GLP-1 receptor, triggering the incretin response. [137,138]
Incretin-based therapies: DPP-IV inhibitors (incretin enhancers)	Sitagliptin, saxagliptin, linagliptin, alogliptin.	Prevent cleavage and inactivation of GLP-1, consequently retaining the insulinotropic activity and inhibitory activity on glucagon. Additionally, they prevent β-cells apoptosis, and increases their proliferation. [137–139]
Sulfonylureas and meglitinides	Sulfonylureas: gliclazide, glipizide, glimepiride, glyburide. Meglitinides: nateglinide, repaglinide	Bind to the sulfonylurea receptor 1 (SUR1) on pancreatic β-cells, leading to increased insulin secretion. [19,23]
α-glucosidase inhibitors	Acarbose, miglitol	Inhibit brush border enzyme α-glucosidase, causing decreased absorption of oligo- and disaccharides and lowered postprandial hyperglycemia. [24,135]
Thiazolidinediones	Pioglitazone, rosiglitazone, troglitazone	Agonists of the peroxisome-proliferator-activated receptor gamma (PPAR-γ) in the adipose tissue, muscle, liver and β-cells. The activation of PPAR-γ results in increased insulin sensitivity and glucose uptake, decreased hepatic gluconeogenesis, reduced free fatty acids release and improved adipogenesis. [135]
SGLT-2 inhibitors	Dapagliflozin, canagliflozin, empagliflozin	Inhibit SGLT2 transporters in the kidneys, leading to increased urinary glucose excretion.[136]

Supplementary Table 1. Drug treatments for T2DM

Pulse	Bioactive component	Characterized compounds/fraction	Digestion model	Bioactivity	Ref.
Chickpea	Protein concentrate (in sunflower oil emulsion)		*In vitro* digestion.	DPP-IV: 3 % (before SGID), 15 % (after SGID)	141
Chickpea	Albumin and globulin fractions (alcalase hydrolysates)	FEI, FEL, FIE FGKG	No *in vitro* digestion	DPP-IV IC_{50} = 0.004.2 α-amylase: 54% α-glucosidase: 56%	142
Chickpea	Ethanol extracts		No *in vitro* digestion	α-amylase IC_{50}: 2.1-3.2 α-glucosidase IC_{50}: 0. 537-0.828	143
Chickpea *var. Kabuli*	Phenolic extracts (methanol)		*In vivo* (rats)	α-glucosidase 36.9 – 43.6 %	144
Chickpea var. Kabuli	Hydrolysed protein isolates	PHPATSGGGL, YVDGSGTPLT, SPQSPPFATPL W	Pepsin and pancreatin digested	α-amylase: 28.4 % DPP-IV IC_{50}: 245 (μg/mol)	145

Chickpea var Blanco Sinaloa 92	Phenolic extracts (ethanol) from SSF flour (*Rhizopus oligosporus*)		No *in vitro* digestion	α-amylase 4.29- 6.50 inhibition index α-glucosidase 4.94 - 8.50 inhibition index	[146]
Faba bean	Phenolic extract (acetone)		No *in vitro* digestion	α-glucosidase: 59.7 - 68.3 % Lipase IC_{50}: 18.5 - 81.43	[147]
Faba bean	Protein isolates		*In vitro* digestion	DPP-IV IC_{50} 0.54 CCK (STC-1 cells): ~4 fold induction GLP-1 (STC-1 cells): ~7.5 fold induction	[148]
Faba bean	Phenolic extract (ethanol)	Hydroxyhydroquinone, gallic acid, epigallocatechin, oxidized glutathione, epigallocatechin, catechin, epigallocatechin gallate, epigallocatechin monogallate, sphingosine	No *in vitro* digestion	α-glucosidase IC_{50} 2.30 (88.3 %)	[149]
Lentil	Purified phenolic extracts (methanol)	Dihydroxybenzoic acid, *p*-ydroxybenzoic acid, catechin glucoside, catechin gallate, epicatechin glucoside, sinapic derivative, procyanidin dimer, syringic acid, *trans-p*-coumaroyl malic acid, *trans-p-*coumaric acid, epicatechin gallate, kaempferol tetraglycoside, kaempferol triglycoside, kaempferol-3-robinoside-7-rhamnoside, quercetin-3-xyloside, procyanidin dimers, quercetin-3-glucoside, flavonoid derivatives, kaempferol-3-glucoside	No *in vitro* digestion	α-glucosidase IC_{50}: 23.1-42.1 Lipase inhibition IC_{50}: 6.26 – 9.26	[150]
Lentil	Water extracts		No *in vitro* digestion	α-amylase: 50.3 – 94.1 % α-glucosidase: 17.1 – 45.0 %	[151]
Lentil	Lentil sprouts phenolic extract (methanol)	Flavan-3-ols, polymeric procyanidins, phenolic acid, flavonols + flavones, isoflavones	No *in vitro* digestion	α-amylase IC_{50}: 88.4 α-glucosidase IC_{50}: 16.0	[152]
Lentil	Vegetable juices supplemented with lentil sprouts and fermented with *L. plantarum*		No *in vitro* digestion	α-amylase IC_{50}: 41 – 149 µM α-glucosidase IC_{50}: 239-350 µM	[153]
Lentil var. castellana	Flour fermented with *L. plantarum*		No *in vitro* digestion	α-glucosidase: 0 – 89.4 % Lipase: 35.8 – 48.9 %	[154]
Bean	Proteins hydrolysates		Pepsin, pancreatin digestion *In vivo* (rats)	α-amylase: 1.3 – 89.1 % α-glucosidase: 22.9 – 89.2 % Hypoglycaemic activity	[155]
Bean, Black-Otomi cultivar	Anthocyanins extract	Delphinidin-3-O-glucoside, petunidin-3-O-glucoside, malvidin-3-Oglucoside	No *in vitro* digestion	α-glucosidase: 37.8% α-amylase: 35.6% DPP-IV: 34.4%	[156]
(Phaseolus vulgaris L.) 12 cultivars	Protein isolate		*In vitro* digestion	α-Amylase: 2.5 – 14.9 % inh rel Acarbose/mg bean protein isolate	[157]
Black turtle bean	Purified phenolic extract (fractions)	Gallic acid, ferulic acid, sinapic acid, syringic acid, myricetin, catechin, epicatechin, quercetin-3-o-glucoside,	No *in vitro* digestion	α-amylase IC_{50}: 0.67-2.69	[158]

		kaempferol-3-o-rutinoside, kaempferol-3-o-glucoside		α-glucosidase IC_{50}: 0.025-0.064 lipase IC_{50}: 0.076 – 0.38	
(Phaseolus vulgaris L., P. lunatus,)	Protein hydrolysates (Alcalase®-Flavourzyme® or pepsin-pancreatin) < 1 kDa		No *in vitro* digestion *In vivo* (rats)	***α***-glucosidase IC_{50}: 0.86 - 75 Inhibition carbohydrates intestinal absorption 19.2 – 40 % Suppression starch-induced postprandial hyperglycaemia (ED_{50} 1.4 - 93 mg kg−1)	159
Bean, Black-Otomi cultivar	Protein hydrolysates (alcalase)	LLPK, QTPF, FFQS, GSLGGH, GSRAH, YVFLS, LALVL, WEVM, FEELN, VYFLS, LKEGGK, LSKSVL, LYELN, ATNPLF, AKSPLF, SGPFGPK, GSPVSSR, TTNPLF, TTGGKGGK, ASATTGVL, SKGSGGGKL, SAKGPPMGAK, SAKGPPTSAK, SARVLAAGAK, SANRLPSAGS, RKLKMRQ, SLKWWDLGS, SLPAGGNRYGK, SRSPAGPPPTEK, ALMLEEYLLE, QQRRRLRLK, EGLELLLLLLAG, DLALLLLAELG, LPPSPERTAAPPF	*In vitro* digestion	DPP-IV: 96.7% α-amylase: 53.4% α-glucosidase: 66.1%	160
Bean var. Black Otomi, BRS-Horizonte, BRS-Pontal, Perola	Protein isolates	KKSSG, TACKD, LSFNT, VKFMT, KMARPV, GHVPP, NPYM, CGPHGA, GGGLHK, KYMKS, SGSYS, GGDEAG, VGTNK, CPGNK, LSGVF, KTYGL, RTLNL, MPHLK, GGNEGA, CPGNK, NPSLP, KGPASK	*In vitro* digestion	DPP-IV inhibition IC_{50}: 0.03–0.87 ***α***-glucosidase: 36.3 - 50.1 %	161
***Bean* var. Black, Pinto, Red, Navy, Great Northern**	Protein isolate	Raw	*In vitro* digestion (SGID)	DPP-IV IC_{50}: 0.093-0.095 α-amyalse 5-36 % rel ac)/mg protein α-glucosidase: 40 – 70 %	162
Kidney bean	Hexane extract	Triacylglycerols (trilinolenin, 1,3-dilinolenoyl-2-linoleoyl glycerol)	No *in vitro* digestion	α-glucosidase: IC_{50} 0.13 – 0.47	163
Bean	Phenolic extract, (in acetone)	Gallic acid, vanillic acid, benzoic acid, chlorogenic acid, l-3dicaffeoylquinic acid, caftaric acid, chicoric acid, catechin, epicatechin, epigallocatechin, spiraeosid, myricetin, quercetin, quercetin 3,4 diglucoside, kaempferol, taxifolin	No *in vitro* digestion	α-amylase IC_{50}: 0.069-0.127 α-glucosidase IC_{50}: 0.039-0.074 Lipase IC_{50}: 0.063-0.103	164
Bean	Protein isolate hydrolysed (Alcalase, Bromelain) < 1kDa	LLSL, QQEG, NEGEAH	No *in vitro* digestion	α-amylase 49.9 ± 1.4 % α-glucosidase 76.4 ± 0.5 % DPP-IV: 55.3 ± 1.6%	165
Chickpea	Protein hydrolysates fractionated (Alcalase, Flavourzyme)		No *in vitro* digestion	DPP-IV IC_{50}: 2.3 – 3.4	166
Lentil	Protein hydrolysates fractionated (Alcalase, Flavourzyme)		No *in vitro* digestion	DPP-IV IC_{50}: 1.8 – 3.2	166

Lentil	Lentil flour Hydrolysed (Savinase) + fermented	Dimer prodelphinidin, (+)-catechin-3-O-glucoside, *trans-p*-coumaric acid derivative, (+)-catechin, trans-*p*-coumaroyl malic acid, *p*-hydroxybenzoic acid, *trans-p*-coumaroyl glycolic acid, vanillic acid, kaempferol dirutinoside, kaempferol rutinoside-hexoside, Isorhamnetin glucuronide, *trans-p*-coumaric acid, kaempferol rutinoside-rhamnoside, kaempferol rutinoside-rhamnoside, kaempferol	*In vitro* digestion	Sucrase: 19.8-81.1 % Maltase: 22.1-37.8 % Lipase: 9.9-90.5 %	[167]
Lentils	Flavonol glycosides	Kaempferol 3-galactoside-7-rhamnoside (1), kaempferol-3- O-β-gulcopyranosyl-(1→2)-[α-rhamnopyranosyl(1→6)]-βgalactopyranosyl-7-O- α-rhamnopyranoside (2), robin (3), kaempferol (4).	No *in vitro* digestion	DPP-IV IC_{50}: 27.9-51.9 µM	[168]
Lupin	Pasta supplemented with lupin protein		*In vivo* (rats)	Significant ↓ food intake; ↓ glycaemia	
Lupin	Lupin kernel flour (12.5 g fibers, 22 g proteins)		*In vivo*	↓ postprandial glycaemia	
Lupine flour	Protein hydrolysates fractionated (Alcalase, Flavourzyme)	RDDVPT, RNTSPQ, HDLPG, NPLL, APVPEM, APLAVR, APENPV	No *in vitro* digestion	DPP-IV IC_{50}: 1.7 – 3.4	[166]
Lupin	Protein (β-conglutin pepsin digested)	LTFPGSAED	No *in vitro* digestion Caco2 cells	DPP-IV IC_{50}: 228 µM (35 % inh.) DPP-IV IC_{50}: 223.2 µM	[169] [170]
Pea	Pea protein hydrolysate (Protamex)		*In vitro* digestion	DPP-IV IC_{50}: 0.73 – 1.1	[171]
Pea	polysaccharide	Glycoprotein PGP2	*In vivo* (rat)	↓body weight, ↓ blood glucose level, ↑insulin secretion, improve insulin resistance, ↑insulin sensitivity	[172]
Pea	Green peas microgreens phenolic extract (methanol)	Flavan-3-ols, polymeric procyanidins, phenolic acid, flavonols + flavones, isoflavones	No *in vitro* digestion	α-amylase IC_{50}: 8.3 α-glucosidase IC_{50}: 8.0	[152]
Pea	Protein hydrolysates fractionated (Alcalase, Flavourzyme)		No *in vitro* digestion	DPP-IV IC_{50}: 2.1 – 4.9	[166]
Pea	Pea proteins		*In vitro* digestion	DPP-IV IC_{50} 0.98-1.46 CCK (STC-1 cells): 6 fold induction GLP-1 (STC-1 cells): 15-40 fold induction	[148]
	Hydrolysed pea protein		*In vitro* digestion	DPP-IV IC_{50} 0.79 CCK (STC-1 cells): ~6 fold induction GLP-1 (STC-1 cells): 60 fold induction	

Supplementary Table 2. Pulse components showing glucose regulating properties. Bioactivity: % inhibition or IC50: mg/mL if not stated differently. Chickpea (Cicer arietinum L.), Faba bean (Vicia faba L.), Lentil (Lens culinaris), Bean (Phaseolus vulgaris L.), Lupin (Lupinus albus), Pea (Pisum sativum).

CHAPTER 2

Bioprocessing of common pulses changed seed microstructures, and improved dipeptidyl peptidase-IV and α-glucosidase inhibitory activities.

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Abstract

Type 2 diabetes mellitus (T2DM) is a leading cause of death globally. T2DM patients experience glucose intolerance, and inhibitors of dipeptidyl peptidase IV (DPP-IV) and α-glucosidase are used as drugs for T2DM management. DPP-IV and α-glucosidase inhibitors are also naturally contained in foods, but their potency can be affected by the food matrix and processing methods. In this study, germination and solid-state fermentation (SSF) were used to alter pulse seed microstructures, to convert compounds into more bioactive forms, and to improve their bioaccessibility. Germination substantially modified the seed microstructure, protein digestibility, contents and profiles of phenolic compounds in all the pulses. it also increased DPP-IV and α-glucosidase inhibitory activities in chickpeas, faba beans and yellow peas. compared to germination, SSF with *Lactobacillus plantarum* changed the content and the profile of phenolic compounds mainly in yellow peas and green lentils because of greater disruption of the seed cell wall. In the same pulses, heat treatment and SSF of flour increased DPP-IV and α-glucosidase inhibitory activities. the results of this study suggest that germination and SSF with *L. plantarum* are effective and simple methods for modulating phenolic and protein profiles of common pulses and improve the action on DPP-IV and α-glucosidase.

Keywords: germination, *L. plantarum*, pulses, dipeptidyl peptidase-IV, α-glucosidase.

1. Introduction

Diabetes is currently one of the ten leading causes of death globally and its morbidity is expected to reach 10.4% of the world population by 2040.[1,2] Type 2 diabetes mellitus (T2DM) is the most common condition, representing about 90% of diagnosed diabetes in high-income countries, and its development is highly influenced by environ-mental and dietary factors.[3] T2DM patients suffer from many metabolic anomalies such as insulin resistance and deficient pancreatic β-cells insulin secretion, which in turn cause difficulty in maintaining blood glucose homeo- stasis and glucose intolerance.[4,5] Decreasing postprandial glucose absorption by inhibiting key digestive enzymes involved in glucose regulation, or by facilitating the action of gut hormones involved in insulin secretion has therefore become a target for many anti-diabetic drugs. The human digestive enzymes, α-glucosidase, α-amylase and dipeptidyl peptidase IV (DPP-IV), play a central role in postprandial glucose regulation. α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is the primary enzyme involved in the hydrolysis of terminal α-1,4-linked glucose moieties from polysaccharides in the human body.[6] DPP-IV is a ubiquitous enzyme that selectively cleave glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). GLP-1 and GIP are known as incretin hormones; they trigger the secretion by 60% of the insulin released during meal consumption, the so-called "incretin response".[7] DPP-IV drastically impair the metabolism of incretin hormones by converting about 85% of the biologically active hormones to their inactive forms in the human body.[8,9] Drugs or food-derived bioactive compounds able to inhibit α-glucosidase and DPP-IV are therefore used as obvious strategy in T2DM management.[10,11]

Pulses are an excellent source of dietary proteins and other nutrients, and play a major role in the diets of many developing countries. They are regarded as low glycaemic index food, and recent *in silico* and *in vitro* studies have shown DPP-IV and α-glucosidase inhibitory properties of peptides and phenolic extracts derived from pulses.[12–19] Moreover, pulses contain other components with beneficial effects on glycaemic regulation, such as complex carbohydrates and resistant starches.[20] Processing of plant material can improve bioavailability of nutrients and bioactive compounds by disrupting the plant cell wall, dissociating nutrient-matrix complexes, or biotransforming them into more active forms. Improved bioaccessibility was indeed observed in processed protein and phenolic extracts from pulses[13–15,21–23] but the bioaccessibility of these components when concurrently present in a food matrix is still not clear. Thermal treatment can either be beneficial or detrimental for nutrient bioavailability, with particular impact on proteins. Heat can inactivate protease inhibitors and denature proteins in seeds, potentially making them more available to digestive enzymes, or also encourage aggregation and prevent enzyme accessibility when starch gelatinization occurs.[14,24,25] Desiccated commercial legumes are in a metabolically dormant state, with seed coats rich in protective compounds (e.g. phenolic compounds, lignin, cutin) which are not digestible for human digestive enzymes. Intactness and porosity of the cell wall therefore play a major role in the bioavailability of micro- and macronutrients of plant seeds.[25–27] Soaking legume seeds (water imbibition) ends their dormancy stage and initiates the germination process, with significant changes in protein profile and dramatic impact on nutrients composition.[28] Germination also triggers the synthesis of phenolic compounds, which are needed for their antioxidant activity in the early stages of germination, and for the structural growth of the plant in later stages.[29,30] Furthermore, germination of legumes has been used for generating bioactive peptides with DPP-IV and α-glucosidase inhibitory activity.[13,31] Fermentation has also been successfully used as a sustainable method for improving protein digestibility, releasing bioactive peptides from the native protein sequence, degrading tannins, increasing soluble phenolic compounds and, thus, improving the bioactivity of plant materials.[23,32–34]

The objective of this study was to investigate the effect of physical and biological processing on the bioaccessibility of nutrients and bioactive molecules with α-glucosidase and DPP-IV inhibitory activities in five common pulses. Soaking, heating, grinding, SSF with *Lactobacillus plantarum*, and germination were used as bioprocessing methods, alone or combined, for treating chickpeas, kidney beans, faba

beans, green lentils, and yellow peas. Detailed study on the microstructure, protein and phenolic profiles of bioprocessed yellow peas and green lentils was conducted to provide further evidence on how the bioprocesses modulate the content and chemical form of specific bioactive molecules. The ultimate goal was to select an optimal bioprocessing method (i.e. the one leading to the highest α-glucosidase and DPP-IV inhibition *in vitro*) to design functional ingredients for T2DM prevention.

2. Material and Methods

2.1 Materials

Chickpeas (*Cicer arietinum* variety Kabuli), Yellow peas (*Pisum sativum*), Faba beans (*Vicia faba*), Red beans (*Phaseolus vulgaris* variety Kidney), green lentils (*Lens culinaris*) were generously donated by Pulse Canada (Manitoba, Canada). α-Glucosidase from *Saccharomyces cerevisiae* (≥100 units/mg protein), dipeptidyl peptidase IV human (recombinant expressed in Sf9 cells), α-amylase (from porcine pancreas, type VI-B, ≥5 units/mg solid), pancreatin (from porcine pancreas, 8xUSP specification), pepsin (from porcine gastric mucosa, ≥250 units/mg solid), Calcoflour white stain (calcofluor white M2R 1 g/L, evans blue, 0.5 g/L), Toluidine blue O, gallic acid, vanillin and catechin were purchased from Millipore Sigma (Burlington, MA, USA). *Lactobacillus plantarum* ATCC 8014 was purchased from Cedarlane (Burlington, ON, Canada). DeMan, Rogosa and Sharpe (MRS) broth, M17 broth and Bacteriological agar were purchased from Oxoid (Nepean, ON, Canada). DC^{TM} Protein Assay Kit II and Ladder Precision Plus Protein dual color standards were purchased from BioRad (Mississauga, ON, Canada). GelCode blue safe protein stain was purchased from Fisher Scientific (Toronto, ON, Canada).

2.2 Preparation of microbial culture

Lactobacillus plantarum (ATCC 8014) culture was reconstituted in MRS broth and stored at −80 °C in 30% (v/v) glycerol. Bacterial cells were propagated twice prior to experimental use, recovered by centrifugation (10000 *g* for 10 min at 4 °C) and washed twice in sterile saline solution (0.9% NaCl, w/v). The obtained suspension was used as inoculum for solid-state fermentation.

2.3 Pulse bioprocessing

Solid state fermentation (SSF). Pulse seeds were prepared in three different ways (i, ii, iii) prior to inoculation with *Lactobacillus plantarum* ATCC 8014. (i) Cracked beans (10 g) were sanitized for 10 min with 2% (v/v) sodium hypochlorite, rinsed 3 times with sterile distilled water and soaked overnight in sterile distilled water. On the following day, cracked seeds were homogeneously inoculated with 1 mL of *L. plantarum* suspension containing 9 $\log_{10}$ colony-forming units (CFU) / mL. (ii) Cracked beans (10 g) were suspended in distilled water (1:2, w/v) and autoclaved at 121 °C for 15 min. After cooling down, the autoclaved beans were then homogeneously inoculated with 2 mL of *L. plantarum* suspension containing 9 $\log_{10}$ colony-forming units (CFU) / mL. (iii) Pulses were ground into flour (10 g), mixed with equal volume (1:1, w/v) of sterile distilled water and homogeneously inoculated with 1 mL of *L. plantarum* suspension containing 9 $\log_{10}$ colony-forming units (CFU) / mL. Fermentation of pulse products was carried out for 48 h at 37 °C. SSF samples were withdrawn at 0 and 48 h for microbiological analysis, then freeze-dried and stored at −20 °C for chemical analysis.

Germination. Pulses (20 g) were sanitized for 10 min with 2% (v/v) sodium hypochlorite and then rinsed 3 times with sterile distilled water and soaked overnight (15 h) at room temperature (23 °C). On the following day, the excess water was removed and pulses were placed in a sterile petri dish covered with filter paper and sprayed with 5 ml distilled water. The germination was carried out at 0, 3 and 5 days at room temperature (21 °C) in the darkness. Seeds were then watered with 2 ml distilled sterile water on day 2 and 4 of germination. Germinated pulses were then frozen at −80 °C, freeze-dried, and ground to a flour for further analysis.

2.4 Microbiological analysis

Fermentation was monitored by withdrawing samples at 0 and 48 h of fermentation using plate count and change in pH to determine the efficiency of the process. *L. plantarum* was counted in acidified

MRS broth (pH 5.8) supplemented with 1.5% (w/v) agar, after anaerobic incubation at 30 °C for 72 h. Total anaerobic population was counted on reinforced clostridial agar (RCA) after anaerobic incubation at 30 °Cfor 48 h. Total aerobic population was counted on plate count agar (PCA) after incubation at 30 °C for 48 h. Cell counts were expressed as $\log_{10}$ CFU/ml. pH was measured after 0 and 48 h of fermentation by means of a pH meter (Fisher brand, USA).

2.5 Simulated gastrointestinal digestion

In vitro simulated gastrointestinal digestion (SGD) was conducted according to the consensus three steps *in vitro* digestion model described by Minekus *et al*.[35] The entire process was performed at 37 °C. In brief, 5 g of ground sample were mixed with 5 mL of solution containing simulated salivary fluids (SSF) and salivary α-amylase (1500 U/ml) and incubated two minutes, with a pH of 7.0. The 10 mL of bolus were then mixed with 10 mL of a solution containing simulated gastric fluids (SGF) and porcine pepsin (25000 U/mL) and incubated for two hours with constant shaking (120 rpm), at a constant pH of 3.0. The gastric chyme was then mixed with an equal amount of a solution made of simulated intestinal fluids (SIF), pancreatin (100 trypsin U/mL), bile extract (10 mM) and incubated for two hours at constant pH of 7.0 and agitation (120 rpm). The amount of pepsin and pancreatin were determined based on their activities, as suggested by the consensus method.[36] Pepsin activity was assessed according to the method described by Anson *et al*.[37,38] Trypsin activity was determined according to the method described by Hummel *et al*.[39]

2.6 Chemical analysis

2.6.1 Determination of total phenolic content (TPC)

Total phenolics were extracted following a modified method from Zhang *et al*.[19] Briefly, 200 mg pulse flour were extracted in the dark with 70% MeOH containing 1% HCl (v/v) using a shaking incubator (Mandel, Canada) at 200 rpm for 3 h at 37 °C. The mixture was then centrifuged 7000 *g* for 15 min and supernatant was stored at −20 °C in the darkness for TPC analysis. Total phenolic content (TPC) in crude extract was determined by a colorimetric reaction using Folin and Ciocalteu's phenol reagent according to the method described by Singleton *et al*.[40], with modifications. Briefly, 50 µL gallic acid standard or pulse extract were mixed with 475 µL of 10-fold diluted Folin-Ciocalteu reagent in an amber microcentrifuge tube and reacted for 5 min at room temperature. A 475 µL of 60 g/L sodium carbonate (Na_2CO_3) solution was then added and incubated 2 h at room temperature before the absorbance was read at 725 nm using a visible-UV microplate reader (Tecan, Switzerland). Calibration was achieved with an aqueous gallic acid solution (31.25–500 µg/mL). The total phenolic content (TPC) was expressed as mg gallic acid equivalents (GAE) per gram dry weight pulse flour (mg GAE/g DW) based on the calibration curve.

2.6.2 Identification of phenolic compounds by HPLC-DAD

Chromatographic analysis were performed using a Breeze 2 HPLC system equipped with a 2998 photodiode detector and Empower 3 data analysis software from Waters Canada (Montreal, QC). The separation was performed on a Waters Spherisorb 5 µm ODS2 4.6 × 150 mm analytical column. The mobile phase consisted of 1% formic acid in water (v/v) (solvent A) and 95% methanol/5% acetonitrile (v/v) (solvent B). Injection volume 20 µL and flow rate was kept at 0.8 mL/min for a total run time of 60 min. A linear gradient solvent (A:B) system was used as follow: 0–5 min (100:0); 5–10 min (90:10); 10–25 min (80:20); 25–35 min (75:25); 35–45 min (70:30); 45–50 min (20–80); 50–60 min, (100:0). Data were collected at 280 and 320 nm. Phenolic compounds were identified by comparing retention time and UV absorption spectra with available commercial standards. Compounds with no standard reference material were tentatively identified by UV spectrum, retention time and by matching with published data.

2.6.3 Soluble protein content and SDS-PAGE profile

Soluble protein concentration of the samples was determined using DC Protein Assay (Bio-rad,

California, USA). In brief, 100 mg of ground sample was weighted, mixed with 1 mL distilled water, and centrifuged for 10 min at 10 000 *g*. Five microliters of appropriately diluted supernatant were then placed in a 96 well plate, mixed with reagent A (alkaline copper tartrate solution) and 200 µL of reagent B (diluted Folin reagent). The plate was shacked for 5 s and allowed to stand for 15 min at room temperature, before reading the absorbance at 750 nm with a microplate reader (Tecan, Switzerland). A standard curve was prepared with bovine serum albumin (BSA) using concentrations of 0.08–1.23 mg/mL in deionised water and measured along with samples. The protein concentration was calculated using a BSA curve (y = 0.2777 × −0.0115, R^2 =0.9874). Proteins from SSF and germination were separated by SDS-PAGE under reducing condition. In brief, protein extracts were prepared with sample buffer to a final concentration of 0.5 mg/mL of proteins, then denatured for 10 min at 95 °C and loaded onto a denaturing gel (6% to 18% acrylamide gradient), prior to electrophoresis (75 min, 120 V). The gel was then stained with GelCode^{TM} Blue Safe Protein Stain according to the manufacturer manual and visualised with ChemiDoc MP Imaging System (Bio-Rad, USA). Bands intensity was quantified using the ImageJ software version 1.x (NIH, USA).

2.6.4 Determination of protein degree of hydrolysis (DH)

Degree of hydrolysis of proteins was determined accord- ing to the method described by Nielson *et al.*[41], with some modifications. In brief, 20 mg of ground sample was weighted, mixed with 1 mL of distilled water, and centrifuged for 10 min at 10 000 *g*. In a 96-well plate, 30 µL of sample (supernatant), standard (serine), or control (deionized water) was mixed with 225 µL of OPA reagent. The mixture was incubated for 2 min at room temperature (23 °C), before reading the absorbance at 340 nm. Degree of hydrolysis was determined with Eq (1) and expressed as meqv Ser-NH_2/g DW.

$$\text{Serine-NH}_2 = \frac{(ODsample - ODblank)}{(ODstandard - ODblank)} \times 0.9516 \frac{meqv}{L} \times 0.1 \times \frac{100}{x} \quad (1)$$

Where serine-NH2= meqv serine NH_2 g sample; X= g sample; 0.1 is the sample volume in litre.

2.7 Determination of enzyme inhibition activity

2.7.1 α-Glucosidase inhibition

α-Glucosidase inhibition was determined according to the method described by Mojica L. (2016)[42], with modifications. In brief, 100 mg of sample was mixed with 1 mL of simulated intestinal fluid (SIF)[35], shaking incubated for 10 min at 37 °C and 90 rpm, and centrifuged for 10 min at 10 000 *g*. The extract was then diluted in phosphate buffer (pH 6.9) to the final assay concentrations of 25, 12.5 and 6.25 mg/200 µL. Fifty microliters of the extract, control (phosphate buffer) or standard (1 mM Acarbose) were then transferred in triplicate in a 96-well microplate. 100 µL of 1 U/mL α-glucosidase solution and the mixture was incubated for 10 min at room temperature (23 °C). Fifty microliters of the substrate solution (5 mM p-nitrophenyl- α-D-glucopyranoside dissolved in 0.1 M sodium phosphate buffer, pH 6.9) was then added to each well, and the mixture was incubated for 5 min at 25 °C, before reading the absorbance at 405 nm in a microplate reader (Tecan, Switzerland). The percentage of α-Glucosidase inhibition was determined with the with Eq (2):

$$\frac{Abs\ test\ (Abs\ test - Abs\ blank) - Abs\ control\ (Abs\ control - Abs\ blank)}{Abs\ control\ (Abs\ control - Abs\ blank)} \times 100 \quad (2)$$

Values for α-glucosidase inhibition were then converted in millieqv of Acarbose (Fig. 1).

2.7.2 DPP-IV Inhibition

DPP-IV inhibition was determined according to the method described by Lacroix *et al*.[43] and Nongonierma & FitzGerald[44], with modifications. In brief, 100 mg of sample was mixed with 1 mL of simulated intestinal fluid (SIF)[35], shaking incubated for 10 min at 37 °C and 90 rpm, and centrifuged for 10 min at 10 000 *g*. The extract was then diluted in Tris-HCl buffer (pH8.0). Twenty-five microliters of the extract, control or standard (Diprotin A) were then transferred in a 96-well microplate followed by addition of 25 µL of substrate (Gly-Pro-p-nitroanilide 1.6 mM) and incubation for 10 min at 37 °C. Fifty microliters of DPP-IV (1852 U/mL)were then added and the mixture was incubated for 60 min at 37 °C. At the end of the incubation, the absorbance was immediately read at 405 nm with a microplate reader (Tecan, Switzerland). DPP-IV inhibition in percent- age was determined as:

$100 \times \{1 - [(A_{405}\ (\text{test sample}) - A_{405}\ (\text{test sample blank})]/[(A_{405}\ (\text{positive control}) - A_{405}$

(negative control)]}, and expressed as milliequivalents of Diprotin A. Positive control was prepared with 25 µL SIF, 25 µL substrate, 50 µL DPP-IV. Negative control was prepared with 75 µL SIF and 25 µL substrate. A standard curve was prepared with increasing concentration (0.5–40 µM) of Diprotin A. Values for DPP-IV inhibition were then converted in millieqv of Diprotin A (Fig. 1).

2.8 Brightfield and fluorescence microscopy

The microstructure of bioprocessed samples was analysed under bright field and fluorescence microscopy. Samples were fixed for 90 min at 37 °C in FAA solution (2:1:17 of 10% formalin, glacial acetic acid, 75% ethanol), as described by Schichnes *et al*.[45] Fixed samples were then dehydrated through a series of ethanol solutions, positioned in molds, embedded in paraffin and mounted on blocks for sectioning. Sections were cut to a thickness of 0.5 μm with a vibratome and each section was positioned and dried on a slide. Before staining, paraffin was removed by ethylene and ethanol cycles, then washed in distilled water and air dried. For Toluidine Blue O staining, a 0.1% aqueous solution was prepared and sections were stained for 15 min, washed with water, and mounted on a glass slide with 25% glycerol solution. Samples were observed with brightfield microscopy (Carl Zeiss, Germany). For Calcofluor staining, a mixture of Calcofluor White Stain (Sigma-Aldrich, St. Louis, USA) and 10% potassium hydroxide (1:1) was placed on the specimen for one minute, before observation under UV light using DAPI channel (Carl Zeiss, Germany). Toluidine blue O is a polychromatic dye that reacts with various constituents of the plant cells, with each reaction leading to a different color. A pinkish-purple color is generated when Toluidine blue O reacts with carboxylated polysaccharides; bright blue when reacting with poly-aromatic compounds (e.g., lignin, tannins), and purplish-greenish blue with nucleic acids.[46] Calcofluor white is a fluorescent stain that strongly binds structures containing cellulose, callose, lignin, and other non- or weakly substituted β-glucans.[46]

2.9 Statistical analysis

Experiments were carried out on three biological replicates, and each assay was further carried out in triplicate. Data were expressed as mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was used to compare different treatments on the same pulse. Tukey's multiple comparison test was carried out to determine any significant differences between different sample treatments. Differences were considered significant when $p < 0.05$. Statistical analyses were carried out using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA).

Results

3.1 Efficiency of bioprocessing

The pH significantly decreased for all samples during SSF, with ground samples showing the most prominent decrease in all the pulses. The pH value dropped from 5.9 ±0.2 to 4.3 ±0.6 in soaked and cracked pulse seed samples, from 6.3 ±0.1 to 4.5 ±0.2 in autoclaved seed samples, and from 6.1 ±0.1 to 3.9 ±0.1 in ground samples. The bacterial population of *L. plantarum* increased to 9.2 log colony forming units (CFU)/mL in soaked samples, to 9.3 log CFU/mL in autoclaved samples and decreased to 6.1 CFU/mL in ground samples. Germination efficiency, calculated as percentage of germinated seeds over the total number of seeds was maximum (100%) for green lentils, both at day 3 and 5 of germination, followed by yellow peas (97.7 ±0.5% and 99.3 ±1.0% at days 3 and 5, respectively) and faba beans (90.0 ±1.7% and 91.9 ±7.6% at days 3 and 5, respectively). Kidney bean and chickpea germination efficiency had higher variability (77.6 ±12.9% and 90.1 ±2.4% at days 3 and 5, respectively for kidney beans and 63.5 ±1.3% and 65.1 ±5.2% for chickpeas), which makes these two germinated pulses less befitting for further application in our study.

3.2 Effect of bioprocessing on bioaccessibility of bioactive compounds and inhibition of glucose-regulating enzymes

3.2.1 Total phenolic content and degree of hydrolysis of proteins

Total phenolic content and protein degree of hydrolysis values for physically and biologically processed pulses, before and after simulated gastrointestinal digestion, are shown in Table 1. Phenolic contents before bioprocessing (germination and solid-state fermentation) differed between pulses, with the highest values observed for faba beans, kidney beans and green lentils. Grinding the pulses to flour prior to bioprocessing led to an increase in total phenolic content (TPC) in faba beans, kidney beans and green lentils. During germination, a gradual and significant increase in TPC before *in vitro* digestion was observed in faba beans and yellow peas, while kidney beans showed the opposite trend. The highest increase was observed for faba beans, with TPC values almost doubling during 5 days of germination. Simulated gastrointestinal digestion led to a significant increase in TPC in all the germinated pulses, but no significant difference in TPC was observed between various germination times of each digested pulse. Germination also induced a significant increase in protein digestibility, measured as degree of hydrolysis (DH). The highest increase prior to *in vitro* digestion was observed in yellow peas, faba beans and green lentils, with digestibility increasing by two or three folds. Simulated gastrointestinal digestion also led to a substantial increase in DH in all the pulses, when compared to the undigested samples. However, the changes in DH caused by germination were not significant after gastrointestinal digestion, with the exception of yellow peas. In fact, digested germinated yellow peas showed the overall highest increase in protein digestibility among all samples.

The pre-treatment applied before SSF substantially influenced the ability of *L. plantarum* to ferment the substrate (i.e., the pre-treated sample). Soaking and grinding increased TPC and DH with SSF in chickpea and yellow pea, while a decrease was observed after SSF of heat-treated samples. A significant increase in TPC during 48 h fermentation with *L. plantarum* was observed for ground green lentil samples and soaked kidney beans, while SSF of heat-treated samples led to a significant decrease in TPC in faba beans. Protein digestibility was higher in soaked and ground unfermented pulses compared to the heat-treated ones. SSF of ground samples led to an increase in DH in all the pulses except for green lentils, with significant values observed for yellow peas. *In vitro* digestion induced a large increase in all TPC and DH values, with the exception of kidney beans, masking the effect of the bioprocessing. Despite the bioprocessing applied, faba beans, kidney beans and green lentils had the highest TPC, while green lentils and yellow peas had the highest values for protein digestibility.

		GERMINATION						SOLID STATE FERMENTATION											
Pulse		Day 0		Day 3		Day 5		S		S + SSF		HT		HT + SSF		G		G + SSF	
		TPC	DH	TPC	DH	TPC	DH	TPC	DH	TPC	DH	TPC	DH	TPC	DH	TPC	DH	TPC	DH
Chickpeas	ND	1.72 ±0.15	20.49 ±3.01^{1}	2.41 ±0.17	24.11 ±3.15^{2}	2.16 ±0.19	32.01 ±5.64^{12}	1.10 ±0.47^{a}	16.52 ±1.26^{123}	2.21 ±0.06	17.04 ±0.18^{45}	1.79 ±0.45^{b}	12.08 ±0.35^{1467}	1.79 ±0.09^{c}	10.57 ±0.65^{2589}	1.86 ±1.04	17.13 ±0.08^{68}	3.04 ±0.10^{abc}	21.18 ±1.00^{379}
	SGID	5.39 ±0.23	103.75 ±2.31	5.43 ±0.20	111.35 ±10.26	4.36 ±0.52	109.60 ±4.94	3.19 ±0.80	97.37 ±8.59	2.44 ±0.26	87.62 ±7.28	2.61 ±0.25	100.92 ±2.04	3.13 ±0.55	102.00 ±17.33	3.18 ±0.24	94.13 ±5.01	3.59 ±0.29	92.36 ±1.19
Faba Bean	ND	2.40 ±0.24 ^{a}	23.93 ±1.38^{1}	3.22 ±0.20 ^{a}	27.49 ±2.35^{2}	4.33 ±0.43 ^{a}	47.44 ±17.71^{12}	2.78 ±0.96^{a}	17.42 ±4.61^{123}	3.40 ±0.91	17.98 ±1.67^{456}	3.75 ±0.34^{bc}	12.66 ±1.30^{1478}	2.59 ±0.24^{bd}	11.07 ±0.77^{259*}	4.05 ±0.69^{a}	19.14 ±1.82^{79}	3.87 ±0.58^{cd}	22.41 ±1.75^{368*}
	SGID	5.71 ±0.62	137.84 ±5.11	7.12 ±0.41	134.42 ±1.65	7.26 ±1.84	138.23 ±4.48	4.80 ±0.44	119.98 ± 6.80	4.33 ±0.38	113.74 ±10.38^{1}	5.51 ±1.99	113.68 ±6.80	5.58 ±2.08	104.00 ±2.93^{2}	6.63 ±0.45	113.10 ±0.24	5.17 ±1.94	143.84 ±27.63^{12}
Kidney Beans	ND	3.24 ±0.42 ^{a}	18.94 ±1.08^{12}	2.65 ±0.92	24.73 ±1.14^{1}	2.52 ±0.13 ^{a}	27.87 ±1.72^{2}	2.14 ±0.81^{abc}	17.78 ±3.91^{12}	3.74 ±0.15^{ade}	16.24 ±2.15^{34}	2.40 ±0.25^{df}	10.34 ±0.89^{1356}	2.15 ±0.48^{egh}	9.53 ±0.44^{2478}	3.35 ±0.30^{bg}	17.16 ±0.13^{57}	4.12 ±0.35^{cfh}	19.10 ±2.55^{68}
	SGID	3.27 ±0.51	79.11 ±9.09	3.07 ±1.00	73.27 ± 5.35	3.61 ±0.52	71.06 ±7.33	3.27 ±0.44	79.41 ±14.97	3.54 ±0.65	98.11 ±4.57^{1}	3.14 ±0.38	95.17 ±17.55^{2}	2.93 ±0.09	85.17 ±3.16^{3}	2.76 ±0.54	83.31 ±13.50^{4}	2.87 ±0.39	51.12 ±33.59^{1234}
Green Lentils	ND	2.41 ±0.08	23.80 ±1.55^{1}	2.62 ±0.10	25.91 ±0.94^{2}	2.77 ±0.20	43.11 ±2.21^{12}	2.11 ±0.40^{abc}	23.57 ±3.99^{12}	2.51 ±0.39^{de}	22.33 ±0.70^{34}	3.99 ±0.47^{adf}	16.89 ±0.81^{1356}	2.90 ±0.19^{g}	16.29 ±1.28^{2478}	3.45 ±0.42^{bc}	24.48 ±2.09^{57}	6.06 ±0.88^{cefg}	23.03 ±0.63^{68}
	SGID	5.46 ±0.25	117.41 ±11.99	5.71 ±0.37	116.02 ± 6.23	4.61 ±0.82	120.42 ±5.70	4.85 ±0.43	118.21 ±19.82	4.02 ±0.78	121.96 ±5.48	4.07 ±1.26	126.91 ±10.47	3.39 ±1.28	122.01 ±6.01	4.12 ±0.75	114.99 ±23.92	3.75 ±0.18	111.31 ±5.66
Yellow Peas	ND	1.41 ±0.06 ^{a}	17.33 ±0.79^{1}	1.94 ±0.27	36.15 ±1.05^{1}	2.31 ±0.11 ^{a}	42.20 ±1.18^{1}	1.98 ±0.13	16.78 ±1.61^{123}	2.56 ±0.23^{a}	17.81 ±0.76^{456}	1.60 ±0.26	9.91 ±0.75^{147}	1.23 ±0.32^{ab}	9.05 ±0.25^{256}	1.91 ±0.22	14.00 ±0.70^{8}	2.68 ±1.00^{b}	24.65 ±0.78^{3678}
	SGID	4.78 ±0.44	98.23 ±6.72^{1}	5.38 ±0.43	102.27 ±3.23^{2}	5.11 ±0.52	125.54 ±8.77^{12}	3.39 ±0.76	90.50 ±16.04	3.10 ±0.47	117.24 ±10.49	2.99 ±0.52	95.35 ±5.28	3.45 ±1.33	87.90 ±5.10	3.35 ±0.16	90.82 ±4.79	4.49 ±0.57	115.56 ±7.02

Table 1. Total phenolic content (TPC), expressed as gallic acid equivalent/g DW, and protein degree of hydrolysis (DH), expressed as milliequivalent Ser-NH2/g DW, of bioprocessed pulses (mean ± SD, n = 3). Germination was performed for 0 (Day 0), 3 (Day 3) and 5 (Day 5) days in the darkness at 23 °C. Solid state fermentation (SSF) with L. plantarum ATCC 8014 for 48 hours at 37 °C. Prior SSF, samples were either soaked (S) overnight, heat treated 121 °C for 15 min (HT), or grinded into flour (G). ND: non digested; SGID: simulated gastrointestinal digestion. For each row and bioprocessing (germination and fermentation), TPC values marked with the same letter are significantly different ($p < 0.05$); DH values marked with n are significantly different ($p < 0.05$).

3.2.2 DPP-IV inhibitory activity

Results on the DPP-IV inhibition assay are reported in Supplementary Table S1. Results converted into diprotin A equivalents are shown in Fig. 1, Panels A and B. The DPP-IV inhibition did not deviate significantly from the average value of 57% inhibition. Regardless the processing method, green lentils and faba beans showed higher DPP-IV inhibitory activity compared to the other pulses. Germination had a negative impact on the bioactivity, with the exception of faba beans. Among the processing methods, the heat treatment appeared to be the most impactful in enhancing the bioactivities. The combination of heat treatmentand SSF with *L. plantarum* appeared to be beneficial only for chickpeas, while a slight decrease in bioactivity was observed for the other pulses. Soaking and grinding of samples had mild effect on DPP-IV inhibition, while their combination with SSF appeared to be beneficial in the case of green lentils and yellow peas. Overall, yellow peas and green lentils were the most susceptible pulses to the activity of *L. plantarum*, with beneficial outcome on their bioactivity. SSF of ground pulses induced an increase in DPP-IV inhibitory activity for green lentils and yellow peas. On the other hand, the combination of grinding and SSF led to a significant decrease in bioactivityfor kidney beans.

3.2.3 α-glucosidase inhibitory activity

Results of the α-glucosidase inhibition assay are reported in Supplementary Tables S2. Results converted in Acarbose equivalents are shown in Fig. 1, Panels C and D. As observed for DPP-IV, unprocessed green lentil showed the highest inhibitory activity against α-glucosidase. α-Glucosidase inhibitoryactivity varied substantially between pulses after germination (Fig. 1, Panel C). In chickpeas, germination for 3 days caused a significant increase of about 36% of the bioactivity, which then slightly decreased at day 5 of further germination. Likewise, germination of kidney beans and yellow peas led to a moderate increase in α-glucosidase inhibitions. Opposite trend was observed for faba beans and green lentils, with a significant decrease of about 24% of the bioactivity after 5 days of germination in the latter case. The pre-treatment applied before SSF had asubstantial influence on α-glucosidase inhibition (Fig. 1, Panel D). SSF of heat-treated samples led to a decrease inbioactivity in all the pulses, while fermentation of ground flour greatly increased the bioactivity, except for chickpeas. The most remarkable impact of SSF was observed in ground green lentil and yellow pea samples, where the bioprocessing led to an increase of 18 % and 17 % of α-glucosidase inhibitory activity, respectively. Heat treatment caused an increase in bioactivity in all the pulses, when compared to the other treatments.

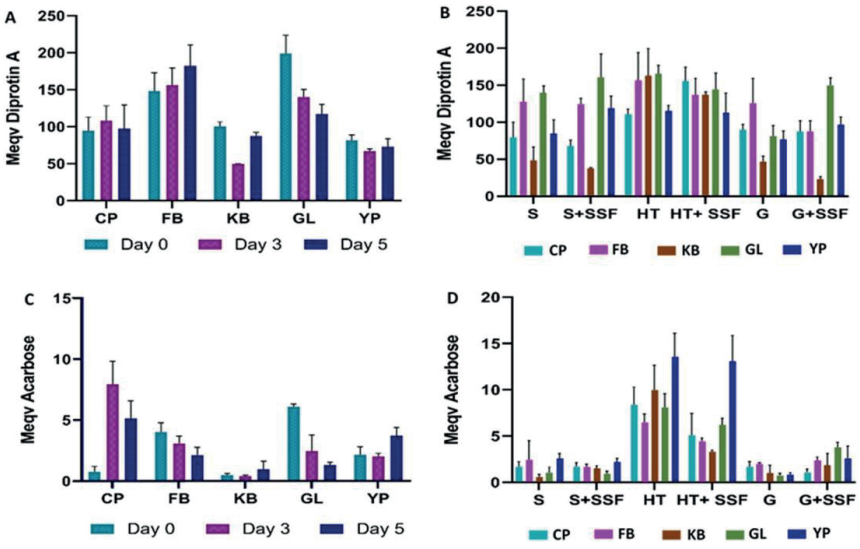

Figure 1. Enzyme inhibitory activity of bioprocessed pulses after in vitro digestion. (Panels A, B): DPP-IV inhibition of germinated (A) and solid state fermented (B) pulse samples. Values are expressed as milliequivalent Diprotin A (mM Diprotin A Eqv/100 mg DW). (Panel C, D): α-glucosidase inhibition of germinated (C) and solid state fermented (D) pulse samples. Values are expressed as milliequivalent Acarbose (mM Acarbose Evq/100 mg DW). CP: chickpea, FB: faba bean, KB: kidney bean, GL: green lentil, YP: yellow pea; S: soaked, SSF: solid state fermentation, HT: heat treatment, G: grinding. Data are expressed as mean ± SD of three independent replicates.

3.3 Changes in phenolic and protein profiles, and microstructures of yellow peas and green lentils

3.3.1 Protein profile (SDS-PAGE)

The protein profiles of fermented and germinated yellow pea extracts are shown in Fig. 2. Panels A and C show protein profiles of the seed samples upon soaking, heat treatment and grinding, alone or in combination with SSF (*L. plantarum*). When considering the unfermented samples (S1, T1, G1), more bands can be observed in the ground seeds (G1), while heat treatment (T1) seemed to have substantially decreased the number and intensity of the protein bands. Yellow pea proteins were characterized by a variety of polypeptide subunits in the molecular weight range of 10 to 97 kDa, with major subunits at 17–19 kDa (albumin fraction), 25 kDa (legumin β), ~30 kDa (lectin), 40 kDa (legumin α), 35 and 50 kDa (vicilin), 70 kDa (convicilin), and 97 kDa (lipoxygenase), which were identified based on literature.[47–50] Albumin, lipoxygenase and legumin alpha were degraded substantially in ground-SSF yellow pea samples (G2), showing a 10- to 26-fold decrease in band intensities. Soaked-SSF pea samples (S2) also showed a 3- to 9-fold decrease in band intensities for the same proteins. SSF appeared to be less effective on heat treated samples (T2) when compared to the other pre-treatments, and this was especially evident in green lentils (Fig. 2C), where no decrease greater than 1-fold was observed for the major proteins. In accordance with the results on protein degree of hydrolysis (Table 1), a combination of grinding and SSF led to the most marked decrease in protein bands, possibly due to extensive proteolytic activity during fermentation. Simulated gastrointestinal digestion heavily affected the intensity of the bands, especially in green lentils, suggesting an intense proteolytic activity of digestive enzymes on pulse proteins. Besides an overall decrease in bands intensity over time, it is interesting to notice the changes in certain groups of proteins, and not others,

during germination. In pea proteins (Fig. 2, Panel B), the major bands of convicilin, vicilin and provicilin (32 kDa) showed a 1.5- fold decrease in band intensity after 3 days of germination. Similar to the pea proteins, the bands appeared very intense in soaked (S1) and ground (G1) samples, and significantly less intense after heat treatment (T1). Similar to the pea proteins, it is possible that the intense heat treatment caused denaturation and aggregation of proteins, with subsequent decrease in solubility. Solid-state fermentation appeared to be very effective in degrading proteins, since only a few bands with weak intensities were present in profiles of S2 and G2 samples. On the other hand, SSF of heat-treated lentils did not significantly affect the protein profile, except for the ~50 kDa, which showed a 2-fold decrease in T2. Germination of green lentils resulted in an overall decrease in band intensities (Fig. 2, Panel D). As expected, simulated gastrointestinal digestion had an intense effect on all samples, with very little protein bands present after the treatment (Fig. 2: S3, T3, G3, DO*, D3*, D5*).

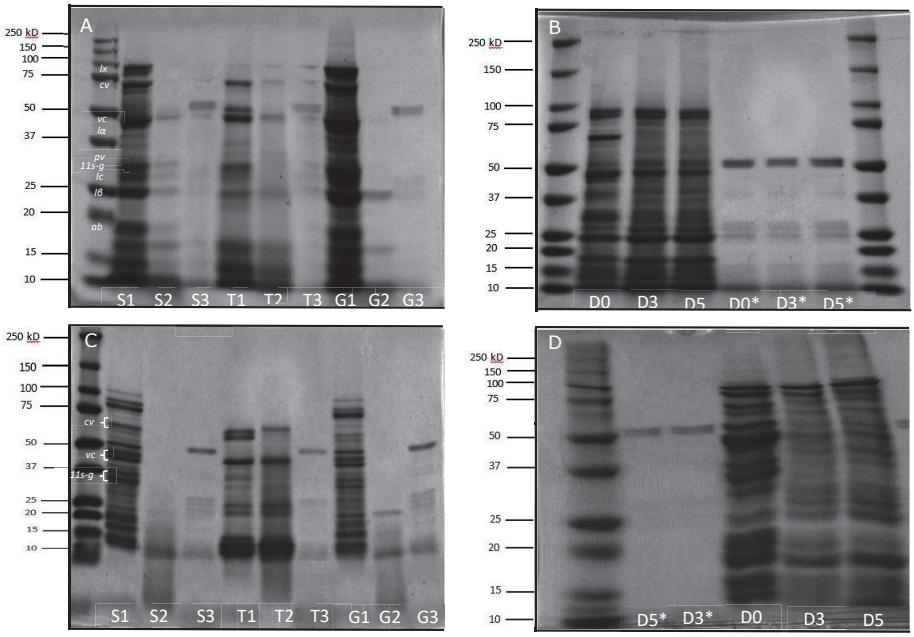

Figure 2. SDS-PAGE profiles of water-soluble extracts obtained from yellow peas (Panels A,B) and green lentils (Panels C,D) by solid state fermentation with Lactobacillus plantarum at 37 °C for 48 h (Panels A,C) and germination (Panel B,D). (Panels A,C): S1–S3: soaking (S1) followed by SSF (S2) and simulated gastrointestinal digestion (S3); A1–A3: autoclaving (A1) followed by SSF (A2) and simulated gastrointestinal digestion (A3); G1-G3: grinding (G1) followed by SSF (G2) and simulated gastrointestinal digestion (G3). Panel B, D: D0-D3: day 0 (D0), 3 (D3) and 5 (D5) of germination at room temperature (23 °C) in the darkness. ab: albumin fraction; lβ: legumin β; lc: lectin; lα: legumin α; pv: provicilin; vc: vicilin; cv: convicilin; lx: lipoxygenase; 11s-g: 11s globulin.

3.3.2 Identification and quantification of phenolic compounds in bioprocessed green lentil and yellow pea

A total of 12 and 11 phenolic compounds were identified in green lentil and yellow pea, respectively (Supplementary Table S3). When looking at the green lentil phenolic profile (Supplementary Fig. S1), compounds with the highest intensities corresponded to flavonoids, with predominance of kaempferol derivatives. In lentils, both germination and fermentation led to a significant decrease in intensities of kaempferol tetraglycoside and kaempferol triglycoside, which corresponded to an

increase in intensities of three flavonoid compounds in the germinated samples and that of the kaempferol glycoside in fermented samples. In yellow peas, the highest peaks were identified to be vanillic acid and flavonoids (luteolin glucoside, kaempferol derivative, and flavonoid derivative). Germination for 5 days caused a substantial increase in protocatechuic acid, vanillic acid, chlorogenic acid and flavonoid derivatives. Two peaks were detected at the beginning of the yellow pea germination (day 0) at approximately R_t of 15 and 18 min, which significantly decreased as germination progressed, while four new peaks appeared at R_t of 14, 15.4, 16.7 and 19 min at day 5 (Supplementary Fig. S1). Peaks corresponding to chlorogenic acid, luteolin glucoside luteolin-6-C-glucoside and flavonoid derivative were not retained after simulated gastrointestinal digestion of yellow pea.

3.3.3 Seed microstructure

The microstructures of the untreated, germinated and SSF yellow pea and green lentil are shown in Figs 3 and 4. In yellow peas, the cuticle (*c*), *linea lucida*, palisade layer (*p*) of osteosclereids (*o*) and macrosclereids, parenchyma (*pr*) and aleurone layer (*al*) could be identified (Fig. 3, Panel A). Germination and solid-state fermentation with *L. plantarum* had an evident effect on the seed coat composition. In both cases, macrosclereids and osteosclereids greatly increased in size, while parenchyma cells disappeared. Interestingly, the palisade cells turned into a lighter pinkish-purple colour as a result of SSF (Fig. 3, Panel C, J), possibly indicating the presence of carboxylated polysaccharides. Moreover, the *linea lucida* was no more evident in solid-state fermented yellow pea seed, which was expected after water imbibition. When considering the cotyledon of yellow pea seeds (Fig. 3, Panel D, E, F), cell wall (*CW*), cell membrane (*CM*), middle lamella (*ML*), starch (*ST*), and proteins (*PT*) could clearly be identified in the soaked seed (Fig. 3, Panel D). Germination caused an increase in cell wall thickness, depletion in starch and protein granules and increase in other protein-sized molecules which greatly stained with toluidine blue, possibly phenolic compounds. On the other hand, SSF with *L. plantarum* caused a more intense depletion of starch and storage proteins, and a breakage of the cell wall in certain regions of the seeds (Fig. 4). The same cellular structures were identified in green lentils (Fig. 3, Panel G-M). SSF with *L. plantarum* caused a similar effect on osteoscleroids, while macrosclereids appeared to be decreased in size in this case. In the cotyledon, bacterial fermentation only depleted starch granules and proteins in certain cells, but not in others. The cell membrane increased in size, and the cytosol turned into a greenish color, indicating a change in compositional factors. Germination did not follow the same trend observed for yellow pea, and both seed coat and cell wall appeared thinned after germination (Fig. 3, Panel H, L).

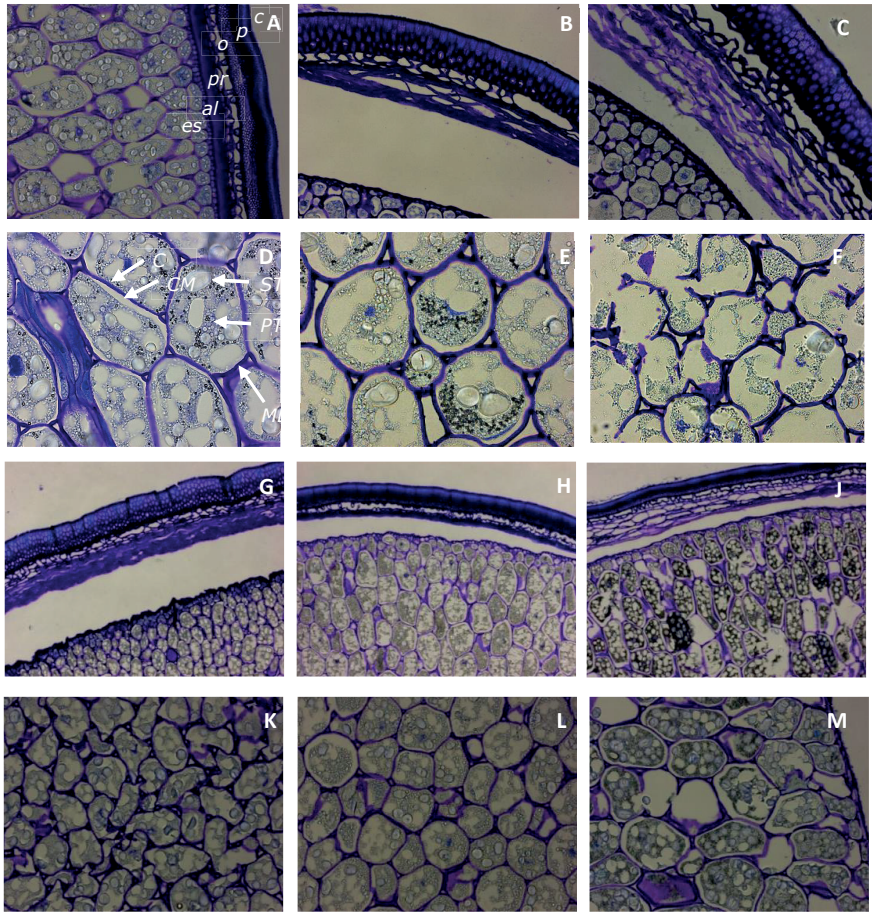

Figure 3. Panel A–F: Yellow pea visualised by Toluidine blue O. Top: seed coat (testa), brightfield, 20×. Bottom: cotyledon, brightfield, 40×. Panels A, D: soaked; B, E: germinated; Panels C, F: solid state fermented with L. plantarum. c: cuticle; p: palisade cells; o- osteosclereids; pr: parenchyma; al: aleurone; es: endosperm; CW: cell wall; CM: cell membrane; ML: middle lamella; ST: starch; PT: protein. Panels G–M: green lentils visualised by Toluidine blue O. Top: seed coat (testa), brightfield, 10×. Bottom: cotyledon, brightfield, 20×. Panels G, K: soaked; Panels H, L: germinated; Panels J, M: solid state fermented with L. plantarum.

Discussion

Digestibility and nutritional value of legume seeds are primarily limited by the presence of anti-nutritional fac-tors and the thick cell wall and seed coat, which represent physical barriers that limit the activity of digestive enzymes.[51] Food bioprocessing can influence the integrity of plant cell walls, dissociate the nutrient-matrix complexes ultimately improving nutrients bioavailability. Fermentation, heat treatment, germination and enzymatic hydrolysis prior to simulated digestion have been related to an increase in the release of antioxidants, DPP-IV inhibitors and improved protein digestibility in pulses.[13–15,21–23] In this paper, five bioprocessed pulse samples were subjected to simulated gastrointestinal digestion, and subsequently tested for their ability to inhibit DPP-IV and α-glucosidase, two key enzymes involved in human blood glucose regulation. The enzymatic assays were performed

in simulated intestinal fluid (SIF)[35] to simulate the human body environment. Yellow peas and green lentils were chosen as representative pulses for further investigation. Among the fermented samples, grinding combined with SSF was chosen as the preferred method, based on the bioactivity. The observed effects of germination and SSF on bioaccessibility of the bioactive compounds in common pulses are discussed separately.

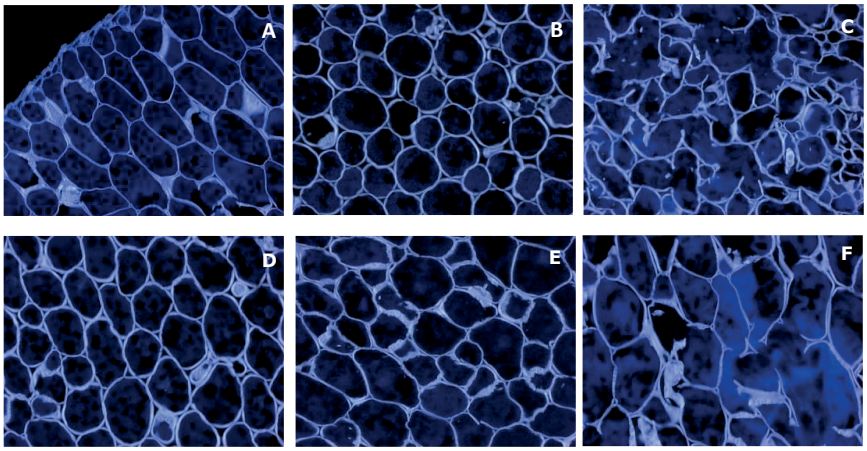

Figure 4. Yellow pea (Panels A–C) and green lentil (Panels D–F) visualised by Calcoflour White Stain, UV light, 20×. (Panels A, D): soaked; (Panels B, E): germinated; (Panels C, F): solid state fermented with L. plantarum.

4.1 Germination

Germination triggers the displacement of storage proteins (legumins and vicilins), *de novo* synthesis of proteins, energy generation, synthesis of primary (amino acids, lipids, sugars) and secondary metabolites, signal molecules, rearrangement of the cellular architecture, and activation of defensive mechanisms to protect the seed from pathogens and oxidative damage caused by reactive oxygen species.[28] Interestingly, the storage protein convicilin that disappeared in the SDS-PAGE (Fig. 2, Panel B), plays a major role in the loss of desiccation tolerance, which initiates germination.[28] Bands that intensified after germination had molecular weight ranges of 27–39 kDa and 50–90 kDa, corresponding to proteins previously reported to increase during pea seed germination.[28] Green lentil protein profile was also characterized by a variety of polypeptide subunits in the molecular weight range of 17–150 kDa, with major subunits at 17–22 KDa (albumin and γ-vicilin fractions), 24–35 kDa (basic and acidic subunits of 11 S globulin), at 48 kDa (vicilin) and 70 kDa (convicilin)[49,52] (Fig. 2, Panel C,D). Moreover, many studies reported that germination significantly increased phenolic compounds in a broad variety of edible seeds. The increase was not always linear, as different classes of phenolic compounds are synthesised in different quantities at different germination stages, according to their physiological functions.[29,30] *De novo* synthesis of flavonoids and phenolic acids can occur during germination, due to the phenylalanine-lyase (PAL) enzyme[53], and possibly occurred in our study for flavonols (kaempferol derivatives), flavanones (naringenin), flavone (luteolin glycosides), hydroxybenzoic acids (syringic and vanillic acid) and hydroxycinnamic acids in yellow pea and green lentil. In fact, no major decreases were observed in phenolic compounds present at the initial stage of the germination (day 0). Similar to our study, an increase in total phenolic and flavonoids content was observed during germination of sweet corn, with a decrease in bound flavonoid content. The

authors further analysed the phenolic profile and observed an increase in syringic acid, hydroxycinnamic acid and ferulic acid, but did not observe any change in phenolic composition.[54] In contrast, we observed a change in the phenolic composition during germination, and this was particularly evident in the yellow pea extract.

When considering the difference observed in the protein profiles (Fig. 2), phenolic profiles (Table 2) and seed microstructures (Figs 3 and 4) of yellow pea and green lentils, it is not surprising that the bioactivity measured (DPP-IV and α-glucosidase inhibition) substantially changed from one pulse to another (Fig. 1, Panels A, C). In fact, bioactivity is strictly related to the bioactive compound profiles, especially for phenolics and peptides. The interaction between enzyme and bioactive molecules, in this case phenolics or peptides, is highly specific and takes place only if specific structural requirements are present. For instance, peptides derived from germinated soybean proteins showed an increased DPP-IV and α-glucosidase inhibitory activities, while peptides generated from proteins of germinated cowpea bean were significantly less bioactive, when compared to their non-germinated forms.[13,31] The phenolic compounds identified in the two legume seeds were greatly different (Table 2), although belonging to the same classes (flavonoids, phenolic acids). This could explain why germination caused a decrease in the bioactivity of green lentils, but not yellow peas (Fig. 1, Panels A, C).

4.2 Solid state fermentation

The metabolic activity of *L. plantarum* on cell wall components was evident in our study, with cell walls greatly disrupted at the end of the fermentation (Fig. 3, Panels C, J, and Fig. 4, Panels C,F), especially in yellow pea seed. SSF also affected the composition of the osteosclereids in the seed coat of both pulses, which resulted in a purple-violet colouration when stained with Toluidine blue. This specific coloration derives by binding of the dye to carboxylated compounds, and carboxylases have indeed been identified in *L. plantarum*.[55] In order to survive in its natural plant material habitat, *L. plantarum* developed a series of adaptive mechanisms, *i.e*. set of enzymes, that allow it to convert phenolics such as tannins into bioactive and less toxic compounds.[34,56] Bioconversion of glycosylated phenolic compounds to their aglycone forms was primarily related to the activity of bacterial β-glucosidase, which is a major enzyme involved in the breakage of the ester bonds between bound phenolic compounds and plant cell wall constituents, with the release of free and bound phenolics.[57,58] *L. plantarum* ATCC8014 indeed exhibited high β-glucosidase activity.[59] Deglycosylation and biotransformation by lactic acid bacteria was reported to significantly improve intestinal barrier integrity *in vitro*, and flavonoid bioavailability *in vivo*.[60] Similarly, in our study, kaempferol glycosides in green lentils were deglycosylated during SSF with *L. plantarum* (Table 2). The same glycoside activity was likely responsible for the significant increase in total phenolic content measured by colorimetric assay (Table 1). The physical form of the substrate greatly affects the outcome of fermentation, making the nutrients required for bacterial growth more or less available. In our study, thermal treatment (121 °C, 15 min) of pulse seeds had a negative impact on bacterial growth. Intense heat treatment cause starch gelation, which impairs enzyme accessibility and could explain the low bacterial growth and low protein hydrolysis observed in all the heat-treated samples in our study. On the other hand, grinding seeds to flour is expected to increase the surface area for bacteria activity and therefore improves bacterial growth. The loss of viability observed during the fermentation of ground pulse samples could be related to the extensive fermentation from *L. plantarum*, providing a significantly higher ΔpH compared to the other pre-treatments, similar to previous findings.[23,57,61,62] When considering the protein profile, SSF led to a considerable decrease in band intensity possibly due to

extensive proteolytic activity of *L. plantarum* on the pulse proteins and increase in protein fragments with a molecular weight below 10 kDa, or protein utilization by the bacteria. Proteolysis could be primarily induced by an acidic activation of endogenous proteinases, followed by the proteolytic activity of the *L. plantarum* to complete the hydrolysis leading to the formation of smaller peptides.[32]

In conclusion, germination and SSF with *L. plantarum* effectively altered the phenolic and protein profiles of yellow pea and green lentil, with improvement of their DPP-IV and α-glucosidase inhibitory activities. The micro- structure of legume seeds was significantly modified by the bioprocesses. Particularly, SSF caused a significant degradation of the plant cell wall, which is a major barrier to nutrients digestibility for humans in plant derived foods. Further studies are needed to investigate the bioavailability of bioactive compounds in the bioprocessed pulses, and their ability to exert DPP-IV and α-glucosidase inhibitory activities *in vivo* after oral consumption.

R_t	Compound	Germination				Fermentation		
Green Lentils		**Day 0**	**Day 3**	**Day 5**	**Day5+ *SGID***	**Flour**	**F + SSF**	**F + SSF + *SGID***
10.211	2,5-Dihydroxybenzoic acid[a]	60.51	54.21	49.07	3.66	66.93	57.08	49.81
11.513	Catechin glucoside[b]	24.23	9.01	7.57	174.54	77.72	63.15	59.30
13.336	Catechin gallate[b]	28.91	27.71	26.98	20.89	34.04	31.94	20.59
16.280	Unknown[b]	ND	ND	8.11	8.06	6.85	5.74	5.05
17.368	Syringic acid[a]	11.35	8.41	8.55	12.39	24.73	7.89	9.70
23.122	*p*-couamric acid[a]	6.58	6.12	6.14	6.74	8.69	88.87	26.62
39.929	Kaempferol tetraglycoside[b]	139.07	112.86	93.94	70.89	125.85	93.35	66.13
40.628	Kaempferol triglycoside[b]	87.28	80.93	76.58	58.89	82.23	64.89	48.87
42.093	Kaempferol glucoside/Luteolin glucoside	ND	ND	7.98	7.04	7.84	42.95	31.58
45.158	Flavonoid derivative/Apigenin methyl ether[b]	5.38	28.14	38.85	4.29	7.58	5.95	ND
45.748	Flavonoid derivative[b]	14.35	22.25	31.36	25.73	16.21	11.45	9.06
46.489	Flavonoid derivative[b]	13.23	41.76	61.34	46.25	16.79	10.86	8.22
Yellow Peas		**Day 0**	**Day 3**	**Day 5**	**Day 5 +SGID**	**Flour**	**F + SSF**	**F + SSF + SGID**
2.711	Protocatechuic acid[b]	18.97	217.40	436.09	505.71	31.69	93.38	426.12
4.803	Dihydroxybenzoic acid[b]	7.98	19.55	27.89	30.63	12.57	22.31	16.01
13.021	Hydroxybenzoic acid[b]	17.63	6.30	6.00	5.35	11.72	10.82	9.81
15.493	Vanillic acid[b]	16.90	13.45	12.88	7.59	55.72	48.71	44.93
16.744	Chlorogenic acid[a]	ND	7.45	8.41	ND	ND	ND	ND
18.857	Luteolin glucoside[b]	20.03	15.65	13.90	4.62	78.82	55.00	ND
19.029	Luteolin glucoside[b]	N.D.	14.99	16.96	12.28	ND	ND	ND
28.17	Ferulic acid[a]	5.48	ND	ND	ND	5.90	ND	ND
42.451	Luteolin-6-C-glucoside/Kaempferol derivative[b]	22.19	31.50	51.96	15.23	26.03	19.37	18.08
47.19	Flavonoid derivative[b]	17.80	73.15	142.90	57.06	28.61	26.09	20.62
47.49	Naringenin[a]	49.28	50.61	54.97	50.54	49.22	53.16	49.24

Table 2. Influence of bioprocessing on the content (µg/g DW) of phenolic compounds in green lentil and yellow pea extracts. [a]Compound quantified as µg/g DW of the corresponding standard. [b]Compound quantified as Meqv rutin/g DW. Germination was carried out for 0 (day 0), 3 (day 3), and 5 (day 5) days, and subsequently subjected to simulated gastrointestinal digestion (Day 5 + SGID). Fermentation was carried out for 0 (flour) and 48 h (F + SSF), and the fermented flour was subsequently subjected to simulated gastrointestinal digestion (F + SSF + SGID).

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Author contributions

C.U., E.D., T.O. and V.F. conceived and designed the project. E.D. and A.T. performed the research and analyzed data, with significant feedback from C.U. and T.O. E.D. wrote the first draft of the manuscript. C.U., A.P., T.O. and V.F. revised the manuscript. E.D. and C.U. revised the manuscript after peer review.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary Material

		GERMINATION			SOLID STATE FERMENTATION					
Pulse	[mg/mL]	Day 0	Day 3	Day 5	S	S +SSF	HT	HT +SSF	G	G +SSF
Chickpea	**25**	39.0 ± 10.1^{ab}	75.3 ± 5.7^{a}	70.3 ± 4.7^{b}	52.9 ± 5.3^{a}	53.2 ± 4.0^{b}	78.0 ± 3.7^{abcd}	68.9 ± 8.9^{abcd}	52.6 ± 6.0^{c}	45.7 ± 5.6^{d}
	12.5	19.2 ± 7.3	55.0 ± 8.7	52.2 ± 4.1	28.1 ± 4.7	29.8 ± 5.6	41.4 ± 2.8	41.0 ± 2.7	31.3 ± 8.4	24.0 ± 2.4
	6.25	12.8 ±5.0	40.8 ± 13.4	43.3 ± 5.2	13.2 ± 6.1	13.2 ± 5.8	17.6 ± 2.9	15.6 ± 5.5	23.2 ± 15.5	17.2 ± 2.8
Faba Bean	**25**	66.6 ± 2.8	62.5 ± 3.2	56.4 ± 5.2	55.1 ± 13.2^{ab}	49.8 ± 3.5^{c}	82.5 ± 14.8^{acde}	68.3 ± 1.3^{bc}	55.7 ± 1.4^{d}	58.5 ± 2.4^{e}
	12.5	27.9 ± 0.6	30.5 ± 5.6	30.9 ± 7.8	32.0 ± 13.1	22.0 ± 3.6	41.8 ± 3.2	40.9 ± 4.1	34.2 ± 9.0	37.5 ± 7.5
	6.25	10.2 ± 4.8	15.6 ± 2.8	19.2 ± 7.4	10.9 ± 13.9	4.7 ± 4.2	17.9 ± 3.4	20.0 ± 6.8	20.8 ± 17.3	23.8 ± 15.7
Kidney Bean	**25**	33.2 ± 4.8	31.4 ± 2.7	40.4 ± 15.8	39.4 ± 15.9^{a}	51.2 ± 3.5^{b}	84.6 ± 13.0^{abcd}	63.6 ± 1.1	45.1 ± 23.1^{c}	50.5 ± 16.0^{d}
	12.5	5.3 ± 4.6	7.7 ± 0.5	13.8 ± 17.0	28.9 ± 12.6	38.2 ± 1.8	65.7 ± 7.4	36.5 ± 2.7	35.8 ± 12.9	11.5 ± 10.7
	6.25	ND	1.2 ± 1.7	8.9 ± 15.4	23.6 ± 8.7	27.5 ± 6.3	61.1 ± 30.6	45.5 ± 8.1	25.3 ± 11.7	1.8 ± 3.1
Green Lentil	**25**	73.3 ± 0.6^{ab}	57.7 ± 7.9^{a}	49.5 ± 2.4^{b}	44.0 ± 8.7^{ab}	42.6 ± 5.7^{cd}	77.5 ± 2.8^{ace}	86.6 ± 22.4^{bdf}	47.6 ± 18.7^{ef}	65.6 ± 2.4^{e}
	12.5	32.1 ± 4.6	24.7 ± 2.7	25.1 ±1.3	26.9 ± 3.1	18.7 ± 6.4	44.1 ± 1.4	45.7 ± 7.3	35.4 ± 3.6	35.5 ± 0.3
	6.25	5.5 ± 8.6	11.6 ± 7.2	8.8 ± 9.1	15.9 ± 9.1	9.1 ± 9.6	30.0 ± 2.8	25.7 ± 6.6	23.4 ± 15.0	14.0 ± 5.1
Yellow Pea	**25**	56.5 ± 5.2	56.0 ± 1.9	61.6 ± 9.7	55.3 ±10.4^{ab}	56.5 ± 3.8^{cd}	88.2 ± 5.0^{acef}	77.8 ± 13.4^{bdgh}	41.0 ± 5.0^{eg}	58.1 ± 9.1^{fh}
	12.5	20.6 ± 5.0	25.1 ± 6.2	35.5 ± 5.4	27.1 ± 2.6	23.4 ± 2.3	57.7 ± 2.3	55.2 ± 7.1	26.4 ± 6.0	36.1 ± 3.8
	6.25	5.3 ± 6.8	12.7 ± 5.3	14.7 ± 0.9	17.2 ± 13.1	ND	20.9 ± 4.7	27.6 ± 6.8	15.0 ± 4.6	16.5 ± 5.1

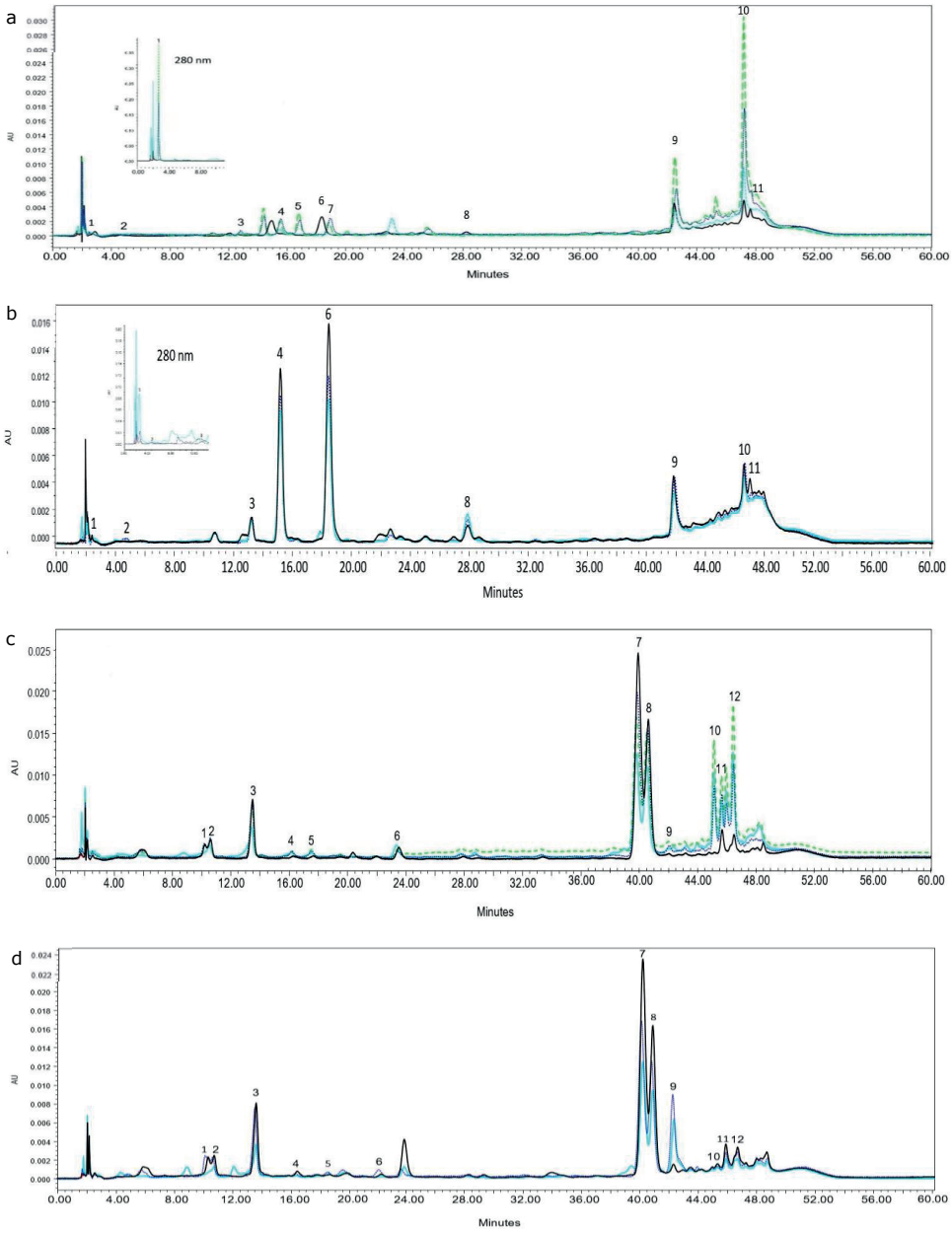
Supplementary Table S1. Effect of bioprocessing on α-glucosidase inhibition in five pulses, at a final concentration of 25 mg/mL (mean ± SD, n=3). Germination was performed for 0 (Day 0), 3 (Day 3) and 5 (Day 5) days in the darkness at 23°C. Solid state fermentation (SSF) with L.plantarum for 48 hours at 37°C. Prior SSF, samples were either soaked (S) overnight, heat treated 121°C for 15 min (HT), or grinded into flour (G). All samples were digested in vitro. α-Glucosidase inhibitory activity was monitored at three different sample concentrations (25, 12.5 and 6.25 mg/mL For each row and bioprocessing (germination and fermentation), values marked with the same letter are significantly different (p < 0.05).

	GERMINATION			SOLID STATE FERMENTATION					
Pulse	**Day 0**	**Day 3**	**Day 5**	**S**	**S +SSF**	**HT**	**HT +SSF**	**G**	**G +SSF**
Chickpea	57.4 ± 3.1[a]	59.6 ± 3.3[ab]	57.5 ± 5.1[b]	57.2 ± 9.3	52.1 ± 2.0[a]	60.6 ± 1.2	65.7 ± 2.0[a]	56.5 ± 1.6	56.1 ± 2.9
Faba Bean	61.1 ± 9.2[a]	65.7 ± 2.6	68.2 ± 2.7[a]	60.8 ± 5.9	63.3 ± 3.5	67.4 ± 6.9[a]	62.6 ± 4.4	61.8 ± 4.8	56.1 ± 3.0[a]
Kidney Bean	58.5 ± 1.0	47.1 ± 0.1	56.4 ± 0.8	45.7 ± 7.1[ab]	42.3 ± 0.7[cd]	66.1 ± 5.4[ace]	63.7 ±0.5[bde]	50.0 ± 9.5[e]	34.0 ± 2.8[ae]
Green Lentil	69.7 ± 2.0[a]	64.0 ± 1.2	61.1 ± 1.8[a]	63.9 ± 1.2	66.0 ± 3.5	66.6 ± 1.3	64.4 ± 2.6	56.9 ± 6.0	65.1 ± 1.1
Yellow Pea	55.1 ± 1.5	51.9 ± 0.8	53.3 ± 2.4	55.3 ± 4.2	61.2 ± 2.4	60.8 ± 1.1	60.2 ± 4.2	54.0 ± 2.6	57.8 ± 2.0

Supplementary Table S2. Effect of bioprocessing on DPP-IV inhibition in five pulses, at a final concentration of 25 mg/mL (mean ± SD, n=3). Germination was performed for 0 (Day 0), 3 (Day 3) and 5 (Day 5) days in the darkness at 23°C. Solid state fermentation (SSF) with L.plantarum for 48 hours at 37°C. Prior SSF, samples were either soaked (S) overnight, heat treated 121°C for 15 min (HT), or grinded into flour (G). DPP-IV inhibition was measured at the sample concentration of 25 mg/mL. All samples were digested in vitro. For each row and bioprocessing (germination and fermentation), values marked with the same letter are significantly different ($p < 0.05$).

Peak no.	Time (min)	Phenolics	Abs of detection	λ_{max} (nm)	Identification
Green Lentil					
1	10.211	2,5-Dihydroxybenzoic acid	320	224, 326	Rt, UV, standard
2	11.513	Catechin glucoside	280	224, 278	UV, λ_{max} ([17],[21])
3	13.336	Catechin gallate	280	224, 277	UV, λ_{max} ([17])
4	16.280	Unknown compound	320	224, 273	Rt, UV
5	17.368	Syringic acid	320	224, 272	Rt, UV, standard
6	23.122	p-coumaric acid	320	225, 271, 308	Rt, UV, standard, λ_{max} ([21])
7	39.929	Kaempferol tetraglycoside	320	224, 265, 346	UV, λ_{max} ([17],[21])
8	40.628	Kaempferol triglycoside	320	224, 265, 346	UV, λ_{max} ([17],[21])
9	42. 093	Kaempferol glucoside/ Luteolin glucoside	320	225, 265, 348	UV, λ_{max} ([17],[21])
10	45.158	Flavonoid derivative/ Apigenin methyl ether	320	225, 269, 328	UV, λ_{max} ([17],[21])
11	45.748	Flavonoid derivative	320	225, 269, 318	UV, λ_{max} ([17])
12	46.489	Flavonoid derivative	320	225, 269, 316	UV, λ_{max} ([17])
Yellow Pea					
1	2.711	Protocatechuic acid	280	215, 264	UV, λ_{max} ([22], [23], [24])
2	4.803	Dihydroxybenzoic acid	280	224, 259	UV, λ_{max} ([17])
3	13.021	Hydroxybenzoic acid	320	224, 274	UV, λ_{max} ([24])
4	15.493	Vanillic acid	320	224, 289	UV, λ_{max} ([22, 25])
5	16.744	Chlorogenic acid	320	224, 328	Rt, UV, standard
6	18.857	Luteolin glucoside	320	228, 284, 320, 364, 396	UV, λ_{max} ([24], [26])
7	19.029	Luteolin glucoside	320	224, 271, 320, 390	UV, λ_{max} ([24], [26])
8	28.17	Ferulic acid	320	224, 274, 320	Rt, UV, standard, λ_{max} ([22], [27])
9	42.451	Luteolin-6-C-glucoside/ Kaempferol derivative	320	225, 266, 346	UV, λ_{max} ([24], [17], [28])
10	47.19	Flavonoid derivative	320	225, 273, 320	UV, λ_{max} ([17])
11	47.49	Naringenin	320		Rt, UV, standard

Supplementary Table S3. Phenolic compounds identified by HPLC-DAD in green lentil and yellow pea extracts, based on retention time (Rt), UV spectra, λmax, or corresponding commercial standards.



Supplementary Figure S4. HPLC chromatograms of phenolic extracts of geminated (a,c) and fermented (b,d) yellow peas and green lentils, as detected at 320 nm, and with detail at 280 nm. Peaks without a number were not identified. Panel a & c, germination: black line: day 0, blue line: day 3, green line: day 5, light blue line: day 5 followed by simulated gastrointestinal digestion. Panel b & d, SSF with L. plantarum: black line: untreated flour, blue line: SSF flour, light blue line: SSF flour followed by simulated gastrointestinal digestion.

CHAPTER 3

Fermented green lentil decreases dipeptidyl peptidase-IV activity without altering glucose transport in human intestinal Caco-2 cells.

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Abstract

The surge in incidence of type 2 diabetes raises the need to rethink food consumption as a strategy to prevent and mitigate it. Food-derived dipeptidyl peptidase-IV (DPP-IV) inhibitors gained attention in recent years due to their high *in vitro* potency and minimal adverse effects. However, there is still missing information on their bioavailability after consumption, physiological stability through the gastrointestinal tract and *in vivo* activity. In our previous study we showed microbial fermentation of green lentil with *L. plantarum* improved DPP-IV inhibition when tested by biochemical assay. Indeed, the aim of this study was to investigate the effect of microbial fermentation on circulating and cell-bound (*in situ*) DPP-IV in polarized Caco-2 cells. The pre-fermented green lentil flour was digested by Infogest *in vitro* protocol and the supernatant was exposed to the luminal side of a Caco-2 cell monolayer. Exposure of the cells to 100 and 200 mg/mL of 72 h fermented lentil flour digest resulted in a significant decrease of the *in situ* DPP-IV activity by 29.7 and 37.3% when compared to the untreated cells, respectively. Microbial fermentation of the lentil flour also improved the inhibition of the circulating DPP-IV (basolateral) in a dose-dependent manner and reached 7.9% when 500mg/mL of 72 h fermented digest were used. Glucose absorption and uptake was not affected. The results of the study confirmed that fermentation with *L. plantarum* is a promising bioprocessing tool for enhancing the DPP-IV inhibitory activity of green lentil flour to design functional foods for type 2 diabetes patients.

Keywords: dipeptidyl peptidase-IV (DPP-IV) inhibition, Caco-2 cells , green lentil, fermentation, *L. plantarum*.

1. Introduction

In the past 40 years, diabetes prevalence has nearly doubled globally, becoming a leading cause of death worldwide and costing almost 12% of the global health expenditure. Type 2 diabetes mellitus (T2DM) is the most common condition, and its prevalence is now increasing at a higher rate in low- and middle-income countries due to westernization of diets.[1,2] In the last decade, novel therapies focusing on incretin hormones and inhibition of glucose absorption were developed. Incretin-based therapies aim at preserving the activity of the endocrine signalling gut hormones glucose insulinotropic peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are referred to as incretin hormones. They are secreted in high amounts after food ingestion and are responsible for the postprandial insulin secretion, decrease in glucagon concentration and glucose regulation. The global response is the so-called "incretin effect".[3] Although secreted after food ingestion, the incretin hormones are quickly cleaved and deactivated by the enzyme dipeptidyl-peptidase 4 (DPP-IV). As T2DM patients experience reduced incretin effect, inhibition of DPP-IV and therefore preservation of the activity of GIP and GLP-1 in these subjects is crucial for post-prandial glucose homeostasis. DPP-IV is an integral membrane proteolytic enzyme ubiquitously present in many human tissues, including the intestinal brush border. The protein is secured to the cell membrane via a short flexible intracellular tail, which is cleaved to produce the circulating soluble DPP-IV.[3–5] Inhibition of intestinal glucose absorption has also been regarded as an interesting target for improving glycaemic control in diabetic patients. Intestinal glucose transport is a concentration-dependent process mediated by two transporters, the sodium-dependent glucose transporter (SGLT1) and the sodium-independent glucose transporter 2 (GLUT2). Diabetic subjects show intestinal GLUT2 and SGLT1 expression levels 3 to 4 times higher than healthy control subjects. The higher levels of GLUT2 is a major driver of the increased glucose absorption observed in diabetic people.[6] Following this observation, GLUT2 and SGLT1 inhibitors are currently being investigated as novel interventions for improving glycaemic control.[7,8]

Besides synthetic DPP-IV inhibitors used in pharmacological therapies, there is a growing interest on food-derived bioactive compounds able to inhibit DPP-IV, GLUT2 and SGLT1. These compounds are primarily peptides present in protein hydrolysates and phenolic compounds, which have been identified in a vast variety of plant or animal-derived products.[9–11] Pulses such as lentils, common beans and lupins were recently reported as valuable source of these bioactive compounds.[12–14] Although present in the pulse seeds and highly active when analysed *in vitro* in their purified form, only limited research has investigated the bioavailability of these molecules in the human body, their stability through the gastrointestinal tract and *in vivo* activity. Moreover, when legumes and derived food ingredients become part of a food matrix, the molecular interactions between food components can highly affect their release from the food, their digestibility or bioaccessibility, and intestinal uptake in the human body.[15,16] Mechanical, physical and chemical processing of food can improve digestibility and bioaccessibility of proteins, phenolics and other components.[16,17] Notably, lactic acid fermentation with selected strains led to the release of free phenolic acids from their bound forms due to the activity of endogenous enzymes, therefore increasing their bioavailability.[18] *Lactobacillus plantarum* species were among the most active in this respect, and its natural dominance of the surface microbiota of plant seeds suggests its adaptation to this ecological niche and its potential application for pulse seed processing [18,19]. In our previous study, we observed that microbial fermentation of green lentil flour with *L. plantarum* ATCC® 8014™ caused disruption of the seed microstructure and greatly modified its protein and phenolic profile, their bioaccessibility and bioactivity. The *in vitro* inhibition of glucose

regulating enzymes DPP-IV and α-glucosidase was also improved.[20] In this study, we investigated if the observed bioactivity was retained in intestinal Caco-2 cells. The epithelial cells were grown into a polarized and differentiated monolayer for 21 days, prior exposure to the green lentil digest. Circulating DPP-IV inhibition was measured in the basolateral fluids collected after the transport study. Membrane-bound DPP-IV activity was measured in the cell free extract obtained from the monolayer. Total phenolic and peptide content was also monitored in the apical and basolateral fluids, as a preliminary evidence of their transport across the monolayer. Moreover, the fermentation time was extended to 72 hours to evaluate the possibility of enhancing the bioactivity of the lentil flour. Lastly, we tested if the bioactive components generated during the fermentation would likewise have an impact on the intestinal glucose transport and uptake.

2. Material and Method

2.1 Materials

Green lentils (*Lens culinaris*) were gifted by Pulse Canada (Manitoba, Canada). *Lactobacillus plantarum* ATCC® 8014™ and human Caco-2 epithelial colonic cells (ATCC® HTB-37™) were purchased from Cedarlane (Burlington, ON, Canada). DeMan, Rogosa and Sharpe (MRS) broth was purchased from Oxoid (Nepean, ON, Canada). Dipeptidyl peptidase IV human (recombinant expressed in Sf9 cells), α-amylase (from porcine pancreas, type VI-B, ≥5 units/mg solid), pancreatin (from porcine pancreas, 8xUSP specification), pepsin (from porcine gastric mucosa, ≥250 units/mg solid) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Millipore Sigma (Burlington, MA, USA). Thincert cell culture inserts for 24-well plate (TC-treated Polyethylene Terephthalate (PET), pore size 0.4 µm) were purchased from Greiner Bio-One (Greiner Bio-One B.V., Alphen Aan Den Rijn, The Netherlands). Characterized foetal bovine serum (FBS) was purchased from Hyclone (VWR International, LLC). Dulbecco's Modified Eagle Medium (DMEM), penicillin (10000 IU)-streptomycin (10000µg/ml) solution, Hank's Balanced Salt solution (HBSS), trypsin-EDTA (0.25%) was purchased from Gibco (Fisher Scientific, Landsmeer, The Netherlands).

2.2 Preparation of bacterial inoculum and solid-state fermented green lentil flour

Lactobacillus plantarum (ATCC® 8014™) was propagated in MRS broth and stored at -80 °C in cryovials containing 30% (v/v) glycerol. Bacterial culture was sub-cultured twice in MRS broth at 37°C for 16 h anaerobically, harvested by centrifugation (10 000 *g*, 10 min) and washed twice with 0.9% saline solution before being used as inoculum. Green lentils (*Lens culinaris*) were ground to flour in a commercial blender (Waring®, USA) and passed through a commercial 40 mesh sieve. Green lentil flour was mixed with equal amount (w/v) of sterile distilled water and inoculated with the starter culture, with an initial cell density of 7.0 log CFU/g of flour paste. Solid state fermentation was carried out for 0, 48 and 72 h at 37 °C, with a constant shaking of 120 rpm. At the end of the fermentation, samples were freeze-dried and stored at -20 °C before simulated gastrointestinal digestion. The efficacy of the fermentation was evaluated by means of bacterial enumeration and pH measurement.

2.3 Simulated gastrointestinal digestion

Simulated gastrointestinal digestion was performed according to the INFOGEST standardized static *in vitro* digestion method with some modifications.[21] All reagents were pre-warmed and maintained at 37 °C during the entire digestion, while the enzyme solutions were kept on ice. The digestion consisted of three consecutive phases (oral, gastric, intestinal). For the oral phase, 5 g of freeze-dried sample were mixed to 3.5 mL of simulated salivary fluids (SSF) stock solution, 0.5 mL of salivary α-amylase solution (1500 U/ml), 25 µL $CaCl_2$ (0.3 M), 0.975 mL distilled water, and incubated at 37 °C for 2 min.

For the gastric phase, 10 ml of oral bolus were mixed to 7.5 ml of simulated gastric fluids (SGF) stock solution, 1.6 ml of pepsin solution (25000 U/ml) 5 µL $CaCl_2$ (0.3 M) and the pH was adjusted to 3 using 3 M and 1 M HCl solution. The total volume was then brought to 20 ml with distilled water, and shake-incubated at 37 °C for 2 h, 90 rpm. For the intestinal phase, 20 ml of gastric chyme were mixed with 11 ml of simulated intestinal fluids (SIF) stock solution, 5 ml of pancreatin solution (800 U/ml), 2.5 ml fresh bile (160mM), 40 µL $CaCl_2$ (0.3 M) and the pH was adjusted to 7 using 3 M and 1 M NaOH solution. The total volume was then brought to 40 ml with distilled water, and shake-incubated at 37 °C for 2 h, 90 rpm. Salivary α-amylase solution was prepared in SSF, pepsin solution was prepared in SGF, pancreatin solution was prepared in SIF, and fresh bile was prepared in distilled water.

2.4 Caco-2 cell proliferation

Caco-2 cells, obtained from the American Type Culture Collection (ATCC), were grown in high glucose DMEM containing L-glutamine, HEPES and phenol red, supplemented with 10% foetal bovine serum (FBS), 100 U/ml of penicillium and 100 µg/ml of streptomycin. Cells were sub-cultured weekly and maintained at 37°C in atmosphere containing 5% CO_2. All the cells used in this study were between passage 30 and 40.

2.5 Cytotoxicity assay

Cytotoxicity of the digested samples was assessed by the MTT assay adapted for Thincert-grown Caco-2 cells. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was dissolved in cell culture medium (DMEM + 10% FBS) at a concentration of 0.5 mg/mL, then filter sterilized and stored at -20 °C. Caco-2 cells were grown on the Thincert inserts for 21 days, then exposed to the test samples for 24 hours. After removal of the test sample, 50 µL of warm MTT medium were added to the apical compartment and incubated for 2 hours at 37 °C and 5% CO_2. The MTT medium was then removed, and the cells gently washed with PBS. The inserts were then placed on a flat surface (inner side of the plate lid), filled with 100 µL of DMSO:EtOH (1:1), and shacked for 10 min at 250 RPM. The suspension was then transferred to a 96-well plate and the absorbance was read at 570 nm in a microplate reader (Tecan, Switzerland). Viability of treated cells was expressed as percentage of the control cells that were exposed to the same amount of DMEM only. There was no cytotoxic effect by any of the concentrations of digest tested and all cells showed viability >95% upon exposure.

2.6 Transport study

The test samples were prepared by dissolving the freeze dried digesta in HBSS at a concentration of 100 and 500 mg/ml. The pH was adjusted to 7.00 with 1M NaOH/HCl. The samples were then filter sterilized through 0.22 µm syringe filter, aliquoted in microcentrifuge tubes and stored at -80 °C till the experiment. On the day of the experiment, an individual test sample tube was thawed and warmed to 37 °C. Transport study was performed according to the method described by Lacroix. et al.[22] with modifications. Caco-2 cells were grown at 80-90% confluence, detached from the culture flask with 0.25% trypsin-EDTA and seeded at a density of 0.225 x 10^6 cells/mL on Thincert cell culture inserts for 24-well plate (TC-treated Polyethylene Terephthalate (PET), pore size 0.4 µm). Cells were grown at 37 °C and 5% CO_2 for 21 days, to allow full differentiation.[23] Media was changed every 2-3 days. Transepithelial Electrical Resistance (TEER) was measured using a Millicell® ERS-2 Volt-Ohm meter (MilliporeSigma, Burlington, MA, USA) for ensuring the integrity of the monolayer. The cell specific resistance (R_{TISSUE}) was then obtained by subtracting the blank resistance (measured on the semipermeable membrane alone) to the resistance measured across the cell monolayer differentiated on the semipermeable membrane (R_{TOTAL}). The $TEER_{reported}$ values were then calculated by multiplying

the R_{TISSUE} by the area of the individual well (1.9 cm^2). On the 21st day, only wells with $TEER_{reported}$ higher than 500 $\Omega \cdot cm^2$ were used for the experiment, as this indicates a proper formation of tight junctions in the Caco-2 monolayer.[24] Growth medium was removed, 300 µL of test sample was placed to the apical side of the monolayer, and 700 µL of HBSS were added to the basolateral side. Plates were then incubated for 2 h at 37 °C in atmosphere containing 5% CO_2. The apical and basolateral solutions were then collected and stored at -80 °C for further analysis.

2.7 Total phenolic content (TPC) and protein determination

Phenolic and protein content of digest, apical and basolateral fluids were determined as previously described [20]. Only wells with a $TEER_{reported}$ value higher than 500 $\Omega \cdot cm^2$ before and after exposure to the test samples were used for the experiment. Total phenolic content (TPC) was measured using the Folin-Ciocalteu reagent assay and expressed as gallic acid equivalent (GA E_{qv})/ g DW. Protein and peptide content were quantified with the Lowry method using bovine serum albumin as the standard. The method was previously described for peptide content of protein hydrolysates.[25]

2.8 Dipeptidyl peptidase-IV activity in fluids and in situ

Dipeptidyl peptidase-IV (DPP-IV) activity was tested in the apical (AP) and basolateral (BL) fluids obtained from the transport study, according to the method previously described[26]. In a 96-well plate, 25 µL of tested fluid, control (HBSS) or standard (Diprotin A) were combined to 25 µL of substrate (Gly-Pro-p-nitroanilide, 1.6 mM) and incubated at 37 °C for 10 min, prior addition of 50 µL of DPP-IV (1852 U/mL). The plate was then incubated at 37°C for 60 min, and the absorbance was immediately recorded at 405 nm in a microplate reader (Tecan, Switzerland). Results were expressed as percentage of DPP-IV inhibition which was calculated according to the formula: 100 X {1- [(Abs_{405} (test sample) – Abs_{405} (test sample blank)] / [(Abs_{405} (positive control) – Abs_{405} (negative control)]}. For the positive control, the tested fluid was replaced with 25 µL of HBSS. For the negative control, 75 µL of HBSS were combined to 25 µL of substrate. For determining the *in situ* DPP-IV inhibition, the test samples were prepared in phenol free DMEM and filter sterilized through 0.22 µm syringe filter. Three concentrations, 100, 200 and 500 mg/mL were assayed. Caco-2 cells were grown of Thincert supports for 21 days at 37 °C and 5% CO_2. Cells were then exposed to the test sample for 2 and 24 hours. After removal of the apical and basolateral fluids, cells were washed with ice-cold PBS, and scraped with 300 µL of phosphate buffer (10 mM, pH 7) containing protease inhibitors, snap frozen in liquid nitrogen and stored at -80 °C. The cell extracts were used for DPP-IV activity and protein content determination. On the day of assay, cell lysates were thawed and passed 10−15 times though a 21G needle syringe in order to obtain a cell-free extract [27]. For DPP-IV activity, 25 µL of substrate were combined to 25 µL of Tris-HCl buffer (100mM, pH 8) in a 96-well plate, and incubated at 37 °C for 10 min. A 50 µL of cell lysate was then added, and the plate was further incubated at 37 °C for 60 min. The absorbance was then recorded at 405 nm in a microplate reader (Tecan, Switzerland). For the sample blanks, 50 µL of Tris-HCl buffer (100mM, pH 8) were combined to 50 µL of cell extract. A standard curve was prepared using the purified DPP-IV enzyme in DMEM, from which the values for the enzyme activity (U/mL) of the DPP-IV contained in the cell lysates were extrapolated. Values were then adjusted based on the protein content.

2.9 Glucose transport and uptake

The transport and uptake of glucose across polarized Caco-2 cells was measured according to the method described by Mojica et al. (2017)[28] with some modifications. Caco-2 cells were grown on the Thincert inserts for 21 days, then the TEER value was recorded and only the well with $TEER_{reported}$ values

higher than 500 Ω cm^2 were used for the experiment. Cells were then equilibrated for 2 hours in glucose-free and phenol-free DMEM at 37°C and 5% CO_2. The media was then replaced with 0.3 mL of sample solution (digesta at 100, 200 or 500 mg/mL suspended in glucose-free, phenol-free DMEM, with 20 mM glucose) at the apical side, and 0.7 mL of glucose-free, phenol-free DMEM at the basolateral side. Phloretin (1mM) was used as standard inhibitor. After 2 to 24 h of incubation, apical and basolateral fluids were collected and stored at -20°C for glucose quantification. The wells were then washed with ice-cold PBS, and the cells were scraped with 300 µL of phosphate buffer (10 mM, pH 7.0) containing protease inhibitors, snap-frozen in liquid nitrogen and stored at -80°C. Glucose content was quantified with a glucose colorimetric assay kit (Cayman, Michigan, USA). Glucose uptake was estimated by subtracting the remaining glucose (in both apical and basolateral sides) at the end of the incubation from the initial amount of glucose used for the assay.

2.10 Statistical analysis

All experiments were conducted at least in triplicate, on cells from different passage numbers (between 30 and 40) independently seeded and grown on the inserts on different weeks. For each replicate, the treatment was carried out on two wells from the same plate (technical replicate) and the resulting measurements were averaged. A two-way ANOVA followed by Tukey's multiple comparison test was performed on values obtained from the DPP-IV inhibition study and the glucose transport and uptake study. GraphPad Prism 8.2.1 was used for data visualization and graphics.

3. Results and Discussion

3.1 Dipeptidyl-peptidase IV inhibition

DPP-IV inhibition was tested *in vitro* in the apical and basolateral fluids collected at the end of the transport study (Fig. 1) and *in situ* in the Caco-2 cells extracted from the Thincert supports (Fig. 2). Test samples were obtained by fermentation of lentil flour with *L. plantarum* for 0, 48 or 72 hours. The microbial suspension was inoculated at 7 log CFU/mL and reached 8.9 – 9.4 and 7.4 – 8.5 log CFU/mL after 48 and 72 h of incubation. Accordingly, the pH decreased from an initial value of 6.4 to 4.2 and 4.0 after 48 and 72 h. The fermented samples were subsequently exposed to *in vitro* gastrointestinal digestion. The concentration of 100 mg/mL represents the ratio of food to digestive fluids of 50:50 (v/v), as recommended by the method.[21] *In vitro* DPP-IV activity was inhibited by 50.2 %, 62.5 % and 67 % when exposed to 100 mg/mL of lentil digest pre-fermented for 0, 48 and 72 h respectively (Fig. 1, A). The activity was mostly retained in the apical fluids collected after 2 h of incubation of the digested samples with the Caco-2 cell monolayer. In the basolateral (BL) compartment, the bioactivity was significantly increased upon incubation with the 48 and 72 h fermented flour. However, the DPP-IV inhibitory activity of the BL fluids was low, ranging between 1.8 and 7.9 % (Fig. 1, B).

In situ DPP-IV activity values are presented in Figure 2. The activity of DPP-IV in cell extracts prior to treatment had an average value of 1500 U/mg protein. In all replicates, the *in situ* DPP-IV activity decreased when the cells were treated with the digested lentil flour. Microbial fermentation of the green lentil flour prior to digestion had a strong effect on this inhibitory activity. The *in situ* inhibition significantly increased with increasing fermentation time of the flour, when a concentration of 100 or 200 mg/mL digesta were tested. After 2 h exposure to 100 and 200 mg/mL of 72 h pre-fermented lentil flour digest, cellular DPP-IV activity was decreased by 29 and 37 % respectively (Fig. 2. A, B). Conversely, unfermented lentil digest led to a 4 and 10 % decrease in activity at the same concentrations. The same trends were observed when the incubation time of the cells with the digest was extended to 24 h, with a limited increase in bioactivity primarily observed for the unfermented

lentil digest (Fig. 2. A, B). Finally, there was a homogeneous response of approximately 28% decrease in activity when the cells were exposed for 2 or 24 h to the highest concentration of 500 mg/mL of digesta (Fig. 2. C). When monitoring the TPC of the BL compartment, we noticed that the phenolic transport did not linearly increase upon exposure to a 5 times higher concentration of the digest (Suppl. Fig. 1. B). This might indicate that the transport is saturable and possibly takes place via the monocarboxylic acid transporter (MCT), as similarly observed for transport of *p*-coumaric acid in Caco-2 cells[29], and could explain the lower *in situ* DPP-IV inhibition we observed at 500 mg/mL when compared to the other concentrations tested (Fig. 2. C). Incubation of the cell monolayer for 2 or 24 h with the digestive fluids (DF) used for performing the *in vitro* digestion caused a decrease of approximately 14 % *in situ* DPP-IV activity (Fig. 2).

While the pre-fermented lentil flour digest bioactivity is a result of multiple bioactive components, we hypothesized that bioactive peptides and phenolic compounds played a major role in it. Accordingly, we could observe that the increase in fermentation time of the green lentil flour led to a 60% increase in peptide transport to the basolateral side (Suppl. Fig. 1, D). However, peptide content in the BL side did not exceeded 30 µg/mL, and the percentage of transport to the BL side was low, with a maximum of 17% in the sample pre-fermented for 72 h (Suppl. Table 1). This was in line with the DPP-IV inhibitory bioactivity and low levels of peptide transport previously reported for plant and milk-derived bioactive peptides or peptidic fractions.[22,30,31] Less than 2% peptide transport across Caco-2 cell monolayers was observed for individual peptides obtained from corn or egg protein hydrolysates.[30,31] On the contrary, up to 8.5% of peptides transport was reported when 25 mg/mL of a casein-derived peptide fraction digest was used.[32] Lacroix et al. (2017) reported that only 0.5% of the milk-derived bioactive peptides tested were transported to the basolateral side, leading to a 12-18 % inhibition of DPP-IV activity, with the only exception of peptide WR which showed 46 % inhibition and a higher intestinal transport.[22] Lammi et al. (2018) investigated the DPP-IV inhibitory property of soybean (Soy1) and lupin (Lup 1) peptides both *in situ* with a Caco-2 cell model, and *ex-vivo*, in blood serum. Similar to our study, both peptide samples exerted a higher inhibitory activity on the membrane DPP-IV than the circulating DPP-IV. *In situ* DPP-IV was inhibited in a dose-dependent manner by up to 50% after one hour of pre-incubation with the peptides. When tested in the serum, Lup1 inhibited DPP-IV activity by 18.1 % and 24.7 % at 100 and 300 µM respectively, while Soy1 decreased the activity by 27.7% and 35.0% at the same concentrations.[13] In a separate study, the author investigated bioactive peptides obtained from phycobiliproteins in spirulina (*Arthrospira platensis*) cyanobacterium. 2.5 and 5.0 mg/mL of hydrolysate produced a DPP-IV inhibition by 82.9 and 95.8 % respectively *in vitro*, by means of biochemical assay, and 34.6 and 44 % respectively of DPP-IV inhibition *in situ*, measured on Caco-2 cells. Extending the incubation time of the peptides with the Caco-2 cell monolayer leads to secondary metabolic activity of the Caco-2 cell enzymes, affecting the hydrolysate composition and related bioactivity.[33] This was reported to either increase [34] or decrease [33] the DPP-IV inhibitory activity of the initial peptides. In our study, longer exposure of the cells to the green lentil digesta minimally impacted the *in situ* DPP-IV inhibition. This was possibly due to the complex nature of the sample. Previous studies showed that the whole protein isolate or the whole phenolic mixture had better retention of the bioactivity compared to their individual components.[22] Whey protein isolates digest was less susceptible to brush border enzyme hydrolysis, and better retained the DPP-IV inhibitory activity after 2 h incubation with Caco-2 cells, when compared to the individual derived peptides.[22] Complex mixtures of protein digests from black bean or maize also showed higher bioactivity in a Caco-2 cellular model when compared to their peptides.[35,36] Moreover, the bioactivity of plant-derived polyphenolic compounds intensified when they were administrated as a mixture rather than as individual

compounds.[37] While potentially more potent, individual peptides are also more susceptible to the enzymatic activity of the digestive and brush border enzymes, and their bioavailability is very limited.[38] Accordingly, novel research is exploring the use of delivery systems such as encapsulation to improve their stability in physiological conditions and fully preserve their bioactivity.[39,40]

Altogether, our results appeared to be aligned with the DPP-IV inhibitory activities reported for bioactive peptides, protein hydrolysates or phenolic extracts, therefore suggesting a major involvement of these components in the bioactivity of the lentil digesta. Moreover, the slight increase in TPC in the BL compartment that we observed upon exposure to the 72 h pre-fermented sample (Suppl. Fig. 1. A,B) could likely relate to the change in the degree of glycosylation of flavonoids and the nature of phenolic acids, which were previously reported as a result of lactic acid fermentation [18,19,41] and can impact the intestinal absorption.[29,42] Congruently, we formerly observed such modifications upon fermentation of the green lentil flour used for this study.[20] Food matrix interactions were also taken into account in our study, by keeping all food components together in the flour digest. By doing so and keeping the concentrations close to the recommended ratio of food to digestive fluids of 50:50 (v/v), we had a more diluted effect of the individual components, when compared to the referenced studies. This further highlights the importance of the observed DPP-IV inhibitory activity, which did not result from a purified and concentrated component, such as peptides, but rather from the whole product itself. This strengthen the likelihood that the observed bioactivity *in vitro* could possibly be exerted *in vivo* upon consumption of simple food products made with the fermented lentil flour. However, the cellular model still offers limitations in term of resembling the human physiological conditions, therefore an *in vivo* trial should follow in order to validate our findings.

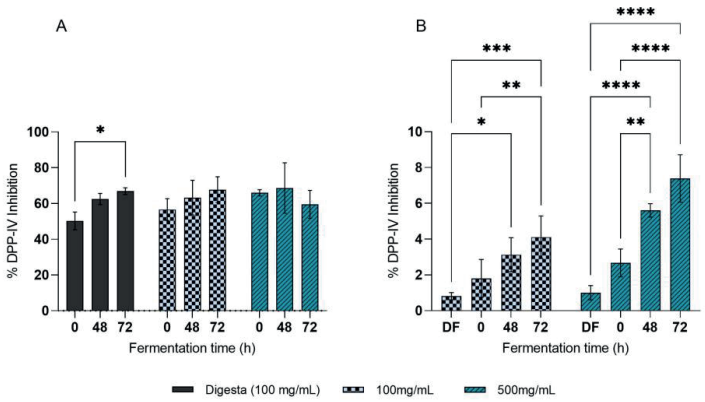

Figure 1. Percentage of DPP-IV inhibition in lentil digesta (A, grey bars), apical (A, chequered and blue bars) and basolateral (B) fluids collected after 2 hours incubation with 100 mg/mL or 500 mg/mL lentil digesta. DF=

digestive fluids. Results are shown as mean values ± SD of four independent replicates. Asterisks (, **, ****) indicate the degree of statistical difference after two-way ANOVA and Tukey's multiple comparison test.*

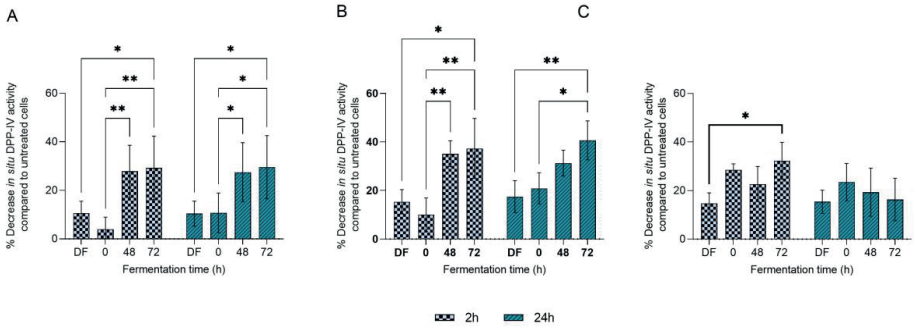

Figure 2. Percentage of decrease in DPP-IV activity in response to exposure to lentil samples pre-fermented for 0, 48, 72 hours and digested, compared to untreated cells. DF= digestive fluids. A: exposure to 100 mg/mL sample; B: exposure to 200 mg/mL sample; C: exposure to 500 mg/mL sample. Results are shown as mean values ± SD of four independent replicates. Asterisks (, **) indicates the degree of statistical difference after two-way ANOVA and Tukey's multiple comparison test.*

3.2 Glucose transport and uptake

Glucose transport and uptake with concurrent exposure to the lentil digest or the standard inhibitor of GLUT2 phloretin were evaluated on polarized Caco-2 cells. Values of glucose uptake are reported in Figure 3. After 2 h, about 90 mg/dL of glucose was taken up by the cells exposed to the fermented samples and phloretin. This was slightly lower compared to the cells exposed to the unfermented sample or the control, which assimilated 107 and 100 mg/mL of glucose, respectively. After 24 h, 339, 320 and 142 mg/mL of glucose were absorbed by the control, lentils exposed and phloretin exposed cells, respectively. While the lentil samples caused a 6% decrease in glucose uptake compared to the control, no difference was observed between fermented and unfermented samples. A significantly greater decrease was observed for samples exposed to phloretin, which is complementary to the glucose transported to the basolateral side (Figure 3.C). When considering the glucose transported to the basolateral side of the polarized monolayer (Figure 3.C), a different trend was observed. An equal amount of glucose was transported by the cells exposed to the lentil digest after 2 h. After 24 h of exposure, a decreasing trend in glucose transport was observed in relation to the fermentation time of the lentil digest, with the 72 h fermented lentils inhibiting ~12% of glucose absorption compared to the unfermented ones. However, there was no apparent decrease in glucose transport when compared to the control. Instead, phloretin significantly inhibited glucose transport after 2 h, and significantly decreased glucose uptake after 24 h exposure. Intestinal glucose transport is a concentration-dependent process primarily mediated by the two transporters sodium-dependent glucose transporter (SGLT1) and sodium-independent glucose transporter 2 (GLUT2) (Suppl. Fig. 2). Since phloretin inhibits GLUT2, its activity was high in the first 2 h exposure, when apical glucose concentration level was still high, and the transport was therefore regulated by GLUT2. When apical glucose level dropped after 24 h exposure, SGLT1 was restored as major regulator of glucose transport and there was no inhibition in the transport (Fig. 3). Since phloretin also decreased the enterocytic glucose uptake, the level of basolateral glucose was 10-fold higher compared to the untreated cells in this case. The mechanism of action of phloretin was previously reported by Zheng et al.[43] The decrease

in basolateral glucose observed in the fermented samples after 24 hours could be related to the increase in *p*-coumaric acid and kaempferol glycoside content caused by the fermentation itself.[20] In fact, flavonoid glycosides were among the most active dietary polyphenols in inhibiting Na-dependent glucose uptake in Caco-2 cells.[44] Similarly, decrease in glucose transport in Caco-2 cells was reported in response to increased caffeic acid, *p*-coumaric acid, quercetin and kaempferol content of a polyphenol herbal extract.[37] Moreover, the later activity of the fermented samples (after 24 h incubation), when the luminal glucose concentrations were low, suggests that SGLT1 was the major transporter inhibited, and not GLUT2 (Fig. 3). Similarly to our results, Chang et al. observed a decrease in SGLT1 activity upon long exposure (2 days) to ginsenoside Rg1 and associated inhibition of glucose transport. Accordingly, there was no effect on the expression level of GLUT2.[45] Lentil peptides can be another contributor to the inhibition of the glucose transporters in Caco-2 cells. Mojica et al. previously reported a significant decrease in glucose uptake by Caco-2 cells, and inhibition of SGLT1 and GLUT2 protein and gene expression upon exposure to black bean protein fractions and hydrolysed isolates.[28,36] Interesting, even in this case SLGT1 protein expression was not affected in the initial 30 min, but significantly inhibited after 24 h. The authors suggested that this could indicate that the peptides could possibly act on the PKC and AMPK pathways and block protein expression and the related transport activity of glucose transporters.[36]

To conclude, glucose absorption and uptake was not improved during two hours of exposure to the digest in our study, and slightly improved after 24 h. This implicates that the hypoglycaemic properties often observed in lentils, and here greatly enhanced by microbial fermentation, are likely due to its activity on DPP-IV, rather than on the inhibition in glucose absorption. This is well in line with the findings by Vella and co-workers who stated that DPP-IV inhibitors do not operate by decreasing glucose absorption in the systemic circulation, but instead induce an increase in GLP-1 concentration that in turn lead to a decrease in postprandial glucose concentration.[46]

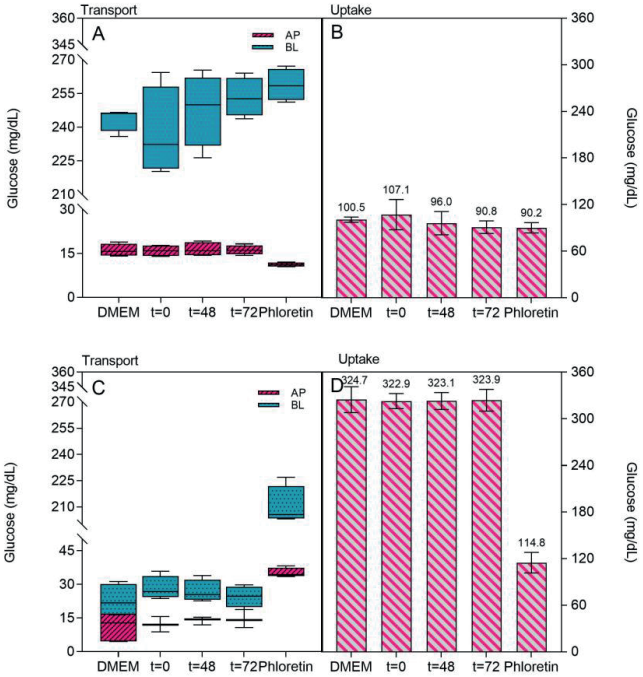

Figure 3. Box and whisker plot of glucose transport and uptake from Caco-2 cells after 2 hours (A, B) and 24 hours (C, D) incubation with DMEM containing 500 mg/mL of lentil flour digest and 20 mM glucose. AP: measurement in the apical fluids; BL: measurements in the basolateral fluids. Results are shown as mean values ± SD of three independent replicates.

4. Conclusion

With the surge in the global incidence of type 2 diabetes, food-derived DPP-IV, SGLT1 and GLUT2 inhibitors have gained attention due to their high *in vitro* potency and minimal adverse effects. In this context, most studies focus on the identification of the individual bioactive components, mainly phenolics and peptides, with the highest potency. Food matrix effect, digestive enzyme activity and limited bioavailability are major constraints often neglected and limiting the application of food derived bioactive compounds in functional food products. Food fermentation has regained popularity in recent years as a natural processing technique for improving sensory, physico-chemical and nutritional properties of foods.[47] In fact, growing consumer's attention on sustainability and personal wellbeing has redirected their choices towards minimally processed and plant-derived food products.[48,49] In this context, it becomes of even more interest to deepen the knowledge of potential health effects of legume fermentation, and tailor design fermentation parameters for the development of functional foods that can help the transition towards healthier and more sustainable lifestyles. Since the objective of our study was to investigate the microbially fermented lentil flour as a candidate for development of functional foods for type 2 diabetic patients, we designed our study taking into account (i) the importance of the food matrix on the bioavailability of bioactive compounds and (ii) the considerable activity of the digestive enzymes. According to this, we did not isolate the independent bioactive components but rather analysed their bioactivity as a whole, to go beyond all the interactions taking place in the food matrix. To take into consideration the activity of the digestive

enzymes we pre-digested the food sample according to the Infogest consensus method. Finally, we used an intestinal cell-based assay with polarized human intestinal Caco-2 cells, to expose the digest to the brush border enzymes activity and to measure the inhibition of both circulating and cell-bounded human DPP-IV. This comprehensive approach increases the confidence that the bioactivity observed in our product can represent the *in vivo* phenomena when the flour is employed in a simple food formulation. Altogether, the results of the study suggest that microbial fermentation of green lentil flour with *L. plantarum* for 72h is an effective strategy for improving both cell-bound and circulating DPP-IV inhibitory activity and potentially improve glucose regulation in diabetic individuals. Data suggests the observed bioactivity derive from the inhibition of DDP-IV enzyme, while glucose transporters are not involved. To the best of our knowledge, this is the first scientific evidence of fermented green lentil flour bioactivity towards *in situ* and circulating DPP-IV. Further research will elucidate the molecules involved in the bioactivity, that we suspect to be primarily the phenolic and peptide fractions. Future studies should also address the *in vivo* response to the product in type 2 diabetic patients.

Acknowledgements

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Conflicts of interest

The authors declare no conflicts of interest.

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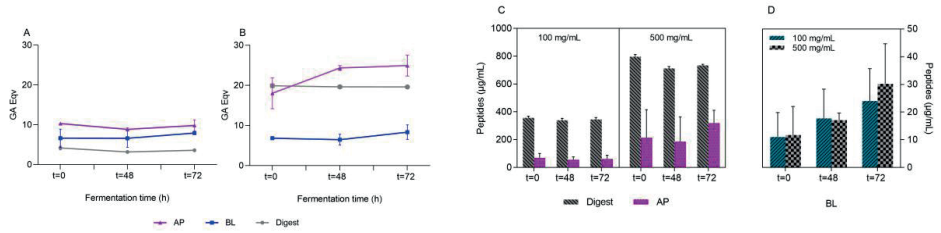
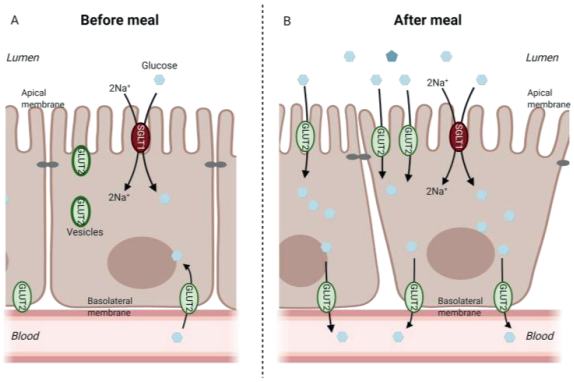
Supplementary Tables and Figures



Supplementary Figure 1. Concentration of total phenolic content (A, B) and peptides content (C, D) in the apical (AP) and basolateral (BL) fluids after incubation with 100 mg/mL and 500 mg/mL of digest. A: TPC, 100mg/mL digest. B: TPC, 500 mg/mL digest. C: peptides content in digest and AP fluids. D: peptide content in BL fluids. Results are shown as mean values ± SD of three independent replicates.

Location	Concentration Digest	t= 0 h	t= 48 h	t=72h
AP	100	79.9 %	74.5 %	67.6 %
	500	48.3 %	48.3 %	62.4 %
BL	100	7.3 %	10.9 %	17.2 %
	500	1.6 %	2.7 %	4.9 %

Supplementary Table 1. Content of peptides in apical (AP) and basolateral (BL) fluids after 2 hours incubation with green lentil digest pre-fermented for 0, 48, 72 hours. Values are expressed as percentage relative to the initial content present in the digest.



Supplementary Figure 2. Representation of the facilitated (Na-dependent) model of dietary glucose absorption in the small intestine. Intestinal glucose transport is a concentration-dependent process mediated by two transporters, the sodium-dependent glucose transporter (SGLT1) on the apical side and the sodium-independent glucose transporter 2 (GLUT2) on the basolateral side. Before a meal and with low level of luminal glucose, SGLT1 is the major regulator of glucose transport in the enterocytes. GLUT2 is primarily located at the basolateral membrane and balances glucose between blood and enterocytes. After meal consumption, when there is high glucose concentration at the apical side of epithelial cells, the amount of apical GLUT2 is greatly increased and it becomes the major regulator of intestinal glucose absorption. Figure created with Biorender and adapted from Kellett and Brot-Laroche.[8]

CHAPTER 4

Impact of gastrointestinal digestion and L. plantarum fermentation on peptides bioaccessibility and transport by human intestinal Caco-2 cells.

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Abstract

Health and environmental concerns are driving consumers interest in plant-proteins. Besides their excellent nutritional profile, pulse proteins have the potential to generate bioactive peptides, included dipeptidyl peptidase-IV (DPP-IV) inhibitors. In this study, green lentils were solid-state fermented with *Lactobacillus plantarum* for 0, 48 and 72 h. Samples were *in vitro* digested (INFOGEST) and their transport across a Caco-2 cell monolayer was investigated. The peptides formed upon gastrointestinal digestion (bioaccessible fraction) and the transported peptides (basolateral fraction) were profiled by LC/MS/MS. After data processing, a total of 515 peptides were identified, originating mainly by parental proteins of the vicilin, convicilin and legumine families. Microbial fermentation led to the formation of more hydrophobic peptides compared to the unfermented samples. This was observed in the peptides of both bioaccessible and bioavailable fraction. 16.1 to 24.5 % of peptides were transported to the basolateral side of Caco-2 cells in more than 60 % of the bioaccessible samples in which they were identified. Peptides with more than 22 amino acids, and with a mass greater than 2000 Da were not transported, suggesting this as size limit for cellular transport. 63 peptides were uniquely identified in the basolateral fraction, resulting from the activity of the brush border enzymes of Caco-2 cells. These peptides were more negatively charged, less hydrophobic and with a higher Boman index value compared to the bioaccessible and transported. Peptides uniquely present in the basolateral fraction of the 72 h fermented samples had a higher potential for DPP-IV inhibitory activity. To the best of our knowledge, this is the first study reporting the implications of *in vitro* gastrointestinal digestion and microbial fermentation with *L. plantarum* on the peptidome of green lentil and its transport across the Caco-2 monolayer.

Keywords: peptides, bioaccessibility, Caco-2, green lentil, *L. plantarum*.

1. Introduction

The growing concerns for public health and environment have driven the attention of consumers and policy makers towards exploring plants as a valuable protein source. Pulses, the legume used for their dry seeds only, are promising candidates in this context, having high protein and low fat content, and being a source of fibers for the gut microflora.[1] Moreover, a wide range of health benefits such as decrease in body weight, prolonged satiety, decrease in circulating atherogenic LDL-C, and improved glucose regulation have been clinically reported in response to pulse consumption in recent years.[2] Bioactivities such as the inhibition of α-glucosidase, α-amylase, dipeptidyl peptidase-IV (DPP-IV) and angiotensin-converting-enzyme (ACE) have also being associated to plant-derived peptides.[3,4] In fact, plant and pulse proteins showed higher content in β-sheets (22-44 %) compared to animal proteins (7-11 %), which confers resistance to gastrointestinal enzymes proteolysis and is favorable for the formation of stable bioactive peptides.[5] Interestingly, fermentation with *Lactobacillus* spp. was recently suggested as an effective tool for production of bioactive peptides due to the high proteolytic activity of the components of this microbial species. In fact, Lactobacilli lack the genetic information for synthetizing certain amino acids (amino acid autotrophy), making them dependent on protein hydrolysis from the environment.[6] In humans, DPP-IV inhibition is related to an increase in post-prandial activity of the incretin hormones, leading to a greater insulin release and better glucose regulation in type 2 diabetic patients.[7] Similarly to drugs, substantial DPP-IV inhibition has been observed in response to the exposure to plant-derived bioactive peptides.[8,9] However, this was often assessed in protein isolates, with little consideration of the food matrix and recent evidence suggests an important role of food matrix in determining the degree of bioaccessibility and absorption of nutrients and bioactive components from food.[10] Moreover, ingested proteins or peptides are exposed to the activity of digestive enzymes in the gastrointestinal tract, and intestinal enzymes produced by the enterocytes. This is also often neglected in studies that investigate food-derived bioactive peptides. Interestingly, we previously observed that solid-state fermentation of green lentil flour with *L. plantarum* led to a significant increase in its circulating and cellular DPP-IV inhibitory activity in Caco-2 cells. In line with the scientific evidence, the observed bioactivity was likely related to the modification in phenolic and/or peptide composition.[11,12] According to this, the aim of this study was to assess the impact of bacterial fermentation and gastrointestinal digestion on the formation of bioaccessible peptides. Green lentil flour was solid-state fermented *L. plantarum* ATCC8014 and subsequently digested following the INFOGEST method. Peptides were identified with LC/MS/MS. Further, a Caco-2 cell model was used to simulate the intestinal absorption of the peptides present in unfermented and fermented products, to assess whether the fermentation would affect the transport of specific classes of peptides with higher bioactivity potential towards DPP-IV.

Keywords: green lentil, peptides, *L. plantarum*, bioaccessibility, Caco-2 transport.

Materials and Methods

2.1 Materials

Green lentils (*Lens culinaris*) were gifted by Pulse Canada (Manitoba, Canada). *Lactobacillus plantarum* ATCC® 8014™, human Caco-2 epithelial colonic cells (ATCC® HTB-37™), Eagle's Minimum Essential Medium (EMEM) were purchased from Cedarlane (Burlington, ON, Canada). All the digestive enzymes were purchased from Millipore Sigma (Burlington, MA, USA). Thincert cell culture inserts for 24-well plate (TC-treated Polyethylene Terephthalate (PET), pore size 0.4 µm) were purchased from Greiner Bio-One (Greiner Bio-One B.V., Alphen Aan Den Rijn, The Netherlands). Fetal bovine serum (FBS), penicillin (10000 IU)-streptomycin (10000 µg/ml) solution, trypsin-EDTA (0.25%) were purchased from

Fisher Scientific (Nepean, ON, Canada). Hank's Balanced Salt solution (HBSS) and chemicals were purchased from Millipore Sigma (Sigma-Aldrich, Oakville, ON, Canada).

2.2 Green lentil fermentation and *in vitro* digestion.

Bacterial inoculum and solid-state fermentation were performed as previously described.[13] In brief, ground green lentils were combined with an equal amount of sterile distilled water and inoculated with *L. plantarum* ATCC® 8014™ at an initial cell density of 7.0 log CFU/g of flour paste. The fermentation was performed for up to 48 h at 37 ^{o}C with continued shaking (120 rpm). To assess the success of the fermentation, samples were withdrawn at multiple time points and analyzed for bacterial enumeration, pH and total titratable acidity. The INFOGEST standardized static *in vitro* digestion method was followed for the simulated gastrointestinal digestion.[14]

2.3 Caco-2 cell proliferation

Caco-2 cells were grown in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillium and 100 µg/ml of streptomycin. Cells were sub-cultured twice a week and maintained at 37°C in atmosphere containing 5% CO_2. All the cells used in this study were between passage 30 and 40.

2.4 Transport study

Test samples were prepared by dissolving the freeze dried digesta in HBSS at a concentration of 100 mg/ml. The pH was adjusted to 7.00 with 1M NaOH/HCl. The samples were then filter sterilized through 0.22 µm syringe filter and store at -80^{o}C until use. On the day of the experiment, samples were thawed and incubated at 37 °C. Transport study was carried out as reported by Lacroix et al. with modification.[15] Shortly, 80-90 % confluent Caco-2 cells were treated with 0.25% trypsin-EDTA, washed and seeded at 0.225 x 10^6 cells/mL in a Transwell™ 24-Well Permeable Support System. After growth for 21 days (37 °C, 5% CO_2), Transepithelial Electrical Resistance (TEER) was determined with a Millicell® ERS-2 Volt-Ohm meter (Millipore Sigma, Burlington, MA, USA). A $TEER_{reported}$ higher than 500 Ω·cm^2 was considered sufficient to guarantee integrity of the cell monolayer.[16] Cells with a lower TEER value were discarded. After removing the media and washing with PBS, 300 µL of test sample were added to the apical side of the monolayer, and 700 µL of HBSS to the basolateral side. Following incubation for 2 h (37 °C, 5% CO_2), apical and basolateral fluids were collected and store at -80^{o}C.

2.5 Protein content and degree of hydrolysis.

Protein content was determined in a microplate using the DC™ Protein Assay (Bio-rad, California, USA) according to the manufacturer instructions. Absorbance was recorded on a Spark microplate reader (Tecan, Switzerland). A standard curve was prepared with bovine serum albumin (BSA) in HBSS. Protein degree of hydrolysis was determined using the o-phthaldialdehyde (OPA) reagent, based on the method described by Nielson et al.[17] Shortly, in a 96-well plate, 30 µL of test sample, standard (serine 0.9516 Meqv/L), or control (HBSS) were combined with 225 µL of OPA reagent and incubated for 2 min at room temperature. The absorbance was then read at 340 nm in a Spark microplate reader (Tecan, Switzerland). Degree of hydrolysis was determined with Eq (1) and expressed as Meqv Ser-NH2/mg protein.

$$Serine - NH2 = \left(\frac{(Abs\ sample - Abs\ blank)}{(Abs\ standard - Abs\ blank)}\right) \times 0.9516 \frac{meqv}{L} \times (3.00E - 04)\ \times \frac{100}{x}$$

Where X= protein percentage sample; $(3.00E-04)$ is the sample volume in litre.

2.6 Peptides profiling by LC-MS/MS

Peptidomic analysis were performed on the basolateral fluids and on the digested test samples used as starting material for the transport study. Samples were standardized to a protein content of 50 µg/mL. 12 µL of reducing agent TCEP (tris(2-carboxyethyl)phosphine, 100mM) were then added, and the mixture was incubated 45 min at 25°C. 12 µL of freshly prepared iodoacetamide (500mM) were then added, and the mixture was incubated 1 hour at 25°C in the dark. Finally, the solution was acidified with 3 µL of formic acid (FA), centrifuged (10000 g, 30 s) to separate the peptides in the supernatant. Obtained peptidic samples were re-suspended with 25 µL of 1% FA in water and 2 µL were injected into the LC/MS/MS. All experiments were performed on an Orbitrap Fusion (Thermo Scientific) coupled to an Ultimate3000 nanoRLSC (Dionex). Peptides were separated on an in-house packed column (polymicro technology), 15 cm x 70 µm ID, Luna C18(2), 3 µm, 100 Å (Phenomenex) employing a water/acetonitrile/0.1% formic acid gradient. Samples were loaded onto the column for 105 min at a flow rate of 0.30 µl/min. Peptides were separated using 2% acetonitrile in the first 7 min and then using a linear gradient from 2 to 38 % of acetonitrile for 70 min, followed by gradient from 38 to 98 % of acetonitrile for 9 min, then at 98 % of acetonitrile for 10 min, followed by gradient from 98 to 2% of acetonitrile for 3 min and wash 10 min at 2 % of acetonitrile. Eluted peptides were directly sprayed into mass spectrometer using positive electrospray ionization (ESI) at an ion source temperature of 250°C and an ion spray voltage of 2.1 kV. The Orbitrap Fusion Tribrid was run in top speed mode. Full-scan MS spectra (m/z 350–2000) were acquired at a resolution of 60 000. Precursor ions were filtered according to monoisotopic precursor selection, charge state (+2 to + 7), and dynamic exclusion (30 s with a ± 10 ppm window). The automatic gain control settings were 4e5 for full FTMS scans and 1e4 for MS/MS scans. Fragmentation was performed with collision-induced dissociation (CID) in the linear ion trap. Precursors were isolated using a 2 m/z isolation window and fragmented with a normalized collision energy of 35%.

2.7 MS Data Processing

MS raw files were analyzed with MaxQuant (version 2.0.1.0)[18] and the Andromeda search engine.[19] Peptides were searched against a UniProt trEMBL fasta file for *Fabeae* (Taxon identifier: 163743, 6225 entries, 23.06.2021) and a UniParc proteome from *Lactobacillus plantarum* (Proteome ID: UP000230784, 2974 entries, 23.06.2021). The default contaminants database was appended with protein sequences from pancreatin enzymes. Default parameters were used if not mentioned otherwise. N-terminal acetylation and methionine oxidation were set as variable modifications, and cysteine carbamidomethylation was set as a fixed modification. Enzyme specificity was set to unspecific with peptide length from 3 to 25 amino acids. The false discovery rate (FDR) was set to 0.01 for both the protein and peptide level, determined by searching against a reverse sequence database. Peptides were identified with an initial precursor mass deviation of up to 10 ppm and a fragment mass deviation of 0.5 Da. The 'Match between runs' algorithm in MaxQuant[20] was performed between all samples to increase peptide identification rate. The identified peptides were quantified using label-free quantification (LFQ). Peptides identified only in 1 out of 3 replicates were discarded. Peptides with > 50 % missing values were removed. Missing values were replaced by LoDs (1/5 of the minimum positive value of each variable). No data filtering was applied. Physico-chemical properties of the peptides were retrieved from the R package "Peptides" using R-4.1.1.[21]

2.8 Statistical analysis

Label free quantification intensities were normalized by auto scaling (mean-centered and divided by standard deviation of each variable) before statistical analysis. Hierarchical clustering was performed with MetaboAnalyst 5.0. Physico-chemical parameters were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. All the statistical analysis were performed with GraphPad Prism 9.0.0. The volcano plot was created with MetaboAnalyst 5.0 using the following parameters: 2.0-fold change (FD) threshold, 0.1 p-value threshold, group variance = equal.

Results and Discussion

3.1 Effect of fermentation and gastrointestinal digestion on peptides formation.

A total of 515 peptides were identified (Supplementary table 1), originating primarily from 15 protein groups (Fig. 1 B). Over 90 % of the parental proteins were legumins, vicilins and convicilins, reflecting the predominance of globulins in lentil seed proteins.[22] No peptides originating from the albumin cysteine rich fraction were identified, although being the second largest protein group in lentils. This could be related to the high resistance to gastrointestinal enzymes previously described for albumins, provided by their high content of disulfide bonds and low molecular weight.[5] Within the vicilin family, vicilin, allergen Len c 1.0101 (Q84UI1), allergen Len c 1.0102 (Q84UI0) and β-lathyrin 1 (A0A3G5BED8**)** were identified. Allergen Len c 1.01 is ~ 50 kD protein previously identified as a major allergen of lentils.[23] Interestingly, over 85 % of the vicilin-derived peptides seemed to originate from this protein. β-lathyrin is a 47 kD protein previously identified as potential allergen in grass pea (*Lathyrus sativus* L.), showing sequence homology with Len c 1.[24] This could be of interest for decreasing the allergenicity of lentil products, similarly to the previously reported enzymatic and bacterial hydrolysis of allergenic proteins in wheat, whey and pulses proteins and decrease in immunoreactive response.[25–29] Within the legumin family, most peptides originated from legumin B, A1 and A2. Fermentation increased the content of peptides formed from the vicilin family of protein (Supplementary Fig 2A). When visualized with a heatmap, clear clustering could be observed between unfermented and fermented samples, and bioaccessible and absorbed peptides (Fig. 1A).

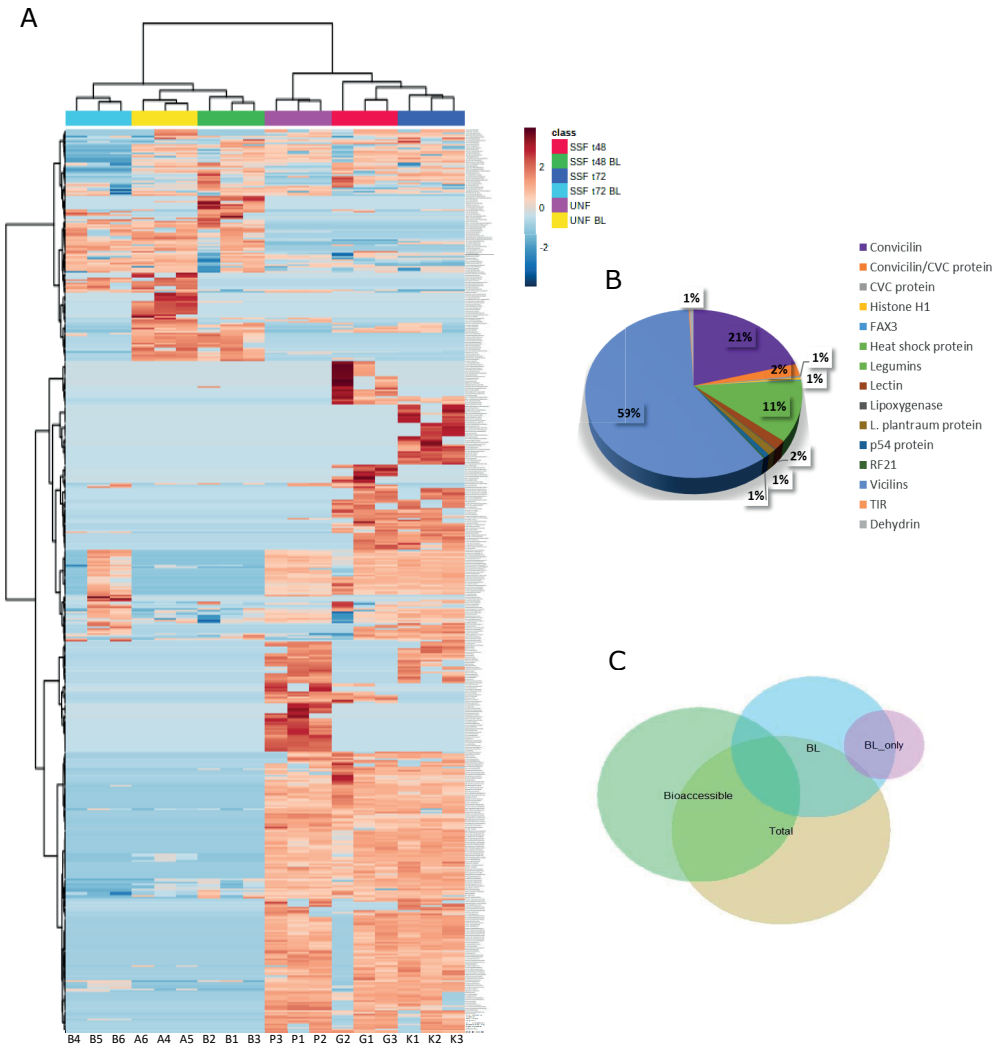

Figure 6. Overview of peptides identified by LC-MS/MS (A), their parental proteins (B), and Venn diagram distribution of the identified peptides (C). UNF: unfermented; t48: 48 h fermentation; t72: 72 h fermentation; BL: peptides identified in the basolateral fluids; BL_only: peptides identified in the basolateral fraction but absent in the bioaccessible fraction.

3.2 Bioaccessible peptides formed upon microbial fermentation and *in vitro* gastrointestinal digestion.

A total of 452 peptides were identified in the bioaccessible (*in vitro* digested) fraction. 136 peptides were exclusively present in the bioaccessible fermented samples, of which 35 only in the 48 h SSF samples and 44 only in the 72 h SSF samples. The hydrophobicity index was significantly higher in fermented samples (Fig. 2A), showing an increasing trend in relation with the fermentation time (Fig. 2C). Hydrophobicity was previously reported to play an important role in peptides bioactivity, particularly towards α-glucosidase, dipeptidyl-peptidase-4 (DPP-4) and angiotensin converting enzyme (ACE).[30] Interestingly, member of the *L. plantarum* species were previously reported to lack

biosynthetic pathway for the branched-chain amino acids Ile, Leu and Val.[31] More specifically, auxotrophy for the amino acids Ile, Leu, Val, Met, Phe, Glu and Trp was described for *L. plantarum* ATCC8014.[32] In our study, we observed that these amino acids were found in higher amount in peptides sequences formed in the fermented samples, particularly in the 72 h fermented, compared to the unfermented samples. As fermentation progresses, the substrate is used and nutrients such as free amino acids become scarce, putatively inducing more extensive protein hydrolysis to generate the required amino acids. This could suggest that nutritional requirements of the bacterial strain drive its specificity in releasing peptides containing the vital amino acids for its survival, for subsequent degradation of the peptide and utilization of the amino acids. On the contrary, the median of the Boman index was lower in fermented samples compared to the unfermented ones (Fig. 2B, D), indicating lower protein-binding potential.[33] In fact, the Boman index measures the solubility of the amino acids present in the peptide sequence and provide a general estimation of the tendency of the peptide to bind to membranes or other protein receptors.[21,34] Amino acids K, L and R were preferred at the cleavage site, as they were the most abundant units before the amino-terminal of peptides. This reflects the activity of trypsin, which cleaves on the C-terminal of lysine (K) and arginine (R), and chymotrypsin, which cleaves on the C-terminal of aromatic and hydrophobic amino acids.[35,36] 229 peptides were present in all samples (UNF, SSF t=48 h, SSF t=72 h), but at different intensities. A volcano plot was used to identify the peptides up/downregulated in the fermented samples (Supplementary Fig. 3). 33 of these peptide sequences were significantly different between unfermented and fermented samples (Supplementary Table 2). The peptides upregulated in the fermented samples derived from vicilin and convicilin protein families, while the peptides upregulated in the unfermented samples derived from vicilins, convicilin, lectin, dehydrin 2/3/b and legumins.

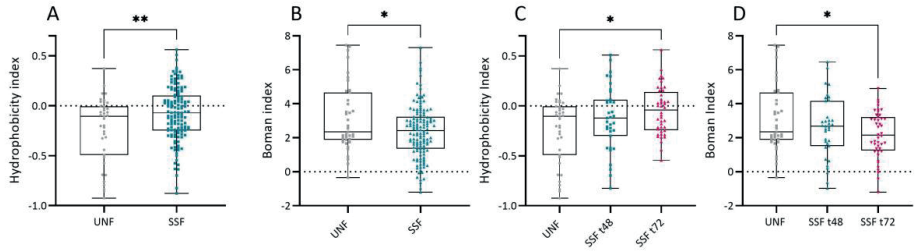

Figure 27. Details of bioaccessible peptides. Hydrophobicity (A) and Boman index (C) of bioaccessible peptides present in unfermented samples (UNF) and in fermented samples (SSF). B-D: same parameters measured in peptides uniquely present in unfermented, 48 h fermented (SSF t48) or 72 h fermented (SSF t72) samples. Asterix show significant difference after one-way ANOVA followed by Tukey's multiple comparisons test.

3.3 Peptides transport across Caco-2 cell monolayer

Transport of proteins and peptides contained in the digested test samples across the Caco-2 cells monolayer was estimated by Lawry method and OPA respectively. The percentage of transport are reported in Supplementary Fig. 1. Protein content ranged between 7.88 and 10.16 mg/mL in the digested samples, and 0.19 – 0.32 in the basolateral fluids. SSF for 72 hours led to a significant increase of 2.03 % of protein transported across the intestinal cells monolayer. Peptide content increased from 0.61 to 2.23 µEqv Ser-NH_2 after SSF for 72 hours, although there was an important variability between replicates.

After standardizing the protein content, transported peptides were profiled by LC-MS/MS. A total of 215 peptides were identified in the basolateral fluids. Of these, 144 were transported from the bioaccessible peptides in the basolateral side, while 63 were exclusively present in the basolateral fraction. These likely originated from the hydrolysis by brush border enzymes. Among the 144 bioaccessible peptides transported to the basolateral side of the Caco-2 monolayer, only 83 peptides (16.1 % of the total bioaccessible peptides available for transport) were transported in more than 60 % of the number of samples in which they were identified in the bioaccessible fraction. The other 61 were transported only as a small fraction of the total amount identified in the bioaccessible fraction and therefore were not taken into account for understanding the physico-chemical propertiess driving transport. When looking at the individual fermentation times, 83/361 peptides (23 %) were absorbed in SSF t=72h, 77/314 (24.5%) in unfermented samples, and 83/338 in SSF t=48 h. This was in line with previous studies on food-derived protein hydrolysates transport studies in Caco-2 cells, were a transport of 9 – 21 % was observed.[37,38] The peptides length and mass distributions as function of peptides absorption are shown in Fig. 3A. With the exception of peptides FFEITPEKNPQLQDLDIFVNSV and NILEAAFNTEYEEIEKVLLEEQEQK in the unfermented samples, a length of 22 amino acids appeared to be the cutoff for Caco-2 cell transport, and 85 % of peptides of 21 amino acids were also not absorbed. More than 74 % of the peptides with a mass greater than 2000 were not absorbed. Interestingly, charge appeared to play a role in peptide absorption, as the peptides in the BL fluids were significantly more negatively charged than the unabsorbed ones. Charge and molecular weight were indeed previously described as important factors in peptide bioavailability.[39] Moreover, absorbed peptides had lower instability index. This index is calculated based on the amino acids composition of the peptide, with the assumption that certain dipeptides occur in different amount and location in unstable proteins *in vivo*.[40] A value of <40 is considered stable.[21] Peptides formed upon brush border activity ("Only in BL") were negatively charged, less hydrophobic and with higher Boman index compared to the absorbed or unabsorbed ones. Following the same trend observed in Fig. 1B, the peptides identified in the basolateral fraction were mainly generated by vicilin, legumin and convicilin families of proteins (Supplementary Fig. 2). This was independent of the fermentation, although fermentation decreased the relative abundance of peptides derived from convicilin and legumins. Similarly to what observed for bioaccessible peptides, the peptides identified in the basolateral fraction of fermented samples were considerably more hydrophobic, with the hydrophobicity value increasing in line with fermentation time (Fig. 4 B). Amino acids I, L, K, F, P, V were present in higher amount in the fermented samples, particularly after 72 h fermentation. The presence of hydrophobic amino acids and P, K, R was previously reported as a predictor of bioactivity, suggesting a beneficial effect of fermentation in this context.[5] Moreover, the peptides present in position P_1, P_2, P_3 and, to a lesser extent, the C-terminal are important predictors of DPP-IV inhibitory activity. We therefore inspected the amino acids present in these positions in the peptides that were unique for each fermentation times (0, 48, 72 h) and we could observe some trends. (Fig. 4 C). In the unfermented samples, L and N were predominant in first position, E and V in second and E, Q, V at the C-terminal. In the 72 h fermented peptides, E, L and S were predominant at the N-terminal, F, I, S and A at the second position, and I, L, S and V at the C-terminal. DPP-IV inhibitors can be either substrate inhibitors, true inhibitors or prodrug inhibitors. Substrate inhibitors preferably have P, A or to a lower extent G, S or T at P_2.[11,12,41] The peculiar IPI belongs to this class. True inhibitors are resistant to the activity of DPP-IV, while prodrug inhibitors are degraded to more inhibitory components. Food-derived DPP-IV inhibitory oligopeptides (≥ 4 a.a.) with considerable activity were observed to commonly have L, G, I in P_1, P, L or K in P_2, A, V, G or P at P_3 and P, L, R at the C-terminus.[41] Moreover, the presence of

hydrophobic amino acids within the bioactive peptides is also commonly encountered. Based on the results of our study, the peptides generated by 72 h fermentation with *L. plantarum* harbor a greater potential for DPP-IV inhibitory activity. Absorption of peptides in the intestinal epithelium takes place via four major routes: (1) passive paracellular transport though tight junctions, (2) active carrier-mediated transport via PepT1, (3) active transcytosis by means of internalization in vesicles, and (4) transcellular passive diffusion. Properties such as size, charge, hydrophobicity, lipophilicity and hydrogen bonding potential are important determinant of the type of transport. For instance, lipophilic peptides prefer transcellular passive diffusion, small di- and tri-peptides are transported via PepT1, while transcytosis seemed preferred for long-chain oligopeptides and hydrophobic peptides.[42] Wang et al. recognized the importance of molecular weight, as the low-molecular weight fraction (<500 Da) of casein-derived peptides were transported across Caco-2 cells via PepT1, while the larger fractions (500-1600 Da) were transported via paracellular route. In this case, 16.23 % of the low MW fraction was absorbed, and ~ 10 % of the larger fractions.[37] Other studies suggested paracellular and transcytosis as preferred routes for transepithelial transport of a 10 and 17 residues peptides.[43,44] Likewise, paracellular and transcytosis transport could have played a major role in the transport of the peptides identified in this study, justifying the size and weight cutoff observed.

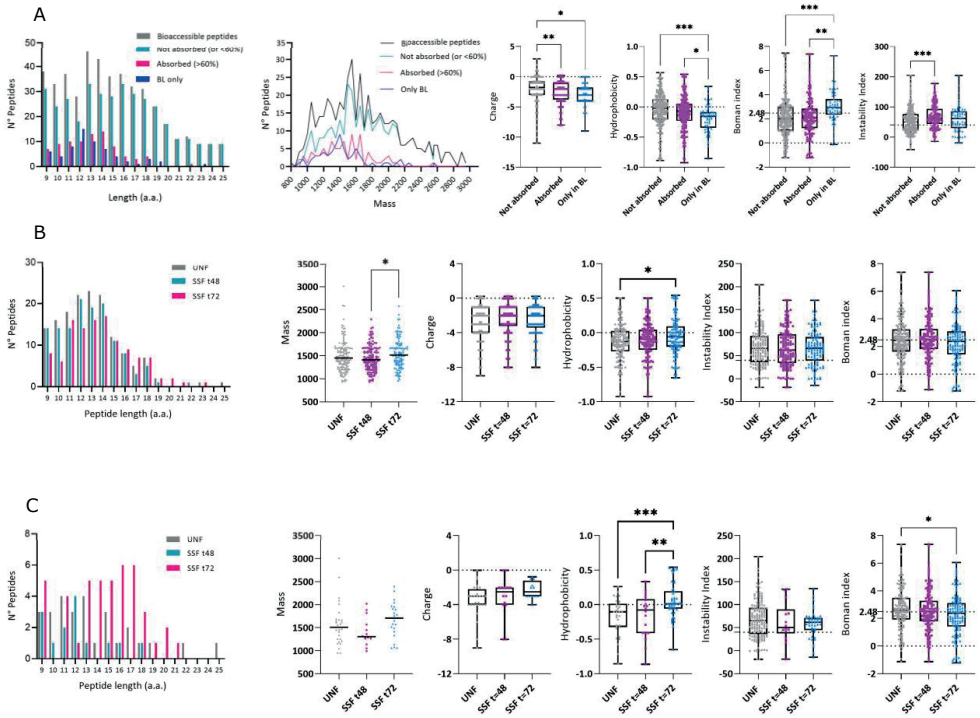

Figure 38. A: physico-chemical properties involved in peptides transport across Caco-2 cell monolayer. "Only in BL" = peptides identified uniquely in the basolateral fraction, putatively formed upon brush border enzymes activity. B: physico-chemical properties of total peptides identified in unfermented (UNF), 48 h microbially fermented (SSF t=48) of 72 h microbially fermented (SSF t=72) green lentil flour. C: physico-chemical properties of peptides identified uniquely in the three fermentation times. Asterix show significant difference after one-way ANOVA followed by Tukey's multiple comparisons test.

Conclusions

To conclude, we provided evidence of the implications of the gastrointestinal digestion and microbial fermentation with *L. plantarum* ATCC8014 on the peptidome of green lentil, and on the peptide transport across a polarized Caco-2 cell monolayer. Microbial fermentation led to the formation of more hydrophobic peptides. Intestinal transport was influenced by peptide length, mass, charge and hydrophobicity. Boman index and instability index also differed among unabsorbed and absorbed samples. Exposure to the brush border enzymes led to the formation of 63 peptides that were absent in the bioaccessible fraction. Among the peptides identified in the basolateral fractions, the ones uniquely present in the 72 h fermented samples had a higher potential for DPP-IV inhibitory activity. To the best of our knowledge, this is the first study reporting the implications of *in vitro* gastrointestinal digestion, brush border enzymes activity and microbial fermentation with *L. plantarum* on the peptidome of green lentil and its transport across the Caco-2 monolayer.

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Conflicts of interest

The authors have no conflicts to declare.

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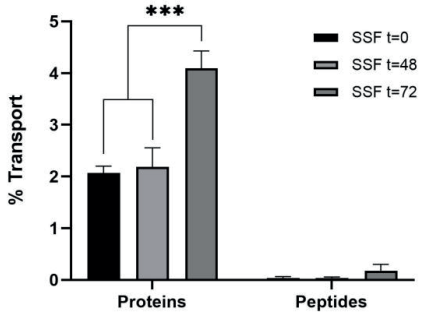
Supplementary Material



Supplementary Figure 1. Percentage of transport across Caco-2 cells monolayer for phenolic compounds (TPC), proteins, and peptides (OPA). Values are mean ± SD of three independent replicates of the treatment. Asterisk (*) represents significant difference after Tukey's multiple comparison test.

ID n°	Sequence	Length	Net charge	Hydroph. Index	Boman Index	Mass	Protein ID
1	VNGVAPGPI	9	-0.02	0.51	-1.12	822.5	Putative oxidoreductase YghA (*L. plantarum*)
2	GVSSESEPF	9	-2.02	0.09	1.76	937.4	Vicilin 47k
3	PHMPSSSSN	9	0.22	-0.11	2.51	942.4	p54 protein
4	LTFPGSAQE	9	-1.02	0.18	0.85	948.5	Convicilin;Convicilin
5	NEGGLLLPH	9	-0.78	0.26	0.16	948.5	Convicilin
6	LPSFSPSPQ	9	-0.02	0.14	0.87	958.5	Legumin B; Minor legumin
7	FLTGSDDNV	9	-2.02	0.11	1.91	966.4	Convicilin;Convicilin
8	SVSSESEPF	9	-2.02	0.02	2.24	967.4	Vicilin; allergen Len c 1.0101/2; beta-lathyrin 1
9	HLPSFSPSP	9	0.22	0.19	0.77	967.5	Legumin B; Minor legumin
10	EKIPGTEQS	9	-1.02	-0.23	2.76	987.5	Dehydrin 2/3/b
11	GVNAENNQR	9	-0.02	-0.48	4.49	1000.5	Cvc protein
12	GINAENNQR	9	-0.02	-0.44	4.39	1014.5	Vicilin; convicilin; beta-lathyrin 1
13	GINAENNER	9	-1.02	-0.43	4.53	1015.5	Convicilin ; Vicilin 47k; Vicilin ; Convicilin; Cvc protein
14	DLDIFVNSV	9	-2.02	0.34	0.73	1020.5	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ; Beta-lathyrin 1
15	KSLPSEFEP	9	-1.02	-0.09	2.01	1032.5	Convicilin ; Cvc protein ;
16	LVNPDDEED	9	-5.02	-0.30	4.16	1044.4	Convicilin
17	SLPSEFEPF	9	-2.02	0.20	1.06	1051.5	Convicilin
18	LPQFTDADF	9	-2.02	0.16	1.43	1052.5	Allergen Len c 1.0101
19	FNLEEGDIM	9	-3.02	0.18	1.43	1066.5	p54 protein
20	FLPQYTDAD	9	-2.02	0.06	1.78	1068.5	Allergen Len c 1.0102 ; vicilin 47k; Vicilin ; Beta-lathyrin 1
21	EETSTQVQR	9	-1.02	-0.55	4.90	1076.5	Allergen Len c 1.0102

22	SDLFENLQN	9	-2.02	-0.10	2.77	1078.5	Convicilin ; Cvc protein ; Convicilin;
23	EQSPGQWRP	9	-0.02	-0.40	3.66	1083.5	Convicilin
24	EVDRLLTNQ	9	-1.02	-0.29	3.48	1086.6	Allergen Len c 1.0102 ; Allergen Len c 1.0101
25	SKIFENLQN	9	-0.02	-0.13	2.42	1091.6	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ;
26	SDQENPFIF	9	-2.02	0.05	2.25	1095.5	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ; Beta-lathyrin 1
27	LIETWNPNN	9	-1.02	0.03	1.90	1099.5	Legumin (Minor small); Legumin A; N-terminal incomplete legumin A1 pre-pro-polypeptide; LegA class
28	AFNTDYEEI	9	-3.02	0.03	2.44	1100.5	Allergen Len c 1.0102 ; Vicilin 47k
29	VVEEEEGEW	9	-5.02	-0.03	2.52	1104.5	Convicilin
30	VFEDNDFET	9	-4.02	-0.07	3.36	1114.4	p54 protein
31	ETWNPNHPE	9	-1.78	-0.27	3.53	1122.5	Legumin (Minor small); Legumin B; Storage protein
32	LEEQENEPH	9	-3.78	-0.42	4.35	1123.5	Convicilin
33	KEDEDEDEE	9	-7.02	-0.88	7.31	1136.4	Legumin (Minor small)
34	EDEDEDEEE	9	-9.01	-0.79	7.45	1137.3	Legumin (Minor small)
35	TEYEEIEKV	9	-3.02	-0.20	2.95	1138.5	Allergen Len c 1.0101
36	NTDYEEIEK	9	-3.02	-0.42	4.35	1139.5	Allergen Len c 1.0102 ; Vicilin 47k
37	EEDEEEKEQ	9	-6.02	-0.85	6.74	1163.4	Convicilin
38	LFENLQNYR	9	-0.02	-0.23	3.10	1195.6	Convicilin; Cvc protein ; Convicilin;
39	LLSGTQNQPS	10	-0.02	-0.02	1.63	1043.5	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ; Beta-lathyrin 1
40	IESEGGLIET	10	-3.02	0.23	0.98	1046.5	Legumin (Minor small); Legumin A; N-terminal incomplete legumin A1 pre-pro-polypeptide; LegA class
41	GFGVNAENNQ	10	-1.02	-0.01	2.16	1048.5	Cvc protein
42	INEGGLLLPH	10	-0.78	0.37	-0.35	1061.6	Convicilin
43	KGVSSESEPF	10	-1.02	-0.07	2.14	1065.5	Vicilin 47k
44	HLPSFSPSPQ	10	0.22	0.08	1.25	1095.5	Legumin B; Minor legumin
45	LVNPDDEEDL	10	-5.02	-0.16	3.25	1157.5	Convicilin
46	EQIEELSKNA	10	-2.02	-0.25	2.99	1159.6	Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ; Beta-lathyrin 1
47	EITPEKNPQL	10	-1.02	-0.20	2.41	1167.6	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
48	EQSPGQWRPS	10	-0.02	-0.38	3.63	1170.5	Convicilin
49	KSLPSEFEPF	10	-1.02	0.03	1.51	1179.6	Convicilin
50	FLPQQTDADF	10	-2.02	0.06	1.84	1180.5	Vicilin
51	TLYENENGHI	10	-1.78	-0.03	2.35	1188.5	Vicilin 47 K ; beta-lathyrin 1
52	DEEEGQEEET	10	-7.02	-0.58	5.68	1193.4	Allergen Len c 1.0101
53	FLPQFTDADF	10	-2.02	0.27	0.99	1199.5	Allergen Len c 1.0101
54	FEITPEKNPQ	10	-1.02	-0.19	2.60	1201.6	Vicilin; Allergen Len c 1.0101/2
55	YLVNPDDEED	10	-5.02	-0.24	3.76	1207.5	Convicilin
56	LTETWNPNHP	10	-0.78	-0.07	2.26	1207.6	Legumin (Minor small); Legumin B; Storage protein
57	EVDRLLTNQK	10	-0.02	-0.42	3.69	1214.7	Allergen Len c 1.0101/2

58	SDQENPFIFK	10	-1.02	-0.11	2.58	1223.6	Vicilin; Allergen Len c 1.0101/2; vicilin; beta-lathyrin 1
59	AFNTDYEEIE	10	-4.02	-0.05	2.88	1229.5	Allergen Len c 1.0102 ; vicilin
60	NTDYEEIEKV	10	-3.02	-0.27	3.51	1238.6	Allergen Len c 1.0102 ; vicilin
61	SDLFENLQNY	10	-2.02	-0.07	2.51	1241.6	Convicilin; Cvc protein
62	AFNTEYEEIE	10	-4.02	-0.03	2.69	1243.5	Allergen Len c 1.0101
63	YQHEGKEEET	10	-2.78	-0.50	4.48	1248.5	Convicilin
64	DDEEEQEEET	10	-8.01	-0.71	6.64	1251.4	Allergen Len c 1.0102
65	LEDQEQEPQH	10	-3.78	-0.49	4.55	1251.5	Allergen Len c 1.0102
66	NTEYEEIEKV	10	-3.02	-0.26	3.32	1252.6	Allergen Len c 1.0101
67	SKIFENLQNY	10	-0.02	-0.09	2.19	1254.6	Allergen Len c 1.0101/2; vicilin
68	KEDEDEDEEE	10	-8.01	-0.86	7.26	1265.4	Legumin (Minor small)
69	EDEDEDEKEE	10	-8.01	-0.86	7.26	1265.4	N-terminal incomplete legumin A1 pre-pro-polypeptide; Legumin A2 primary translation product
70	FNTDYEEIEK	10	-3.02	-0.26	3.62	1286.6	Allergen Len c 1.0102; vicilin
71	EEQENEPHQR	10	-2.78	-0.83	6.45	1294.6	Convicilin
72	LSPGDVLVIPA	11	-1.02	0.54	-1.22	1079.6	Allergen Len c 1.0101; vicilin
73	LSPGDVVIIPA	11	-1.02	0.57	-1.22	1079.6	Convicilin ; Cvc protein ;
74	GDTIKLPAGTI	11	-0.02	0.27	0.09	1084.6	Vicilin; Allergen Len c 1.0101/2 ;
75	LNIGSSSSPDI	11	-1.02	0.18	1.21	1088.5	N-terminal incomplete legumin A1 pre-pro-polypeptide; Legumin A2 primary translation product
76	GLHLPSFSPSP	11	0.22	0.29	0.10	1137.6	Legumin B; Minor legumin
77	NASSDLNLIGF	11	-1.02	0.27	0.76	1149.6	Allergen Len c 1.0101/2; vicilin
78	EINEGGLLLPH	11	-1.78	0.27	0.31	1190.6	Convicilin
79	SVSSESEPFNL	11	-2.02	0.04	1.99	1194.5	Vicilin; allergen Len c 1.0101/2; beta-lathyrin 1
80	EIKEGSLLLPN	11	-1.02	0.11	0.78	1211.7	Allergen Len c 1.0101/2; beta-lathyrin 1
81	GFGINAENNQR	11	-0.02	-0.21	3.24	1218.6	Vicilin; convicilin; beta-lathyrin 1
82	NFLAGEEDNVI	11	-3.02	0.17	1.46	1219.6	Vicilin; allergen Len c 1.0101/2; beta-lathyrin 1
83	EIKEGSLLLPH	11	-0.78	0.15	0.60	1234.7	Vicilin
84	DLDIFVNSVEI	11	-3.02	0.33	0.77	1262.6	Allergen Len c 1.0101/2; beta-lathyrin 1
85	GLTETWNPNHP	11	-0.78	-0.02	1.97	1264.6	Legumin (Minor small); Legumin B; Storage protein
86	NILEAAFNTEY	11	-2.02	0.19	1.20	1283.6	Allergen Len c 1.0101
87	EQIEELSKNAK	11	-1.02	-0.36	3.22	1287.7	Allergen Len c 1.0101 ; vicilin ; Beta-lathyrin 1
88	FLPQQTDADFI	11	-2.02	0.18	1.23	1293.6	Vicilin
89	SDQDNPFIFES	11	-3.02	-0.06	2.94	1297.5	Vicilin type C
90	DLNFTPNEFDS	11	-3.02	-0.07	2.97	1297.5	Lipoxygenase
91	EEETSSESQER	11	-4.02	-0.70	6.12	1309.5	Convicilin
92	FLPQFTDADFI	11	-2.02	0.37	0.45	1312.6	Allergen Len c 1.0101
93	FEITPEKNPQL	11	-1.02	-0.07	1.92	1314.7	Vicilin; Allergen Len c 1.0101/2
94	SFNTGYEEIEK	11	-2.02	-0.13	2.72	1315.6	Vicilin
95	YLVNPDDEEDL	11	-5.02	-0.13	2.97	1320.6	Convicilin
96	VLLEDQEQEPQ	11	-4.02	-0.21	2.90	1326.6	Allergen Len c 1.0102

97	FLPQYTDADFI	11	-2.02	0.28	0.73	1328.6	Allergen Len c 1.0102 ; vicilin ; beta-lathyrin 1
98	TLFENENGHIR	11	-0.78	-0.17	3.21	1328.6	Convicilin; Cvc protein
99	QEEETSTQVQR	11	-2.02	-0.59	5.13	1333.6	Allergen Len c 1.0102
100	VLLEEQENEPH	11	-3.78	-0.15	2.75	1335.6	Convicilin
101	LTETWNPNHPE	11	-1.78	-0.13	2.68	1336.6	Legumin (Minor small); Legumin B; Storage protein
102	AFNTDYEEIEK	11	-3.02	-0.18	3.12	1357.6	Allergen Len c 1.0102 ; Vicilin
103	LLEDQEQEPQH	11	-3.78	-0.35	3.69	1364.6	Allergen Len c 1.0102
104	AFNTEYEEIEK	11	-3.02	-0.17	2.95	1371.6	Allergen Len c 1.0101
105	EEDEDEDEKEE	11	-9.01	-0.85	7.22	1394.5	Legumin A2 primary translation product
106	SDLFENLQNYR	11	-1.02	-0.29	3.64	1397.7	Convicilin; Cvc protein;
107	SKIFENLQNYR	11	0.98	-0.32	3.35	1410.7	Allergen Len c 1.0101/2 ; Vicilin
108	QEEKEEEEEEK	11	-6.01	-0.89	6.47	1434.6	Legumin B
109	GDTIKLPAGTIA	12	-0.02	0.30	-0.07	1155.6	Vicilin; Allergen Len c 1.0101/2 ;
110	NGLHLPSFSPSP	12	0.22	0.20	0.65	1251.6	Legumin B; Minor legumin
111	GLHLPSFSPSPQ	12	0.22	0.20	0.55	1265.6	Legumin B; Minor legumin
112	LAGEEDNVISQI	12	-3.02	0.15	1.36	1286.6	Vicilin; Allergen Len c 1.0101/2 ; beta-lathyrin 1
113	NEGKGDFELVGQ	12	-2.02	-0.06	2.11	1291.6	Allergen Len c 1.0101 ; Vicilin
114	LLSGTQNQPSFL	12	-0.02	0.17	0.70	1303.7	Allergen Len c 1.0101/2
115	GVSSESEPFNLR	12	-1.02	-0.12	2.71	1320.6	Vicilin
116	GFGINAENNQRN	12	-0.02	-0.26	3.52	1332.6	Convicilin;Convicilin ; Beta-lathyrin 1
117	GFGINAENNERN	12	-1.02	-0.25	3.63	1333.6	Convicilin ; Vicilin 47k; Vicilin ; Convicilin; Cvc protein
118	SVSSESEPFNLR	12	-1.02	-0.17	3.07	1350.6	Vicilin; Allergen Len c 1.0101/2 ; beta-lathyrin 1
119	EIKEGSLLLPNY	12	-1.02	0.12	0.73	1374.7	Allergen Len c 1.0101/2; beta-lathyrin 1
120	DLDIFVNSVEIK	12	-2.02	0.18	1.17	1390.7	Allergen Len c 1.0101/2; beta-lathyrin 1
121	EDNVISQIQRPV	12	-1.02	-0.15	2.80	1396.7	Allergen Len c 1.0101/2; beta-lathyrin 1
122	SREQIEELSKNA	12	-1.02	-0.43	4.02	1402.7	Allergen Len c 1.0101 ; Vicilin; Beta-lathyrin 1
123	AYLANRDDNEDL	12	-3.02	-0.33	3.99	1407.6	Allergen Len c 1.0101/2; Vicilin ; Beta-lathyrin 1
124	SRNPIYSNKFGK	12	2.97	-0.34	3.12	1409.7	Vicilin; Allergen Len c 1.0101/2 ; vicilin ; beta-lathyrin 1
125	SFNTGYEEIEKV	12	-2.02	-0.03	2.15	1414.7	Vicilin
126	SDQDNPFIFESK	12	-2.02	-0.18	3.16	1425.6	Vicilin type C
127	FLTLFENENGHI	12	-1.78	0.24	1.04	1432.7	Convicilin; Cvc protein ;
128	AFNTDYEEIEKV	12	-3.02	-0.08	2.53	1456.7	Allergen Len c 1.0102 ; Vicilin 47k
129	FFEITPEKNPQL	12	-1.02	0.03	1.51	1461.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
130	FQTLYENENGHI	12	-1.78	0.00	2.17	1463.7	Vicilin 47 K ; Beta-lathyrin 1
131	AFNTEYEEIEKV	12	-3.02	-0.06	2.37	1470.7	Allergen Len c 1.0101
132	QTLYENENGHIR	12	-0.78	-0.31	3.66	1472.7	Vicilin 47 K ; Beta-lathyrin 1
133	HGKEEDEEEKEQ	12	-4.78	-0.76	5.83	1485.6	Convicilin
134	KEEAWTLFEKHS	12	-0.78	-0.18	2.51	1503.7	TIR-similar-domain-containing protein TSDC
135	FNTEYEEIEKVL	12	-3.02	-0.03	2.11	1512.7	Allergen Len c 1.0101

136	KEDEDEDEEEEE	12	-10.01	-0.84	7.18	1523.5	Legumin (Minor small)
137	GDTIKLPAGTIAY	13	-0.02	0.30	-0.05	1318.7	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k
138	VNGVAPGPIWTPL	13	-0.02	0.50	-1.13	1319.7	Putative oxidoreductase YghA (*L. plantarum*)
139	AIVIVTVNEGKGY	13	-0.02	0.37	-0.30	1361.8	Allergen Len c 1.0102
140	SVEINEGGLLLPH	13	-1.78	0.30	0.21	1376.7	Convicilin
141	NGLHLPSFSPSPQ	13	0.22	0.12	1.02	1379.7	Legumin B; Minor legumin
142	LLVNEGKGNLELV	13	-1.02	0.22	0.22	1396.8	Convicilin ; Cvc protein
143	AVLTVLNSNDRNS	13	-0.02	-0.10	2.55	1401.7	Allergen Len c 1.0102
144	NFLTGSDDNVISQ	13	-2.02	0.04	2.14	1408.6	Convicilin
145	AILTVLNSNDRNS	13	-0.02	-0.08	2.49	1415.7	Allergen Len c 1.0101 ; Beta-lathyrin 1
146	ELAFPGSAQEVDR	13	-2.02	-0.06	2.28	1417.7	Vicilin
147	DLAIPVNRPGQLQ	13	-0.02	0.00	1.52	1419.8	Allergen Len c 1.0101
148	NFLAGEEDNVISQ	13	-3.02	0.06	1.92	1434.7	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k; Beta-lathyrin 1
149	ELAFPGSSHEVDR	13	-1.78	-0.09	2.62	1442.7	Vicilin 47 K ; Beta-lathyrin 1
150	KGVSSESEPFNLR	13	-0.02	-0.22	2.93	1448.7	Vicilin 47k
151	YLGGNPETEFPET	13	-3.02	0.05	1.74	1452.6	Legumin J
152	AGLTETWNPNHPE	13	-1.78	-0.03	2.05	1464.7	Legumin (Minor small); Legumin B
153	GFGINAENNERNF	13	-1.02	-0.14	3.12	1480.7	Convicilin ; Vicilin 47k; Vicilin; Cvc protein
154	EIKEGSLLLPNYN	13	-1.02	0.05	1.18	1488.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
155	VSREQIEELSKNA	13	-1.02	-0.32	3.40	1501.8	Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ; Beta-lathyrin 1
156	FLPQQTDADFILV	13	-2.02	0.32	0.35	1505.8	Vicilin
157	GLTETWNPNHPEL	13	-1.78	0.01	1.81	1506.7	Legumin (Minor small); Legumin B; Storage protein
158	TIFLPQQTDADFI	13	-2.02	0.26	0.86	1507.8	Vicilin
159	LVNPDDEEDLRVV	13	-4.02	-0.15	3.03	1511.7	Convicilin
160	EASFNTGYEEIEK	13	-3.02	-0.12	2.68	1515.7	Vicilin
161	EITPEKNPQLQDL	13	-2.02	-0.21	2.57	1523.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
162	FLPQFTDADFILV	13	-2.02	0.48	-0.31	1524.8	Allergen Len c 1.0101
163	TIFLPQFTDADFI	13	-2.02	0.41	0.20	1526.8	Allergen Len c 1.0101
164	GKFFEITPEKNPQ	13	-0.02	-0.13	2.13	1533.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
165	SRSDQDNPFIFES	13	-2.02	-0.26	3.90	1540.7	Vicilin type C
166	TLFLPQYTDADFI	13	-2.02	0.32	0.44	1542.8	Allergen Len c 1.0102 ; vicilin 47k; Vicilin ; Beta-lathyrin 1
167	SRNPIYSNKFGKF	13	2.97	-0.22	2.65	1556.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ; Beta-lathyrin 1
168	EAAFNTDYEEIEK	13	-4.02	-0.16	3.03	1557.7	Allergen Len c 1.0102 ; Vicilin 47k
169	FEITPEKNPQLQD	13	-2.02	-0.20	2.72	1557.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
170	EVDRLLTNQKQSH	13	0.22	-0.43	3.88	1566.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101

171	DDEEEQEEETSTQ	13	-8.01	-0.63	5.99	1567.6	Allergen Len c 1.0102
172	AFNTDYEEIEKVL	13	-3.02	0.01	1.95	1569.8	Allergen Len c 1.0102 ; Vicilin 47k
173	YLVNPDDEEDLRV	13	-4.02	-0.22	3.35	1575.7	Convicilin
174	RSDQDNPFIFESK	13	-1.02	-0.36	4.06	1581.7	Vicilin type C
175	AFNTEYEEIEKVL	13	-3.02	0.02	1.81	1583.8	Allergen Len c 1.0101
176	FLTLFENENGHIR	13	-0.78	0.03	2.11	1588.8	Convicilin ; Cvc protein
177	FFEITPEKNPQLQ	13	-1.02	-0.03	1.82	1589.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
178	FQTLYENENGHIR	13	-0.78	-0.19	3.15	1619.8	Vicilin 47 K ; Beta-lathyrin 1
179	FNTEYEEIEKVLL	13	-3.02	0.06	1.57	1625.8	Allergen Len c 1.0101
180	SKIFENLQNYRLL	13	0.98	-0.10	2.08	1636.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ;
181	KEDEDEDEEEEEE	13	-11.01	-0.84	7.15	1652.6	Legumin (Minor small)
182	SVEKEDKNDEWHR	13	-1.78	-0.69	5.56	1670.8	Heat shock protein
183	GDTIKLPAGTIGYL	14	-0.02	0.34	-0.34	1417.8	Vicilin
184	GDTIKLPAGTIAYL	14	-0.02	0.35	-0.40	1431.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k
185	DLAIPVNNPGQLES	14	-2.02	0.12	1.16	1465.7	Allergen Len c 1.0102
186	LVNEGKGNLELVGF	14	-1.02	0.25	0.27	1487.8	Convicilin ; Cvc protein ; Convicilin;
187	TVNEGKGDFELVGQ	14	-2.02	0.02	1.70	1491.7	Allergen Len c 1.0101 ; Vicilin
188	VLDLAIPVNRPGQL	14	-0.02	0.21	0.38	1503.9	Allergen Len c 1.0101
189	SFLLSGTQNQPSFL	14	-0.02	0.22	0.63	1537.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101
190	SVEINEGGLLLPHY	14	-1.78	0.30	0.20	1539.8	Convicilin
191	NFLAGEEDNVISQI	14	-3.02	0.16	1.43	1547.7	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
192	AVLTVLNSNDRNSF	14	-0.02	-0.01	2.16	1548.8	Allergen Len c 1.0102
193	AGEEDNVISQIQRP	14	-2.02	-0.18	2.98	1554.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
194	EINEGGLLLPHYNS	14	-1.78	0.16	0.97	1554.8	Convicilin
195	ELAFPGSSHEVDRL	14	-1.78	0.00	2.08	1555.8	Vicilin 47 K ; Beta-lathyrin 1
196	NLLGFGINAENNER	14	-1.02	-0.06	2.41	1559.8	Convicilin ; Cvc protein
197	DTGSSNNQLDQMPR	14	-1.02	-0.41	4.13	1561.7	N-terminal incomplete legumin A1 pre-pro-polypeptide
198	AILTVLSPNDRNSY	14	-0.02	0.01	1.84	1561.8	Convicilin ; Cvc protein
199	AILTVLNSNDRNSF	14	-0.02	0.02	2.10	1562.8	Allergen Len c 1.0101 ; Beta-lathyrin 1
200	KELAFPGSSHEVDR	14	-0.78	-0.19	2.83	1570.8	Vicilin 47 K ; Beta-lathyrin 1
201	EIKEGSLLLPNYNS	14	-1.02	0.04	1.34	1575.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
202	AILTVLKPDDRNSF	14	-0.02	-0.02	1.92	1587.9	Vicilin
203	GFGINAENNERNFL	14	-1.02	-0.05	2.54	1593.8	Convicilin ; Vicilin 47k; Vicilin; Cvc protein
204	FNRAEDLATAMEAR	14	-1.02	-0.21	3.14	1593.8	Energy-coupling factor transporter transmembrane protein EcfT (*L. plantarum*)
205	EIKEGSLLLPHYNS	14	-0.78	0.06	1.20	1598.8	Vicilin
206	GNLELVGFKNEQQE	14	-2.02	-0.12	2.26	1603.8	Convicilin
207	EASFNTGYEEIEKV	14	-3.02	-0.03	2.20	1614.7	Vicilin
208	SLNTKYDTIEKVLL	14	-0.02	0.01	1.30	1635.9	Convicilin

209	EITPEKNPQLQDLD	14	-3.02	-0.26	3.01	1638.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
210	DEEEGQEEETTKQV	14	-6.02	-0.51	4.74	1649.7	Allergen Len c 1.0101
211	EAAFNTDYEEIEKV	14	-4.02	-0.07	2.52	1656.8	Allergen Len c 1.0102 ; Vicilin 47k
212	VNPDDEEDLRVVDF	14	-5.02	-0.20	3.57	1660.8	Convicilin
213	EEEGQEEETTKQVQ	14	-5.02	-0.50	4.51	1662.7	Allergen Len c 1.0101
214	DDEEEQEEETSTQV	14	-8.01	-0.51	5.28	1666.6	Allergen Len c 1.0102
215	SRSDQDNPFIFESK	14	-1.02	-0.35	4.02	1668.8	Vicilin type C
216	EAAFNTEYEEIEKV	14	-4.02	-0.06	2.39	1670.8	Allergen Len c 1.0101
217	FEITPEKNPQLQDL	14	-2.02	-0.11	2.17	1670.9	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
218	YLVNPDDEEDLRVV	14	-4.02	-0.13	2.82	1674.8	Convicilin
219	KEDEEKVVEEEEGE	14	-7.01	-0.51	4.66	1676.7	Convicilin
220	AFNTDYEEIEKVLL	14	-3.02	0.09	1.46	1682.8	Allergen Len c 1.0102 ; Vicilin 47k
221	AFNTEYEEIEKVLL	14	-3.02	0.10	1.33	1696.9	Allergen Len c 1.0101
222	FFEITPEKNPQLQD	14	-2.02	-0.10	2.31	1704.8	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
223	FELVGQRNENQQEQ	14	-2.02	-0.42	4.14	1717.8	Allergen Len c 1.0101
224	SRQEEDEDEDEKEE	14	-8.01	-0.92	7.37	1765.7	Legumin A2 primary translation product
225	FDKRSDLFENLQNY	14	-1.02	-0.31	3.66	1787.8	Convicilin ; Cvc protein
226	LSPGDVLVIPAGHPV	15	-0.78	0.48	-0.92	1469.8	Allergen Len c 1.0101
227	LSPGDVVIIPAGHPV	15	-0.78	0.50	-0.92	1469.8	Convicilin ;Cvc protein
228	LSPGDVFVVPAGHPV	15	-0.78	0.47	-0.73	1489.8	Allergen Len c 1.0102
229	LSPGDVFVIPAGHPV	15	-0.78	0.49	-0.79	1503.8	Vicilin 47 K ; Beta-lathyrin 1
230	ASSNLNLLGFGINAK	15	0.98	0.25	0.27	1517.8	Convicilin
231	TGGTVITDDLGLELK	15	-2.02	0.19	0.73	1530.8	60 kDa chaperonin (*L. plantarum*)
232	VSSVEINEGGLLLPH	15	-1.78	0.32	0.14	1562.8	Convicilin
233	TGSDDNVISQIENPV	15	-3.02	0.01	2.24	1586.7	Convicilin
234	NLERGDTIKLPAGTI	15	-0.02	0.00	1.63	1596.9	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k
235	VNSVEIKEGSLLLPN	15	-1.02	0.16	0.70	1610.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
236	DLAIPVNNPGQLESF	15	-2.02	0.19	0.88	1612.8	Allergen Len c 1.0102
237	VLDLAIPVNRPGQLQ	15	-0.02	0.14	0.72	1631.9	Allergen Len c 1.0101
238	AGEEDNVISQIQRPV	15	-2.02	-0.10	2.51	1653.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
239	SVEINEGGLLLPHYN	15	-1.78	0.22	0.63	1653.8	Convicilin
240	DLAIPVNRPGQLQSF	15	-0.02	0.07	1.35	1653.9	Allergen Len c 1.0101
241	LAGEEDNVISQIQRP	15	-2.02	-0.10	2.46	1667.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
242	IESEGGLIETWNPNN	15	-3.02	0.06	1.82	1671.8	Legumin (Minor small); Legumin A; N-terminal incomplete legumin A1 pre-pro-polypeptide; LegA class
243	SVEIKEGSLLLPNYN	15	-1.02	0.11	0.98	1674.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
244	TIFLPQQTDADFILV	15	-2.02	0.37	0.14	1719.9	Vicilin
245	EASLNTKYDTIEKVL	15	-1.02	-0.07	1.88	1722.9	Convicilin
246	EASFNTGYEEIEKVL	15	-3.02	0.04	1.73	1727.8	Vicilin
247	EITPEKNPQLQDLDI	15	-3.02	-0.15	2.48	1751.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101
248	TLFLPQYTDADFILV	15	-2.02	0.42	-0.22	1754.9	Allergen Len c 1.0102 ; vicilin 47k; Vicilin ; Beta-lathyrin 1

249	EAAFNTDYEEIEKVL	15	-4.02	0.00	2.03	1769.8	Allergen Len c 1.0102 ; Vicilin 47k
250	LVNPDDEEDLRVVDF	15	-5.02	-0.11	3.01	1773.8	Convicilin
251	DEEEGQEEETTKQVQ	15	-6.02	-0.53	4.79	1777.7	Allergen Len c 1.0101
252	EAAFNTEYEEIEKVL	15	-4.02	0.01	1.90	1783.9	Allergen Len c 1.0101
253	FEITPEKNPQLQDLD	15	-3.02	-0.16	2.61	1785.9	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
254	HAIVKVSREQIEELR	15	0.22	-0.24	2.98	1806.0	Convicilin
255	FFEITPEKNPQLQDL	15	-2.02	-0.02	1.83	1817.9	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
256	EEEGQEEETTKQVQR	15	-4.02	-0.64	5.21	1818.8	Allergen Len c 1.0101
257	SNRFQTLYENENGHI	15	-0.78	-0.23	3.40	1820.8	Vicilin 47 K ; Beta-lathyrin 1
258	SRSDQDNPFIFESKR	15	-0.02	-0.49	4.74	1824.9	Vicilin type C
259	RPSHGKEEDEEEKEQ	15	-3.78	-0.78	5.88	1825.8	Convicilin
260	DEEEQEEETSTQVQR	15	-6.02	-0.64	5.71	1835.8	Allergen Len c 1.0102
261	KEDEEKVVEEEEGEW	15	-7.01	-0.42	4.20	1862.8	Convicilin
262	LSPGDVLVIPAGHPVA	16	-0.78	0.49	-0.98	1540.9	Allergen Len c 1.0101
263	SPGDVLVIPAGHPVAI	16	-0.78	0.51	-0.98	1540.9	Allergen Len c 1.0101
264	LSPGDVVIIPAGHPVS	16	-0.78	0.46	-0.65	1556.9	Convicilin
265	LSPGDVFVVPAGHPVA	16	-0.78	0.48	-0.80	1560.8	Allergen Len c 1.0102
266	LSPGDVFVIPAGHPVA	16	-0.78	0.50	-0.85	1574.8	Vicilin 47 K ; Beta-lathyrin 1
267	NASSDLNLIGFGINAK	16	-0.02	0.20	0.80	1632.8	Allergen Len c 1.0102 ; Allergen Len c 1.0101
268	NLERGDTIKLPAGTIA	16	-0.02	0.04	1.41	1667.9	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k
269	LSGTQNQPSFLSGFSK	16	0.98	0.05	1.36	1696.8	Allergen Len c 1.0101
270	SLTDTGSSNNQLDQMP	16	-2.02	-0.15	2.75	1706.7	N-terminal incomplete legumin A1 pre-pro-polypeptide
271	TGSDDNVISQIENPVK	16	-2.02	-0.08	2.45	1714.8	Convicilin
272	VLDLAIPVNRPGQLQS	16	-0.02	0.12	0.89	1719.0	Allergen Len c 1.0101
273	DLAIPVNNPGQLESFL	16	-2.02	0.25	0.52	1725.9	Allergen Len c 1.0102
274	SVEINEGGLLLPHYNS	16	-1.78	0.20	0.81	1740.9	Convicilin
275	SVEIKEGSLLLPNYNS	16	-1.02	0.09	1.13	1761.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
276	LAGEEDNVISQIQRPV	16	-2.02	-0.02	2.05	1766.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
277	DLAIPVNRPGQLQSFL	16	-0.02	0.13	0.96	1767.0	Allergen Len c 1.0101
278	AFPGSAQEVDRLLENQ	16	-2.02	-0.08	2.31	1772.9	Vicilin
279	LTDTGSSNNQLDQMPR	16	-1.02	-0.29	3.47	1775.8	N-terminal incomplete legumin A1 pre-pro-polypeptide
280	AGEEDNVISQIQRPVK	16	-1.02	-0.18	2.70	1781.9	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
281	SVLEASLNTKYDTIEK	16	-1.02	-0.07	1.97	1809.9	Convicilin
282	EASLNTKYDTIEKVLL	16	-1.02	0.01	1.45	1836.0	Convicilin
283	SKPHTIFLPQFTDADF	16	-0.78	0.13	1.32	1862.9	Allergen Len c 1.0101
284	SILEAAFNTDYEEIEK	16	-4.02	0.01	2.06	1870.9	Allergen Len c 1.0102
285	EAAFNTDYEEIEKVLL	16	-4.02	0.07	1.59	1882.9	Allergen Len c 1.0102 ; Vicilin 47k
286	EAAFNTEYEEIEKVLL	16	-4.02	0.08	1.47	1896.9	Allergen Len c 1.0101
287	FEITPEKNPQLQDLDI	16	-3.02	-0.06	2.14	1899.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101
288	EITPEKNPQLQDLDIF	16	-3.02	-0.06	2.14	1899.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101
289	NILEAAFNTEYEEIEK	16	-4.02	-0.02	2.14	1911.9	Allergen Len c 1.0101

290	FFFVSFGNMPVHNRSQ	16	1.22	0.15	1.37	1912.9	Hypothetical chloroplast RF21
291	SNKFLTLFENENGHIR	16	0.22	-0.13	2.69	1918.0	Convicilin; Cvc protein
292	DTIEKVLLEEQENEPH	16	-4.78	-0.22	3.06	1921.9	Convicilin
293	FFEITPEKNPQLQDLD	16	-3.02	-0.07	2.26	1932.9	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
294	DEEEGQEEETTKQVQR	16	-5.02	-0.65	5.43	1933.9	Allergen Len c 1.0101
295	KSNRFQTLYENENGHI	16	0.22	-0.31	3.54	1948.9	Vicilin 47 K ; Beta-lathyrin 1
296	SNRFQTLYENENGHIR	16	0.22	-0.37	4.12	1976.9	Vicilin 47 K ; Beta-lathyrin 1
297	FFEVTPEKKYPQLQDL	16	-1.02	-0.07	1.71	1981.0	Convicilin
298	SKIFENLQNYRLLEYK	16	0.98	-0.21	2.47	2057.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin ;
299	DLSAPDLDNSGGGGSIK	17	-2.02	0.04	1.66	1601.8	FAX3
300	LSPGDVLVIPAGHPVAI	17	-0.78	0.54	-1.21	1653.9	Allergen Len c 1.0101
301	LSPGDVVIIPAGHPVAI	17	-0.78	0.56	-1.21	1653.9	Convicilin; Cvc protein
302	LSPGDVVIIPAGHPVSI	17	-0.78	0.51	-0.90	1669.9	Convicilin
303	LSPGDVFVVPAGHPVAI	17	-0.78	0.53	-1.04	1673.9	Allergen Len c 1.0102
304	AKLSPGDVFVVPAGHPV	17	0.22	0.36	-0.43	1688.9	Allergen Len c 1.0102
305	AKLSPGDVFVIPAGHPV	17	0.22	0.38	-0.48	1702.9	Vicilin 47 K ; Beta-lathyrin 1
306	AAVDTLPPVVNESEEPT	17	-4.02	0.10	1.39	1766.9	Histone H1
307	TFFIAPVDTKPQTGGGY	17	-0.02	0.24	0.48	1797.9	Lectin
308	LLSGTQNQPSFLSGFSK	17	0.98	0.11	0.99	1809.9	Allergen Len c 1.0101
309	NLERGDTIKLPAGTIAY	17	-0.02	0.05	1.34	1831.0	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k;Vicilin
310	TGSDDNVISQIENPVKE	17	-3.02	-0.12	2.70	1843.9	Convicilin;Convicilin
311	VLDLAIPVNRPGQLQSF	17	-0.02	0.19	0.66	1866.0	Allergen Len c 1.0101
312	EEESEEQNEGNSVLSGF	17	-6.02	-0.18	3.30	1882.8	Legumin (Minor small)
313	LAFPGSAQEVDRLLENQ	17	-2.02	-0.02	1.88	1886.0	Vicilin
314	VNSVEIKEGSLLLPNYN	17	-1.02	0.11	1.02	1888.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
315	SVLEASLNTKYDTIEKV	17	-1.02	0.00	1.62	1909.0	Convicilin
316	NFLAGEEDNVISQIQRP	17	-2.02	-0.06	2.38	1929.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
317	NLLGFGINAENNERNFL	17	-1.02	0.04	1.91	1934.0	Convicilin ;Convicilin;Cvc protein ;
318	SILEAAFNTDYEEIEKV	17	-4.02	0.07	1.70	1970.0	Allergen Len c 1.0102
319	SKPHTIFLPQFTDADFI	17	-0.78	0.20	0.95	1976.0	Allergen Len c 1.0101
320	EITPEKNPQLQDLDIFV	17	-3.02	0.00	1.78	1998.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101
321	GKFFEITPEKNPQLQDL	17	-1.02	-0.08	1.89	2003.0	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
322	NILEAAFNTEYEEIEKV	17	-4.02	0.05	1.78	2011.0	Allergen Len c 1.0101
323	FEITPEKNPQLQDLDIF	17	-3.02	0.01	1.84	2046.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101
324	FFEITPEKNPQLQDLDI	17	-3.02	0.01	1.84	2046.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101
325	FEVTPEKKYPQLQDLDL	17	-2.02	-0.12	2.01	2062.1	Convicilin
326	DEEEGQEEETTKQVQRY	17	-5.02	-0.60	5.12	2096.9	Allergen Len c 1.0101
327	KSNRFQTLYENENGHIR	17	1.22	-0.44	4.20	2105.0	Vicilin 47 K ; Beta-lathyrin 1
328	DDEEEQEEETSTQVQRY	17	-7.02	-0.60	5.56	2113.9	Allergen Len c 1.0102
329	TEYEEIEKVLLEEQEQK	17	-5.02	-0.30	3.16	2136.0	Allergen Len c 1.0101

330	QWRPSHGKEEDEEEKEQ	17	-3.78	-0.69	5.38	2139.9	Convicilin
331	LSPGDVVIIPAGHPVSIS	18	-0.78	0.47	-0.66	1757.0	Convicilin
332	LSPGDVLVIPAGHPVAIN	18	-0.78	0.47	-0.77	1768.0	Allergen Len c 1.0101
333	ARLSPGDVLVIPAGHPVA	18	0.22	0.33	-0.14	1768.0	Allergen Len c 1.0101
334	VSSVEINEGGLLLPHYNS	18	-1.78	0.23	0.68	1927.0	Convicilin
335	SATTGAEFAAHEVHSWSF	18	-1.54	0.24	0.99	1933.9	Lectin
336	NLERGDTIKLPAGTIAYL	18	-0.02	0.11	0.99	1944.1	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k
337	NSFNLERGDTIKLPAGTI	18	-0.02	0.01	1.75	1945.0	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Vicilin 47k
338	DALEPDNRIESEGGLIET	18	-5.02	-0.10	2.71	1956.9	Legumin (Minor small); Legumin A; N-terminal incomplete legumin A1 pre-pro-polypeptide; LegA class
339	TGSDDNVISQIENPVKEL	18	-3.02	-0.06	2.28	1957.0	Convicilin
340	NFLTGSDDNVISQIENPV	18	-3.02	0.09	1.80	1960.9	Convicilin
341	VNSVEIKEGSLLLPNYNS	18	-1.02	0.10	1.15	1975.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
342	VLDLAIPVNRPGQLQSFL	18	-0.02	0.23	0.35	1979.1	Allergen Len c 1.0101
343	VNSVEIKEGSLLLPHYNS	18	-0.78	0.12	1.04	1998.0	Vicilin
344	LAFPGSAQEVDRLLENQK	18	-1.02	-0.10	2.09	2014.0	Vicilin
345	SVLEASLNTKYDTIEKVL	18	-1.02	0.05	1.26	2022.1	Convicilin
346	AGEEDNVISQIQRPVKEL	18	-2.02	-0.15	2.51	2024.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
347	FLPQYTDADFILVVLSGK	18	-1.02	0.34	-0.10	2025.1	Allergen Len c 1.0102 ; vicilin 47k; Vicilin ; Beta-lathyrin 1
348	NFLAGEEDNVISQIQRPV	18	-2.02	0.00	2.03	2028.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
349	NSVEIKEGSLLLPNYNSR	18	-0.02	-0.11	2.21	2032.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
350	DVIVKVSREQIEELSKNA	18	-1.02	-0.16	2.53	2056.1	Allergen Len c 1.0101
351	LVNPDDEEDLRVVDFVIS	18	-5.02	0.03	2.20	2073.0	Convicilin
352	SILEAAFNTDYEEIEKVL	18	-4.02	0.13	1.33	2083.0	Allergen Len c 1.0102
353	EITPEKNPQLQDLDIFVN	18	-3.02	-0.04	2.05	2112.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101
354	NEDVIVKVSREQIEELSK	18	-2.02	-0.24	3.00	2114.1	Allergen Len c 1.0101
355	NILEAAFNTEYEEIEKVL	18	-4.02	0.10	1.40	2124.1	Allergen Len c 1.0101
356	FEITPEKNPQLQDLDIFV	18	-3.02	0.07	1.51	2145.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101
357	FGKFFEITPEKNPQLQDL	18	-1.02	-0.01	1.62	2150.1	Vicilin; Allergen Len c 1.0102 ; Allergen Len c 1.0101
358	FFEITPEKNPQLQDLDIF	18	-3.02	0.08	1.57	2193.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101
359	SNRFQTLYENENGHIRLL	18	0.22	-0.22	3.12	2203.1	Vicilin 47 K ; Beta-lathyrin 1
360	FFEVTPEKKYPQLQDLDL	18	-2.02	-0.05	1.73	2209.1	Convicilin
361	NTEYEEIEKVLLEEQEQK	18	-5.02	-0.33	3.36	2250.1	Allergen Len c 1.0101
362	AKLSPGDVFVVPAGHPVAI	19	0.22	0.43	-0.73	1873.0	Allergen Len c 1.0102
363	ARLSPGDVLVIPAGHPVAI	19	0.22	0.38	-0.39	1881.1	Allergen Len c 1.0101
364	NASSDLNLIGFGINAKNNQ	19	-0.02	0.04	1.67	1989.0	Allergen Len c 1.0102 ; Allergen Len c 1.0101
365	NNPGQLESFLLSGTQNQPS	19	-1.02	-0.03	1.92	2030.0	Allergen Len c 1.0102
366	NRPGEPQSFLLSGNQNQPS	19	-0.02	-0.21	2.83	2069.0	Vicilin
367	LDALEPDNRIESEGGLIET	19	-5.02	-0.03	2.31	2070.0	Legumin (Minor small); Legumin A; N-terminal incomplete legumin A1 pre-pro-polypeptide; LegA class
368	NRPGQLQSFLLSGTQNQPS	19	0.98	-0.13	2.29	2071.0	Allergen Len c 1.0101

369	VSSVEINEGGLLLPHYNSR	19	-0.78	0.08	1.43	2083.1	Convicilin
370	NFLTGSDDNVISQIENPVK	19	-2.02	0.01	1.99	2089.0	Convicilin
371	AFPGSAQEVDRLLENQKQS	19	-1.02	-0.20	2.71	2116.1	Vicilin
372	SVLEASLNTKYDTIEKVLL	19	-1.02	0.11	0.93	2135.2	Convicilin
373	LAGEEDNVISQIQRPVKEL	19	-2.02	-0.08	2.12	2137.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
374	NFLAGEEDNVISQIQRPVK	19	-1.02	-0.08	2.21	2156.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
375	SITKFSPDQKNLIFQGDGY	19	-0.02	-0.01	1.75	2157.1	Lectin
376	SILEAAFNTDYEEIEKVLL	19	-4.02	0.18	1.00	2196.1	Allergen Len c 1.0102
377	EITPEKNPQLQDLDIFVNS	19	-3.02	-0.05	2.12	2199.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101
378	NILEAAFNTEYEEIEKVLL	19	-4.02	0.15	1.07	2237.1	Allergen Len c 1.0101
379	FEITPEKNPQLQDLDIFVN	19	-3.02	0.03	1.78	2259.1	Allergen Len c 1.0102 ; Allergen Len c 1.0101
380	FFEITPEKNPQLQDLDIFV	19	-3.02	0.13	1.28	2292.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101
381	FFEVTPEKKYPQLQDLDLL	19	-2.02	0.01	1.38	2322.2	Convicilin
382	NTEYEEIEKVLLEEQEQKS	19	-5.02	-0.32	3.36	2337.1	Allergen Len c 1.0101
383	AFNTEYEEIEKVLLEEQEQ	19	-6.02	-0.13	2.63	2340.1	Allergen Len c 1.0101
384	RSDQENPFIFKSNRFQTLY	19	0.98	-0.29	3.47	2389.2	Vicilin 47 K ; Beta-lathyrin 1
385	FNTEYEEIEKVLLEEQEQK	19	-5.02	-0.25	3.02	2397.2	Allergen Len c 1.0101
386	LSPGDVVIIPAGHPVSISAS	20	-0.78	0.45	-0.52	1915.0	Convicilin
387	GDAMVNILSGEAEITIEGTI	20	-4.02	0.34	0.35	2032.0	Cupin domain protein (*L. plantarum*)
388	AAVDTLPPVVNESEEPTAKP	20	-3.02	0.05	1.37	2063.0	Histone H1
389	DLAIPVNNPGQLESFLLSGT	20	-2.02	0.26	0.42	2084.1	Allergen Len c 1.0102
390	AIVIVTVNEGKGDFELVGQR	20	-1.02	0.17	0.95	2143.2	Allergen Len c 1.0101 ; Vicilin
391	LAGEEDNVISQIQRPVKELA	20	-2.02	-0.05	1.92	2208.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101
392	LVNEGKGNLELVGFKNEQQE	20	-2.02	-0.10	2.04	2244.1	Convicilin
393	NFLAGEEDNVISQIQRPVKE	20	-2.02	-0.11	2.44	2285.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
394	EITPEKNPQLQDLDIFVNSV	20	-3.02	0.01	1.81	2298.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101
395	NEDVIVKVSREQIEELSKNA	20	-2.02	-0.22	2.95	2299.2	Allergen Len c 1.0101
396	TLNEVVPLKDVVPEWVRIGF	20	-1.02	0.22	0.54	2309.3	Lectin
397	NEGKGDFELVGQRNENQQEQ	20	-3.02	-0.44	4.19	2318.1	Allergen Len c 1.0101
398	FEITPEKNPQLQDLDIFVNS	20	-3.02	0.02	1.86	2346.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101
399	FFEITPEKNPQLQDLDIFVN	20	-3.02	0.08	1.54	2406.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101
400	AFNTEYEEIEKVLLEEQEQK	20	-5.02	-0.20	2.78	2468.2	Allergen Len c 1.0101
401	SRSDQENPFIFKSNRFQTLY	20	0.98	-0.28	3.47	2476.2	Vicilin 47 K ; Beta-lathyrin 1
402	RSDQENPFIFKSNRFQTLYE	20	-0.02	-0.31	3.64	2518.2	Vicilin 47 K ; Beta-lathyrin 1
403	NNPGQLESFLLSGTQNQPSFL	21	-1.02	0.08	1.36	2290.1	Allergen Len c 1.0102
404	NFLTGSDDNVISQIENPVKEL	21	-3.02	0.02	1.89	2331.2	Convicilin
405	DIFVNSVEIKEGSLLLPNYNS	21	-2.02	0.16	1.03	2350.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
406	NFLAGEEDNVISQIQRPVKEL	21	-2.02	-0.05	2.09	2398.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
407	NEDVIVKVSREQIEELSKNAK	21	-1.02	-0.28	3.07	2427.3	Allergen Len c 1.0101
408	VTSYTLNEVVPLKDVVPEWVR	21	-1.02	0.12	1.03	2442.3	Lectin
409	FEITPEKNPQLQDLDIFVNSV	21	-3.02	0.07	1.58	2445.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101

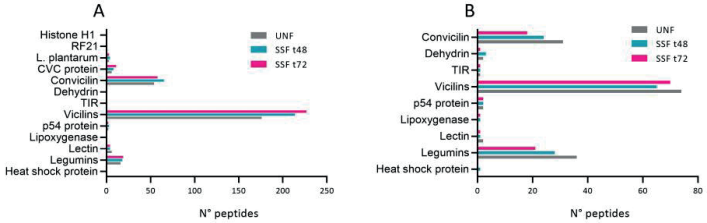
410	FFEITPEKNPQLQDLDIFVNS	21	-3.02	0.07	1.63	2493.2	Allergen Len c 1.0102 ; Allergen Len c 1.0101
411	EQSPGQWRPSHGKEEDEEEKE	21	-4.77	-0.57	4.80	2510.1	Convicilin
412	EAAFNTEYEEIEKVLLEEQEQ	21	-7.01	-0.13	2.62	2540.2	Allergen Len c 1.0101
413	AFNTEYEEIEKVLLEEQEQKS	21	-5.02	-0.20	2.81	2555.2	Allergen Len c 1.0101
414	ASSNLNLLGFGINAENNERNFL	22	-1.02	0.05	1.78	2406.2	Convicilin; Cvc protein
415	KELAFPGSAQEVDRLLENQKQS	22	-1.02	-0.23	2.68	2486.3	Vicilin
416	DIFVNSVEIKEGSLLLPNYNSR	22	-1.02	0.04	1.66	2506.3	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
417	SGFSKNILEAAFNTEYEEIEKV	22	-3.02	0.03	1.76	2517.2	Allergen Len c 1.0101
418	TVNEGKGDFELVGQRNENQQEQ	22	-3.02	-0.36	3.74	2518.2	Allergen Len c 1.0101
419	EITPEKNPQLQDLDIFVNSVEI	22	-4.02	0.04	1.73	2540.3	Allergen Len c 1.0102 ; Allergen Len c 1.0101
420	FFEITPEKNPQLQDLDIFVNSV	22	-3.02	0.12	1.37	2592.3	Allergen Len c 1.0102 ; Allergen Len c 1.0101
421	NILEAAFNTEYEEIEKVLLEEQ	22	-6.02	0.03	1.80	2623.3	Allergen Len c 1.0101
422	SLNTKYDTIEKVLLEEQENEPH	22	-3.78	-0.21	2.83	2628.3	Convicilin
423	EQSPGQWRPSHGKEEDEEEKEQ	22	-4.77	-0.59	4.83	2638.2	Convicilin
424	AFNTDYEEIEKVLLEDQEQEPQ	22	-7.02	-0.20	3.01	2666.2	Allergen Len c 1.0102
425	EAAFNTEYEEIEKVLLEEQEQK	22	-6.02	-0.19	2.76	2668.3	Allergen Len c 1.0101
426	LSPGDVVIIPAGHPVSISASSNL	23	-0.78	0.39	-0.23	2229.2	Convicilin
427	VYVAEDQLSAGPMLITPLANGTE	23	-3.02	0.28	0.25	2388.2	Helix-turn-helix domain-containing protein (*L. plantarum*)
428	DLDIFVNSVEIKEGSLLLPNYNS	23	-3.02	0.15	1.10	2578.3	Allergen Len c 1.0102 ; Allergen Len c 1.0101 ; Beta-lathyrin 1
429	LGVFNSKEYDKTSQTVAVEFDTF	23	-2.02	0.04	1.75	2624.3	Lectin
430	VNEGKGNLELVGFKNEQQEREDN	23	-3.02	-0.35	3.60	2645.3	Convicilin
431	EITPEKNPQLQDLDIFVNSVEIK	23	-3.02	-0.03	1.90	2668.4	Allergen Len c 1.0102 ; Allergen Len c 1.0101
432	FEITPEKNPQLQDLDIFVNSVEI	23	-4.02	0.09	1.53	2687.4	Allergen Len c 1.0102 ; Allergen Len c 1.0101
433	EAAFNTEYEEIEKVLLEEQEQKS	23	-6.02	-0.19	2.78	2755.3	Allergen Len c 1.0101
434	SLNTKYDTIEKVLLEEQENEPHQ	23	-3.78	-0.24	2.95	2756.4	Convicilin
435	LSPGDVVIIPAGHPVSISASSNLN	24	-0.78	0.35	0.06	2343.2	Convicilin
436	SKPHTIFLPQQTDADFILVVLSGK	24	0.22	0.19	0.62	2653.4	Vicilin
437	SKPHTIFLPQFTDADFILVVLSGK	24	0.22	0.27	0.26	2672.5	Allergen Len c 1.0101
438	LVNEGKGNLELVGFKNEQQEREDN	24	-3.02	-0.29	3.24	2758.4	Convicilin
439	GKFFEITPEKNPQLQDLDIFVNSV	24	-2.02	0.07	1.45	2777.4	Allergen Len c 1.0102 ; Allergen Len c 1.0101
440	FEITPEKNPQLQDLDIFVNSVEIK	24	-3.02	0.02	1.69	2815.5	Allergen Len c 1.0102 ; Allergen Len c 1.0101
441	EASLNTKYDTIEKVLLEEQENEPH	24	-4.78	-0.20	2.80	2828.4	Convicilin
442	EAAFNTDYEEIEKVLLEDQEQEPQ	24	-8.01	-0.19	2.97	2866.3	Allergen Len c 1.0102
443	NILEAAFNTEYEEIEKVLLEEQEQ	24	-7.01	-0.04	2.16	2880.4	Allergen Len c 1.0101
444	DLAIPVNNPGQLESFLLSGTQNQPS	25	-2.02	0.11	1.18	2638.3	Allergen Len c 1.0102

445	AIVVLLVNEGKGNLELVGFK NEQQE	25	-2.02	0.13	0.84	2739.5	Convicilin
446	KSKPHTIFLPQFTDADFILVVL SGK	25	1.22	0.20	0.47	2800.6	Allergen Len c 1.0101
447	TETTSFSITKFSPDQKNLIFQG DGY	25	-1.02	0.00	1.92	2823.4	Lectin
448	SILEAAFNTDYEEIEKVLLEDQ EQE	25	-8.01	-0.06	2.37	2954.4	Allergen Len c 1.0102
449	EASLNTKYDTIEKVLLEEQEN EPHQ	25	-4.78	-0.23	2.91	2956.4	Convicilin
450	SLPSEFEPFNLRSQNPKYSNK FGKF	25	1.98	-0.18	2.47	2960.5	Convicilin
451	FFEITPEKNPQLQDLDIFVNS VEIK	25	-3.02	0.07	1.51	2962.5	Allergen Len c 1.0102 ; Allergen Len c 1.0101
452	NILEAAFNTEYEEIEKVLLEEQ EQK	25	-6.02	-0.10	2.30	3008.5	Allergen Len c 1.0101
	Only in basolateral side						
453	AKLSPGDVF	9	-0.02	0.22	0.33	932.5	Allergen Len c 1.0102, vicilin, beta-lathyrin 1
454	VNEGKGDFE	9	-2.02	-0.16	2.85	993.4	Allergen Len c 1.0101, vicilin, beta-lathyrin 1
455	NTNYEEIEK	9	-2.02	-0.41	4.12	1138.5	Beta-lathyrin 1, vicilin
456	YLAGNHEQE	9	-1.78	-0.12	2.55	1059.5	LegA class
457	PSFSPSPQL	9	-0.02	0.14	0.87	958.5	Legumin B, Storage protein, Minor legumin
458	LANRDDNED	9	-3.02	-0.65	6.05	1060.4	Beta-lathyrin 1, allergen Len c 1.0102, vicilin
459	SFNTGYEEIE	10	-3.02	0.01	2.43	1187.5	Vicilin
460	QVEEQTEEKD	10	-4.018	-0.60	5.11	1233.5	Convicilin
461	ETWNPNHPEL	10	-1.78	-0.14	2.69	1235.6	Legumin (Minor small), Storage protein, Legumin B
462	NAAWDPSNKE	10	-1.02	-0.27	3.18	1130.5	Lectin
463	KEEVKVEVEDD	11	-4.02	-0.41	3.97	1317.6	Heat shock protein
464	FNTDYEEIEKV	11	-3.02	-0.14	2.92	1385.6	Allergen Len c 1.0102, vicilin
465	LTGSDDNVISQ	11	-2.02	0.01	2.20	1147.5	Convicilin
466	EVESSDTIDNV	11	-4.02	-0.08	3.10	1206.5	Polyubiquitin; Ubiquitin
467	GTKPEYGSTNT	11	-0.02	-0.18	2.58	1153.5	Dehydrin 2/3/b
468	FNTEYEEIEKV	11	-3.02	-0.13	2.75	1399.6	Allergen Len c 1.0101
469	EDNDFETKIDT	11	-4.02	-0.36	4.47	1325.6	p54 protein
470	EEEEGEWRGSQ	11	-4.02	-0.50	4.88	1334.5	Convicilin
471	KVLLEDQEQEPQ	12	-3.02	-0.32	3.12	1454.7	Allergen Len c 1.0102
472	AAFNTEYEEIEK	12	-3.02	-0.10	2.55	1442.7	Allergen Len c 1.0101
473	ASFNTGYEEIEK	12	-2.02	-0.07	2.34	1386.6	Vicilin
474	EGSLLLPNYNSR	12	-0.02	-0.10	2.19	1361.7	Allergen Len c 1.0101/2; vicilin; beta-lathyrin 1
475	FLTGSDDNVISQ	12	-2.02	0.11	1.77	1294.6	Convicilin
476	YNAAWDPSNKER	12	-0.02	-0.41	3.91	1449.7	Lectin
477	VKGGLSIISPPE	12	-0.02	0.29	-0.13	1195.7	LegA class
478	NDDEGSEPRVPG	12	-3.02	-0.37	4.18	1270.5	Convicilin
479	DALEPDNRIESE	12	-4.02	-0.36	4.26	1386.6	N-terminal incomplete legumin A1 pre-pro-polypeptide, legumin A2 primary translation product, legumin A, LegA class
480	VVEEEEGEWRGS	12	-4.02	-0.21	3.34	1404.6	Convicilin
481	ISLTDTGSSNNQ	12	-1.02	-0.09	2.68	1235.6	N-terminal incomplete legumin A1 pre-pro-polypeptide, Legumin A
482	VEINEGGLLLPH	12	-1.78	0.34	-0.06	1289.7	Convicilin

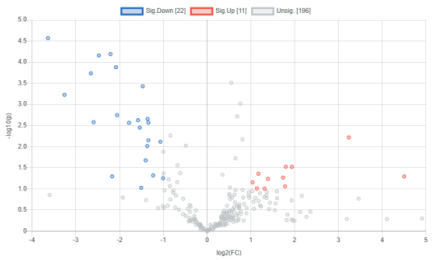
483	WMYNDQDTPVIA	12	-2.02	0.12	1.41	1451.6	Legumin A, legumin A2 primary translation product, LegA class
484	LTETWNPNHPEL	12	-1.78	-0.03	2.04	1449.7	Legumin (Minor small), Storage protein
485	EITPEKNPQLQD	12	-2.02	-0.31	3.20	1410.7	Vicilin, Allergen Len c 1.0101/2
486	NIGSSSSPDIYNP	13	-1.02	0.04	1.92	1349.6	N-terminal incomplete legumin A1 pre-pro-polypeptide, legumin A2 primary translation product
487	EEESEEQNEGNSV	13	-6.02	-0.43	4.73	1478.6	Legumin (Minor small)
488	LEPDNRIESEGGL	13	-3.02	-0.16	2.88	1427.7	N-terminal incomplete legumin A1 pre-pro-polypeptide, LegA class, legumin A2 primary translation product, legumin A
489	ALEPDHRVESEAG	13	-2.78	-0.17	2.97	1408.7	Legumin (Minor small); Legumin B
490	REESDEEEEQEEE	13	-9.01	-0.86	7.22	1665.6	Beta-lathyrin 1
491	YQHEGKEEETSSE	13	-3.78	-0.47	4.49	1551.6	Convicilin
492	VVEEEEGEWRGSQ	13	-4.02	-0.26	3.51	1532.7	Convicilin
493	SVSSESEPFNLRS	13	-1.02	-0.17	3.10	1437.7	Vicilin; Allergen Len c 1.0101/2; Beta-lathyrin 1
494	TDTGSSNNQLDQM	13	-2.02	-0.26	3.50	1409.6	N-terminal incomplete legumin A1 pre-pro-polypeptide
495	VFEDNDFETKIDT	13	-4.02	-0.13	3.25	1571.7	p54 protein
496	KVVEEEEGEWRGSQ	14	-3.02	-0.35	3.66	1660.8	Convicilin
497	GTKPEYGSTNTGSG	14	-0.02	-0.09	2.14	1354.6	Dehydrin 2/3/b
498	ALEPDNRIESEGGL	14	-3.02	-0.10	2.55	1498.7	N-terminal incomplete legumin A1 pre-pro-polypeptide; legumin A; legumin A2 primary translation product; LegA class
499	NLERGDTIKLPAGT	14	-0.02	-0.10	2.10	1483.8	Vicilin, Allergen Len c 1.0101/2; Cvc protein
500	LVNPDDEEDLRVVD	14	-5.02	-0.21	3.44	1626.8	Convicilin
501	DALEPDNRIESEGG	14	-4.02	-0.24	3.52	1500.7	LegA class; N-terminal incomplete legumin A1 pre-pro-polypeptide; legumin A2 primary translation product; legumin
502	AGLTETWNPNHPEL	14	-1.78	0.05	1.56	1577.7	legumin (Minor small), legumin B
503	SLTDTGSSNNQLDQM	15	-2.02	-0.16	2.93	1609.7	N-terminal incomplete legumin A1 pre-pro-polypeptide
504	NDDEGSEPRVPGQRE	15	-3.02	-0.57	5.16	1683.7	Convicilin
505	NALEPDHRVESEAGL	15	-2.78	-0.13	2.69	1635.8	Legumin (Minor small); legumin B
506	LDALEPDNRIESEGG	15	-4.02	-0.15	2.96	1613.7	LegA class; N-terminal incomplete legumin A1 pre-pro-polypeptide; legumin A2 primary translation product; legumin A
507	IESEGGLIETWNPNNR	16	-2.02	-0.10	2.64	1827.9	N-terminal incomplete legumin A1 pre-pro-polypeptide; legumin A2 primary translation product; legumin A
508	DALEPDNRIESEGGLI	16	-4.02	-0.06	2.47	1726.8	LegA class; N-terminal incomplete legumin A1 pre-pro-polypeptide; legumin A2 primary translation product; legumin A
509	HAGDLGNINVGDDGTVS	17	-2.78	0.13	1.56	1639.7	Superoxide dismutase
510	VESEAGLTETWNPNHPEL	18	-3.78	0.01	1.93	2021.9	Legumin (Minor small); legumin B
511	KEDEEKVVEEEEGEWRGS	18	-6.01	-0.50	4.46	2163.0	Convicilin
512	ISLTDTGSSNNQLDQMPR	18	-1.02	-0.19	3.00	1975.9	N-terminal incomplete legumin A1 pre-pro-polypeptide
513	LGGNPETEFPETQEEQQGR	19	-4.02	-0.30	3.51	2145.0	Legumin J
514	KEDEEKVVEEEEGEWRGSQ	19	-6.01	-0.50	4.52	2291.0	Convicilin
515	DALEPDNRIESEGGLIETWN PNN	23	-5.02	-0.14	2.89	2582.2	N-terminal incomplete legumin A1 pre-pro-polypeptide; LegA class;

	legumin A; legumin A2 primary translation product

Supplementary Table 3. Physico-chemical properties of identified peptides and parental protein ID.



Supplementary Figure 2. Parental proteins of peptides in the bioaccessible (A) and basolateral fraction (B) of unfermented and fermented samples. UNF= unfermented; SSFt48= solid-state fermented with L. plantarum for 48 h; SSFt72= solid-state fermented for 72 h.



Supplementary Figure 3. Volcano plot of the bioaccessible peptides present both in unfermented and solid-state fermented but at different intensities. Red dots indicate peptides that were significantly higher (p-value threshold 0.1) in the fermented samples, while blue dots show peptides that were significantly higher in the unfermented samples.

Peptide sequence	FC	Log2(FC)	Raw.pval	-$\log_{10}$(p)	Protein ID	FASTA Header
Upregulated in SSF						
FFEITPEKNPQLQ	9.441	3.2389	0.0060634	2.2173	Q41677; Q84UI0; Q84UI1	Vicilin; Allergen Len c 1.0101/2
FFEITPEKNPQLQDLDIFVNSVEIK	3.4802	1.7992	0.029919	1.5241	Q84UI0; Q84UI1	Allergen Len c 1.0101/2
DLDIFVNSVEIK	3.8297	1.9372	0.030054	1.5221	Q84UI0; Q84UI1; A0A3G5BED8	Allergen Len c 1.0101/2
AILTVLSPNDRNSY	2.2567	1.1742	0.043881	1.3577		Convicilin (Fragment); Cvc protein (Fragment); Convicilin;
DLDIFVNSVEI	22.729	4.5064	0.050771	1.2944	Q84UI0; Q84UI1; A0A3G5BED8	Allergen Len c 1.0101/2; Beta-lathyrin 1 (Fragment)
EASFNTGYEEIEKV	3.3398	1.7398	0.05414	1.2665	Q41677	Vicilin
EITPEKNPQLQDLDIFVNSVEIK	2.624	1.3917	0.05793	1.2371	Q84UI0; Q84UI1	Allergen Len c 1.0101/2
FFEITPEKNPQLQDLD	2.0571	1.0406	0.070003	1.1549	Q41677; Q84UI0; Q84UI1	Vicilin; Allergen Len c 1.0101/2

FQTLYENENGHI	3.4377	1.7814	0.087053	1.0602	D3VND9; D3VNE1; D3VNE2; Q702P0; Q702P1; Q43626; Q9M3X5; D3VND7	Vicilin 47 K (fragment); Beta-lathyrin 1 (Fragment)
Downregulated in SSF						
SATTGAEFAAHEVHSWSF	0.080533	-3.6343	2.70E-05	4.5679	A0A173G7D5	Lectin
TETTSFSITKFSPDQKNLIFQGDGY	0.21665	-2.2065	6.44E-05	4.191	A0A173G7D5	Lectin
EKIPGTEQS	0.18024	-2.472	6.98E-05	4.156	O04118; C4TP26; O04117; G3KBC0	Dehydrin 2/3/b
VNGVAPGPI	0.23603	-2.083	0.00013234	3.8783	UPI0001AFFF95	
KEDEDEDEEEEE	0.15872	-2.6555	0.00018471	3.7335	O24294	Legumin (Minor small)
YLGGNPETEFPET	0.36088	-1.4704	0.00037225	3.4292	Q41032	Legumin J (Fragment)
EQSPGQWRPSHGKEEDEEEKEQ	0.10465	-3.2563	0.00059444	3.2259	Q9M3X8; B0BCI8; B0BCI9; B0BCI6; B0BCI5; B0BCI7	Convicilin (Fragment)
FEITPEKNPQLQDLDIFV	0.2405	-2.0559	0.0018019	2.7443	Q84UI0; Q84UI1	Allergen Len c 1.0101/2
TFFIAPVDTKPQTGGGY	0.39012	-1.358	0.0022047	2.6567		Lectin
RSDQENPFIFKSNRFQTLYE	0.33535	-1.5762	0.0023649	2.6262	D3VND9; D3VNE1; D3VNE2; Q702P0; Q702P1; Q43626; Q9M3X5; D3VND7	Vicilin 47 K (fragment); Beta-lathyrin 1 (Fragment)
TLFLPQYTDADFILV	0.16584	-2.5921	0.0026436	2.5778	Q84UI0; D3VND9; D3VNE1; D3VNE2; Q702P1; Q43626; Q9M3X5; D3VND7; A0A3G5BED8	Allergen Len c 1.0102 (Fragment); vicilin 47k); Beta-lathyrin 1 (Fragment)
LIETWNPNN	0.39465	-1.3413	0.0027119	2.5667	Q03971; Q41676; Q99304; Q9T0P5	Legumin (Minor small); Legumin A; N-terminal incomplete
FEITPEKNPQLQDLDIFVNS	0.29086	-1.7816	0.0027344	2.5631	Q84UI0; Q84UI1	Allergen Len c 1.0101/2
AFNTEYEEIE	0.3445	-1.5374	0.0035441	2.4505	Q84UI1	Allergen Len c 1.0101 (Fragment)

IESEGGLIET	0.3949	-1.3404	0.0070999	2.1487	Q03971; Q41676; Q99304; Q9T0P5	Legumin (Minor small); Legumin A; N-terminal incomplete
LTETWNPNHPE	0.47774	-1.0657	0.0076637	2.1156	O24294; Q43674; Q41703; Q43671	Legumin (Minor small); Legumin B; Storage protein
DALEPDNRIESEGGLIET	0.38794	-1.3661	0.0096825	2.014	Q03971; Q41676; Q99304; Q9T0P5	Legumin (Minor small); Legumin A; N-terminal incomplete
SLNTKYDTIEKVLL	0.37843	-1.4019	0.021069	1.6764	Q9M3X8	Convicilin (Fragment)
RPSHGKEEDEEEKEQ	0.42595	-1.2313	0.047985	1.3189	Q9M3X8; B0BCI8; B0BCI9; B0BCI6; B0BCI5; B0BCI7	Convicilin (Fragment)
FEITPEKNPQ	0.22244	-2.1685	0.050636	1.2955	Q41677; Q84UI0; Q84UI1	Vicilin; Allergen Len c 1.0101/2
FLPQYTDADFILVVLSGK	0.49844	-1.0045	0.055818	1.2532	Q84UI0; D3VND9; D3VNE1; Q702P1; Q43626; Q9M3X5; D3VND7; A0A3G5BED8	Allergen Len c 1.0102 (Fragment); vicilin 47k; Vicilin (Fragment); Beta-lathyrin 1 (Fragment)
TLNEVVPLKDVVPEWVRIGF	0.35245	-1.5045	0.094155	1.0262	A0A173G7D5; Q8RW33; Q703U3;Q703U2; Q93X42	Lectin
FFEITPEKNPQLQDLDIFVN	2.2031	1.1395	0.097972	1.0089	Q84UI0; Q84UI1	Allergen Len c 1.0101/2
AGEEDNVISQIQRPV	2.4903	1.3163	0.098794	1.0053	Q84UI0; Q84UI1; A0A3G5BED8	Allergen Len c 1.0102 (Fragment); Allergen Len c 1.0101

Supplementary Table 2. Up and downregulated peptide sequences among the 229 bioaccessible peptides present in all samples (volcano-plot). Data were auto-scaled. Missing values were estimated by 1/5 of the minimum positive value of each variable.

Protein IDs	Origin	Majority protein IDs	Fasta headers
Q84UI1	Lens culinaris	Q84UI1	Allergen Len c 1.0101 (Fragment)
Q9M3X8; B0BCI8; A0A6G7K501; A0A6G7K4Z2; A0A6G7K4X8; A0A6G7K4W7; A0A6G7K4V3; A0A6G7K4U8; A0A6G7K4T7; A0A6G7K4V9; B0BCL7; B0BCK1	Lens culinaris	Q9M3X8	Convicilin (Fragment)
Q84UI0	Lens culinaris	Q84UI0	Allergen Len c 1.0102 (Fragment)
Q03971	Vicia faba	Q03971	N-terminal incomplete legumin A1 pre-pro-polypeptide (Fragment)
Q41703	Vicia sativa	Q41703	Legumin B
Q41677	Vicia narbonensis	Q41677	Vicilin
Q41674; B0BCL6; B0BCL8; B0BCL3; B0BCK3	Vicia narbonensis	Q41674; B0BCL6	Convicilin; Convicilin (Fragment)
A0A173G7D5; Q8RW33; Q703U3; Q703U2; Q93X42; B5A8N6; I3VZ38; Q8RVW9; Q8RVW8; E2IPT8; E2IPT7; E2IPT6; E2GEP6	Lens culinaris	A0A173G7D5	Lectin

D3VND9; D3VNE1; D3VNE2; Q702P0; Q702P1; Q43626; Q9M3X5	Pisum sativum	D3VND9; D3VNE1; D3VNE2; Q702P0; Q702P1; Q43626; Q9M3X5	Vicilin 47k; Vicilin (Fragment);
O24294; Q43674; REV__Q99304	Pisum sativum; Vicia faba	O24294	Legumin (Minor small)
B0BCI9; B0BCI6; B0BCI5; B0BCI7	Lens culinaris	B0BCI9; B0BCI6; B0BCI5; B0BCI7	Convicilin (Fragment);
A0A3G5BED8	Lathyrus sativus (White vetchling)	A0A3G5BED8	Beta-lathyrin 1 (Fragment)
Q41032	Pisum sativum	Q41032	Legumin J (Fragment)
Q40117	Lens culinaris	Q40117	Vicilin type C (Fragment)
O49927; Q9AVP7	Pisum sativum; Vicia faba	O49927	p54 protein
I0B569	Vicia faba	I0B569	Vicilin
O04118; C4TP26; O04117; C4TP27; G3KBC0	Pisum sativum; Vicia monantha;	O04118; C4TP26; O04117; C4TP27	Dehydrin 3; Dehydrin a; Dehydrin 2; Dehydrin b
Q43671	Vicia faba	Q43671	Storage protein
Q8S4X3	Pisum sativum	Q8S4X3	TIR-similar-domain-containing protein TSDC
Q99304	Vicia faba	Q99304	Legumin A2 primary translation product
B0BCK0	Lathyrus hirsutus (Rough pea) (Hairy vetchling)	B0BCK0	Cvc protein (Fragment)
B0BCJ3	Pisum sativum	B0BCJ3	Convicilin (Fragment)
D3VND7	Pisum sativum	D3VND7	Vicilin 47k
Q41676; Q41036	Vicia narbonensis; Pisum sativum	Q41676	Legumin A
Q9T0P5	Pisum sativum	Q9T0P5	LegA class
Q8H1A6	Pisum sativum	Q8H1A6	Heat shock protein
UPI0001AFFF95	*L. plantarum*	UPI0001AFFF95	Putative oxidoreductase YghA
O24295; O24470	Pisum sativum	O24295; O24470	Lipoxygenase
Q9AT20; Q9AT19; Q9AT18; Q84NF8	Lens culinaris	Q9AT20; Q9AT19; Q9AT18; Q84NF8	Histone H1 (Fragment)
Q41035		Q41035	Minor legumin (Fragment)
Q9LKJ2; A0A650DSK7; A0A650DSI7; A0A650DSH2; A0A650DSG1; A0A650DSF1; A0A650DSF0; A0A650DS79; A0A650DS68; A0A650DS58; A0A650DS49; A0A650DS38; A0A650DS12; A0A650DRR7	Lens culinaris	Q9LKJ2	Phosphoglycerate kinase
UPI00000109F0	*L. plantarum*	UPI00000109F0	60 kDa chaperonin
UPI0000010A23	*L. plantarum*	UPI0000010A23	Glyceraldehyde-3-phosphate dehydrogenase
A0A6G8QS08	*Vavilovia formosa*	A0A6G8QS08	Hypothetical chloroplast RF21
A0A7H0S597	Pisum sativum	A0A7H0S597	FAX3
UPI00019F5083	*L. plantarum*	UPI00019F5083	Cupin domain protein
A0A1S6R6N4	Pisum sativum	A0A1S6R6N4	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)
Q9ZRW9; Q9SB19; Q9ZRI6; Q39257	Vicia faba; Pisum sativum	Q9ZRW9; Q9SB19; Q9ZRI6; Q39257	Polyubiquitin; Ubiquitin
UPI0001B0024B	*L. plantarum*	UPI0001B0024B	helix-turn-helix domain-containing protein
A2ICP9	Pisum sativum	A2ICP9	GTP-binding nuclear protein
Q8H1A4	Pisum sativum	Q8H1A4	DEAD box RNA helicase
UPI0000010B06	*L. plantarum*	UPI0000010B06	Energy-coupling factor transporter transmembrane protein EcfT

Q9M3X6; B0BCJ7; B0BCJ8; B0BCJ6; B0BCK4	Pisum sativum; Lathyrus sativus (White vetchling)	Q9M3X6; B0BCJ7; B0BCJ8; B0BCJ6; B0BCK4	Convicilin; Cvc protein (Fragment); Convicilin (Fragment)
A0A0K0L1U6	Pisum sativum Lathyrus sativus (White vetchling)	A0A0K0L1U6	Alpha-1,4 glucan phosphorylase (Fragment)
A0A2L1J0R5; Q5DWE8	Pisum sativumLathyrus sativus (White vetchling)	A0A2L1J0R5; Q5DWE8	Superoxide dismutase (Fragment); Superoxide dismutase [Cu-Zn]
A3F6K1	Pisum sativum	A3F6K1	Tumor protein-like protein (Fragment)
Q6R6M7	Pisum sativum	Q6R6M7	Isocitrate dehydrogenase [NADP]
Q712V4	Pisum sativum	Q712V4	Histone H3 (Fragment)
Q8LSN7; Q8LSN6; Q9SQJ3; Q9SQF9; Q76KV8	Pisum sativum	Q8LSN7;Q8LSN6;Q9SQJ3 ;Q9SQF9;Q76KV8	Short-chain alcohol dehydrogenase SAD-C (Fragment);Short-chain alcohol dehydrogenase SAD-A (Fragment);Short-chain alcohol dehydrogenase;
Q9SM58	Pisum sativum	Q9SM58	Ketol-acid reductoisomerase
Q9T0N0	Pisum sativum	Q9T0N0	14-3-3-like protein
UPI0000010894	*L. plantarum*	UPI0000010894	Acetate kinase
UPI0000010E67	*L. plantarum*	UPI0000010E67	Elongation factor Tu
UPI00019F4F08	*L. plantarum*	UPI00019F4F08	Acid shock protein; heat shock protein; Hsp20/alpha crystallin family protein
UPI0001B00021	*L. plantarum*	UPI0001B00021	Glutathione biosynthesis bifunctional protein GshAB
UPI0004886462	*L. plantarum*	UPI0004886462	MFS transporter

Supplementary Table 3. Parental proteins of the identified peptides.

CHAPTER 5

Microbial fermentation improves aroma and decreases flatulence factors while preserving the potential prebiotic functionality of yellow pea and green lentil.

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Abstract

Legumes are an excellent source of nutrients, bioactive compounds and dietary fibers, and ideal candidates for the development of prebiotic and functional foods. However, beany off-flavour and flatulence remain major limitations to their adoption in Western diets. Lactic acid fermentation provides the opportunity to improve flavour and digestibility of plant ingredients. Additionally, the ability to convert raffinose family oligosaccharides (RFOs) to other classes of oligosaccharides of certain bacterial strains provides the opportunity to decrease the flatulence formation while retaining the prebiotic functionality. In this study, we investigated the ability of *Lactobacillus plantarum* to ferment green lentil and yellow pea flours for up to 96 h, and its implications for aroma, RFOs profile and potential prebiotic effect. We observed that moderate fermentation of green lentil for 24 h to 48 h had the dual ability to significantly decrease the RFOs while improving the aroma profile via depletion of the compounds responsible of grassy, beany off-flavour and formation of compounds conferring pleasant aroma attributes. Moreover, we observed an increase in beneficial commensal bacteria such as members of the Lactobacillus spp. and Roseburia spp. that was retained in the 48 h pre-fermented flour. In yellow pea, RFOs were efficiently consumed during the initial 24 h of fermentation, but the effect on the aroma was not straightforward. In fact, while important off-flavour compounds were depleted, other unpleasant molecules were formed. Overall, we provided evidence that a mild fermentation with *L. plantarum* can be an effective bioprocessing approach to improve the acceptability of green lentil and yellow pea, and to promote their inclusion in staple foods and daily diets.

Keywords: green lentil, yellow pea, *L. plantarum*, beany flavour, bifidogenic, raffinose family oligosaccharides.

1. Introduction

Legumes are an excellent but still underutilized source of proteins, minerals, vitamins, phytochemicals and prebiotic carbohydrates. While traditionally included in diets of Southeast Asia, Sub-Saharan Africa and Latin America, their inclusion in Western diets remains low. Moreover, the global westernization of diets and economic transition has led to an overall decrease in legume consumption.[1,2] Despite their high nutritional value, legumes have historically been considered as the "protein of the poor man" in the Asian culture, and they are erroneously perceived as a low quality food source in the Western world.[2] The associated intestinal discomfort, limited knowledge of food applications, longer cooking time and the presence of beany off-flavour are additional obstacles to their adoption in the Western world.

With the surge in cardiovascular and metabolic diseases and a recognized urgency to transition towards plant-based protein sources, the attention of government and the scientific community has been re-directed towards legumes consumption in recent years. Particular attention has been given to pulses, which are legumes used for their dry seeds only, such as lentils and peas. *In vitro* and clinical evidence show a clear link between legume consumption and decrease in blood lipids, LDL-cholesterol and body weight, and increase in satiety, therefore decreasing the risk factors for metabolic syndrome.[3–6] Improved glycaemic control in diabetic people and decrease in risk factors for cardiovascular disease were also observed.[3,4] However, the increase in scientific evidence has yet to result in a shift in consumer trends towards pulse consumption.[1] A major obstacle for legume adoption is the flatulence generated during gut fermentation. About 25 to 65% of the sugar fraction of legumes is constituted of raffinose family oligosaccharides (RFOs), which can produce gases in the gut and therefore lead to flatulence.[4] RFOs are sucrose-based carbohydrates composed of galactose residues connected to the glucose moiety of sucrose via α-1,6- glycosidic linkages. These are synthesised by the plant starting from sucrose and stored in the seeds as reserve carbohydrates, stress protectants (heat, cold, salinity, drought) and transport sugars.[7,8] The synthesis involved the initial formation of galactinol (Gol), donated to sucrose for the formation of raffinose (Suc-$[Gal]_1$). Further addition of galactosyl units leads to the formation of stachyose (Suc-$[Gal]_2$) and verbascose (Suc-$[Gal]_3$[7,8] Plants accumulate RFOs mainly in storage organs upon maturity and desiccation. While the amount of RFOs can vary according to genotype and growing environment, they are the major non-digestible oligosaccharide in pea and lentil seeds, accounting for 2.3-9.6% and 1.8-7.5% of the seed weight, respectively.[9] When needed, plant α-galactosidase sequentially catalyses the depolymerisation of RFOs to sucrose. Since humans and monogastric animals lack the enzyme α-galactosidase, RFOs pass undigested through the small intestine and reach the colon in large amount, where they are fermented by gut bacteria with accumulation of CO_2, hydrogen and methane. This is the major cause of discomfort, abdominal pain and cramps often experienced upon consumption of pulses, which limits their consumption in large amounts. At the same time, the oligosaccharide fraction of pulses is also a major contributor to their prebiotic functionality. In fact, the colonic microbiota adapts to an environment rich in oligo- and polysaccharides being *Bifidobacteria* and *Lactobacilli* among the beneficial species that can benefit from RFOs consumption. In fact, substrates that selectively enhance their growth conferring a health benefit are considered prebiotic.[10] Previous studies on legumes and legume-derived oligosaccharides have shown contrasting results in this respect, suggesting that the prebiotic functionality is greatly affected by the type of legume, the fraction used and its pre-treatment.[11–16]

The second major limitation to pulse consumption is the flavour. Beany, earthy, green, grassy and muddy are typical off-flavours described for lentils and peas.[17] These flavours are mainly related to oxidation of unsaturated fatty acids, or to compounds generated during heating. Compounds such as hexanal, 1-hexanol, 1-penten-3-ol, (E)-2-nonenal, 3-methyl-1-butanol and 1-octen-3-ol were previously identified as contributor of the beany off-flavour of pulses.[17,18] Lactic acid bacteria is traditionally used to ferment a wide range of plant material, with formation of specific aroma profiles based on the substrate and the microbe used. Interestingly, certain lactic acid bacteria have been reported to contain the pathway needed for RFOs metabolism. *L. plantarum* is an abundant bacterium on the surface microbiota of legumes, suggesting its adaptation to this niche and that it could therefore be a good candidate for legume fermentation. In line with this hypothesis, the *melA* locus and all the related genes involved in α-galactosidase metabolism were characterized in *L. plantarum* ATCC 8014.[19] We therefore considered that *L. plantarum* fermentation of lentils and peas could have a dual beneficial effect of decreasing the flatulence forming RFOs on one hand, and improving their aroma profile on the other hand.

The aim of this study was to assess how the microbial fermentation of green lentil and yellow pea flour with *L. plantarum* would affect the aroma profile, assessed by chromatographic quantification of the volatile compounds present in the flour and the ones generated with microbial fermentation, and the content and profile of the major RFOs raffinose, stachyose and verbascose. Moreover we *in vitro* digested (INFOGEST) the fermented samples and subsequently performed colonic batch fermentation to evaluate the short-chain fatty acids (SCFA) production in presence or absence of RFOs from the lentil flours. Finally, we used a complex continuous fermentation model of the proximal colon to evaluate the impact of the L*. plantarum* "pre-fermentation" of lentil flour on its potential prebiotic activity. The impact on the bacterial communities was analysed by 16sRNA metagenomic sequencing and SCFA analysis.

2. Material and Methods

2.1 Preparation of bacterial inoculum and solid-state fermented green lentil and yellow pea flours

Lactobacillus plantarum (ATCC® 8014™) was propagated in MRS broth and stored at -80°C in cryovials containing 30% (v/v) glycerol. Bacterial culture was sub-cultured twice in MRS broth at 37°C for 16 h anaerobically, harvested by centrifugation (10 000 *g*, 10 min) and washed twice with 0.9% saline solution before being used as inoculum. Green lentil (*Lens culinaris*) and yellow pea (*Pisum sativum*) seeds were ground to flour in a commercial blender (Waring®, USA) and passed through a commercial 40 mesh sieve. The legume flour was mixed with equal amount (w/v) of sterile distilled water and inoculated with the starter culture, with an initial cell density of 7.0 log CFU/g of flour paste. Solid-state fermentation (SSF) was carried out in triplicate for 0, 24, 48, 72 and 96 h at 37°C, with a constant shaking of 120 rpm. At the end of the fermentation, SSF flour was aliquoted for volatile analysis and stored at -20°C till analysis. The remaining SSF flour was freeze-dried and stored at -20°C before simulated gastrointestinal digestion. The efficacy of the fermentation was evaluated by means of bacterial enumeration on MRS agar, titratable acidity (TTA) and pH as previously reported.[20,21]

2.2 Static *in vitro* digestion

Simulated gastrointestinal digestion was performed according to the INFOGEST standardized static *in vitro* digestion method.[22] All reagents were pre-warmed and maintained at 37°C during the entire digestion, while the enzyme solutions were kept on ice. The digestion consisted of three consecutive

phases (oral, gastric, intestinal). For the oral phase, 5 g of freeze-dried sample were mixed to 3.5 mL of simulated salivary fluids (SSF) stock solution, 0.5 mL of salivary α-amylase solution (1500 U/mL, 25 µL $CaCl_2$ (0.3 M), 0.975 mL distilled water, and incubated at 37°C for 2 min. For the gastric phase, 10 mL of oral bolus were mixed to 7.5 mL of simulated gastric fluids (SGF)stock solution, 1.6 mL of pepsin solution (25000 U/mL) 5 µL $CaCl_2$ (0.3 M) and the pH was adjusted to 3 using 3 M and 1 M HCl solution. The total volume was then brought to 20 mL with distilled water, and shake-incubated at 37°C for 2 h, 90 rpm. For the intestinal phase, 20 mL of gastric chyme were mixed with 11 mL of simulated intestinal fluids (SIF) stock solution, 5 mL of pancreatin solution (800 U/mL), 2.5 mL fresh bile (160 mM), 40 µL $CaCl_2$ (0.3 M) and the solution was adjusted to pH 7 using 3 M and 1 M NaOH solution. The total volume was then brought to 40 mL with distilled water, and shake-incubated at 37°C for 2 h at 90 rpm. Salivary α-amylase solution was prepared in SSF, pepsin solution was prepared in SGF, pancreatin solution was prepared in SIF, and fresh bile was prepared in distilled water.

2.3 Volatile compounds analysis

Four grams of the pulse flours were placed in a 20 mL glass vials sealed with an aluminium cap with PTFE/silicone septa. The vials were incubated at 60^{o}C for 5 min in an autosampler heating block. The volatiles were then extracted with a DVB/CAR/PDMS SPME fibre inserted in the headspace for 50 min at 60 ^{o}C according to the method described by Xu et al. with modifications.[17] The SPME fibre was then injected in the GC-MS injector port and desorbed for 3 min at 250^{o}C. All the analyses were performed on a Trace GC Ultra combined with a DSQ II mass spectrometer (Thermo Scientific, Waltham, USA). The injection port was operated in splitless mode. Volatiles were separated on a ZB-Wax column (60 m × 0.25 mm i.d., 0.25 µm film thickness). Vials were agitated for 60 seconds prior extraction. SPME had extraction time of 50 min and desorption time of 10 min. Oven temperature program was set as follows: Initial temperature 40 °C, final temperature 200 ^{o}C, rate 10 ^{o}C/ min, hold time 5 min. PTV was operated in splitless mode, base temperature 250 ^{o}C, split flow 20 mL / min, splitless time 5 min, surge pressure 3 kPa. Helium carrier gas was used at a flow rate of 2 mL/min. The MS was operated in full scan mode, mass range 33.0 – 250.0, source temperature 225 ^{o}C, positive polarity, scan rate: 699.83.

The identity of the volatile compounds was determined considering three parameters: (1) the comparison of fragmentation profile with NIST 14 MS library and an RSI > 750; (2) the calculated retention index (RI), which were determined for every compound using a C7 to C40 saturated alkanes standard (certified reference material, 1000 µg/mL each component in hexane, Sigma Aldrich), then matched to the reference value extrapolated from the NIST 14 library; and (3) volatile compounds previously identified in literature for green lentil, yellow pea seeds or other foods, or from fermentation with *L. plantarum*.

2.4 RFOs extraction and composition analysis

The content of raffinose, stachyose and verbascose was measured in digested yellow pea and green lentil flour at different fermentation times. RFOs extraction was performed according to the method described in the commercialized RFOs quantification kit (Megazyme, Bray, Ireland) with minor modifications.[23] In brief, 250 mg of digest was combined with 25 mL of sodium acetate buffer (20 mM, pH 4.5) and allowed to extract for 15 min at room temperature. After extraction, 10 mL of suspension were centrifuged (1000 *g*, 10 min). The supernatant was then filtered through a 0.45 µM membrane filter and stored at 4^{o}C until chromatographic analysis. Standards were prepared in the same buffer at a concentration of 5-75 µg/mL.

An ICS-5000 Ion Chromatography HPLC system (ThermoFisher Scientific, Waltham, MA, USA), equipped with a Dionex CarboPac PA-1 column (2 × 250 mm) in combination with a Dionex CarboPac PA-1 guard column (2 × 50 mm) was used for the separation of carbohydrates and oligosaccharides at 30°C. For detection of components, the system was equipped with a pulsed electrochemical detector in pulsed amperometric detection mode. Raffinose, stachyose and verbascose were used as standards and separated with aid of the Dionex CarboPac PA-1 column. A flow rate of 0.25 mL min−1 was applied, and the column was equilibrated with 21 mM NaOH. Elution was performed by mixing eluent A (500 mM NaOAc in 100 mM NaOH), eluent B (100 mM NaOH) and eluent C (H2O) as follows: 0 min, (0:21:79); 20 min, (0:21:79); 20.1 min, (0:50:50), 50 min, (0:40:60); 70 min, (25:50:25); 70.1 min, (0:21:79); 90 min, (0:21:79). All analysis were performed in duplicate. Data were processed using Chromeleon 7.2 sr5® software (Thermo Scientific, Waltham, MA, USA). Oligosaccharide concentrations were expressed as µg/mL extract.

2.5 *In vitro* colonic batch fermentation

In vitro batch fermentation was performed as previously described.[24] In brief, 43 mL of sterile buffered colon medium (5.22 g/L K2HPO4, 16.32 g/L KH2PO4, 2 g/L NaHCO3, 2 g/L yeast extract, 2 g/L peptone, 1 g/L mucin, 0.5 g/L L-cysteine HCL, 2 mL/L tween-80) were combined to 20 mL of sample (0.6 g digesta obtained as described in 2.4 dissolved in sterile distilled water) in glass penicillin bottles. The bottles were then covered with a rubber cap, sealed with an aluminium ring, and flushed with nitrogen for 30 minutes in order to create and anaerobic environment. A 7 mL of microbiota inoculum (prepared form fresh faeces was then injected into each penicillium bottle, and the fermentation was carried out for 30 h at 37 ^{o}C, with constant shaking of 130 rpm. Samples were withdrawn during the fermentation for a maximum of 10 % (v/v) of the total volume. After collection, samples were centrifuged (5 min, 9000 *g*), and supernatant and pellet were stored separately for SCFA and bacterial sequencing respectively. The human faecal inoculum was prepared by combining 40 g of fresh faeces to 200 mL phosphate buffer (8.8 g/L K2HPO4, 6.8 g/L KH2PO4, 0.1 g/L sodium thioglycolate). The mixture was then homogenized into a stomacher for 10 min at speed 300, and centrifuged for 2 min at 500 *g*. The faeces were obtained from 2 healthy donors (one male and one female, aged 25-30 years old) who had not been treated with antibiotics for the previous 3 months, stored in anaerobiosis and used within 4 h after collection. All fermentations were repeated in duplicate. Gas production and pH were used both as quality control parameters, and for comparing the speed of fermentation when the different substrates were used. Gas production was assessed with a manometer, while the pH was measured with a pH meter.

2.6 Short chain fatty acids analysis

Short chain fatty acids (SCFA) concentrations were determined as described by Ladirat et al.[25] with minor modifications. Acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid and isobutyric acid were used as standard solutions (0.01-0.45 mg/mL in water). Supernatants collected from the *in vitro* colonic fermentation were thawed and filtered through a 15 cm 0.2µM RC filter. 50 µL of internal standard solution (0.45 mg/mL 2-ethylbutyric acid in 0.3M HCl and 0.9M oxalic acid) was added to 100 µL of the standard solution/ supernatants, mixed and transferred into GC vial. A Shimatdzu GC-2014AFSC system coupled with a flame-ionization gas detector (FID) (Shimadzu, 's-Hertogenbosh, The Netherlands) was used to quantify the SCFA levels by injecting 1 µL in a Restek Stabilwax column (30 m x 0.32 mm x 0.5 µm, T Max 240C, Restek, Santa Clara, CA, USA). Nitrogen was supplied as carrier gas at a flow rate of 10 mL/min. The pressure of air, H2 and N2 as makeup gas were 260, 30 and 30 mL/min, respectively. The initial oven temperature was 100^{o}C, increased to 180^{o}C at 8^{o}C/min, held at this

temperature for 1 min, increased to 200°C at °C/min and held at this temperature for 5 minutes. Data were processed using Chromeleon 7.2® software (Thermo Scientific, Waltham, MA, USA). SCFA concentrations were expressed as mmol/L supernatant.

2.7 *In vitro* continuous fermentation system of proximal colon

2.7.1 Fecal microbiota extraction and immobilization on gum beads

Fecal samples were provided by two healthy donors (30 - 40 years old) with no history of antibiotic use in the previous two months, and with body mass index was lower than the 95th percentile for age. Sample collection was approved by The University of Ottawa Research Ethics Board (certificate H-02-18-347; 29/07/2019). Within an hour of fecal collection, fecal microbiota was extracted by 2 g of feces, diluted in reduced peptone water (0.1 %, pH 7), homogenized with a stomacher and used for immobilization in a polymer solution of gellan (2.5% w/v) and xanthan (0.25% w/v) gums and sodium citrate (0.2 %, w/v) in anaerobic conditions, as previously described.[26]

2.7.2 *Ex vivo* model of large intestine fermentation

60 mL of gum beads with immobilized microbiota were used to inoculate a 1 L BioFlo® 120 vessel (Eppendorf, Mississauga, ON, Canada) containing 140 mL of Macfarlane culture medium prepared as previously described.[27] For the initial 48 h, the fermentation was performed in batch mode, by replacing 100 mL of the inoculating reactor (IR) medium with fresh one every 12 h. After 48 h, and to the end of the experiment, the system was set to continuous mode. The continuous fermentation system was then maintained in the IR for 15 days, which was previously shown to be the required time for the NuGUT system to stabilize the microbiota.[28] After 15 days, the IR was used to inoculate 3 DASGIP® bioreactors (CR, ER1 and ER2 in Fig.1, Eppendorf, Mississauga, ON) with a volume of 100 mL each. The system was then operated with the following settings: pH 5.7, 37 °C, 120 rpm stirring, 8 h retention time. The anaerobic environment was maintained by flushing of N_2 and CO_2 at a 0.9:0.1 ratio in the headspace. In each sub-reactor, fresh medium and IR stabilized inoculum were added at rates of 11.88 and 0.62 mL/h respectively, while the waste was removed at a rate of 12.5 mL/h, achieving a retention time of 8 h. The temperature of the IR was controlled with a heat blanket (Eppendorf, Mississauga, ON). The sub-reactors were allowed to stabilize for 48 h before addition of test samples. The CR sub-reactor was used as medium control and no additional test sample was added. ER1 was supplemented with unfermented lentil flour, ER2 was supplemented with 48 h solid-state fermented (SSF) and digested lentil flour. Both samples were prepared from the end product of the INFOGEST *in vitro* gastrointestinal digestion. The digestion product was separated in the supernatant and pellet fraction by centrifugation. The supernatant was filter sterilized, while the pellet was autoclaved with dry cycle. After sterilization, the two fractions were combined and re-suspended. 7 mL of the sterilized unfermented and 48 h SSF digesta were added to ER1 and ER2 respectively every 8 h for 3 days (72 h). Every 4 h, 2 mL of samples were withdrawn from IR, CR, ER1 and ER2, centrifuged (14000 *g*, 5 min) and stored at -80 till analysis. The pellet was used for DNA extraction and sequencing, while the

supernatant was used for short-chain fatty acids analysis. The experiment was conducted in duplicate for each donor.

2.7.3 Bacterial composition analysis (DNA extraction and 16sRNA sequencing)

DNA extraction was performed using a Fast DNA Spin Kit (MP Biomedicals, Solon, OH, USA) and a Bead Mill-24 Homogenizer (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer protocol, with modifications previously described.[28] The extracted DNA was quantified with a Qubit fluorometer (Invitrogen; Carlsbad, CA, USA) and stored at −20 ◦C until analysis. Sequencing was performed with a Illumina MiSeq platform (NuGUT Research Platform, University of Ottawa). The V3-V4 regions of the 16S rRNA gene were amplified using dual-barcoded primers, and the amplicon library was constructed according to the Illumina standard protocol. The amplicon libraries were pooled, and paired end sequencing was performed with 600 bp MiSeq Reagent Kit v3 (Illumina; San Diego, CA, USA) according

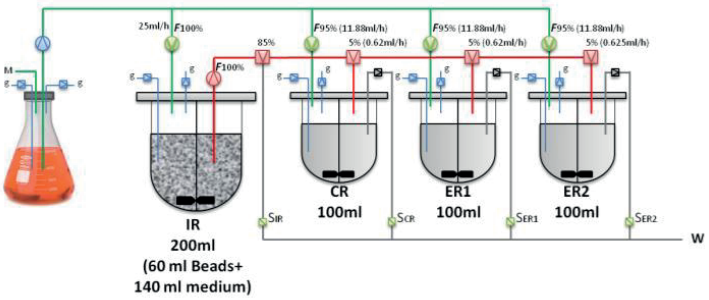

Figure 9. NuGUT Research Platform, University of Ottawa. From Mottawea et al. (2020)[28]

to the manufacturer protocol. Sequences were filtered for quality and clustered in operational taxonomic unites (OTU) based on 97%-similarity using closed-reference OTU picking against Greengenes database (v13.8) from Second Genome Inc. (South San Francisco, CA, USA) as the reference database via QIIME 1.9.0 software. Contaminant OTU were identified and removed if their mean abundance in negative and extraction reagents controls was ≥25% of their mean abundance in the samples.

2.8 Statistical analysis

For the volatile compounds, statistical analysis (principal component analysis [PCA], partial least square analysis, hierarchical clustering analysis) were performed with the online tool MetaboAnalyst (https://www.metaboanalyst.ca/) with the following parameters: data filtering = none, data scaling= auto scaling, and with R software and GraphPad Prism 9. For microbiome data, data analysis was performed with MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) using the following filtering steps: minimum count of 4 and 20 % prevalence in samples (low count filter), 10 % percentage to remove based on inter-quantile range (low variance filter). Data were normalized according to the following parameters: no rarefying, total cum scaling (TSS), no data transformation. Alpha-diversity was estimated with Chao1 and Shannon index. To identify the bacterial species and families significantly different among treatments, Mann-Whitney/Kruskal-Wallis test was used, using a p-value cut-off of 0.05.

3. Results and discussion

3.1 Efficacy of the solid-state fermentation

Efficacy of the solid-state fermentation was monitored by bacterial enumeration on MRS agar plates, pH and titratable acidity changes. Results are presented in Supplementary Fig. 1. The initial cell density after inoculation with the starter culture was 7.0 log CFU/g of flour paste. The bacterial population reached its maximum after 1 day (24 h) of fermentation, with a greater than 2 log increase for both green lentil and yellow pea flours. From day 3 of the fermentation (72 h), the viable bacteria showed a gradual decrease over time, which was sharper in yellow pea compared to green lentil. The pH decreased from an initial value of 6.4 to 4.0 within the first day (24 h), then remained stable until the end of the fermentation. The decrease in pH corresponded to an increase in titratable acidity, which was highest at day 2 (48 h) of fermentation. The increase in CFU and titratable acidity and decrease in pH were in line with values previously reported for other legumes.[29]

3.2 Volatiles composition

Volatile compounds identified in the fermented green lentil and yellow pea flours are presented in Table 1. In green lentils, 59 compounds were identified, and their peak areas were extracted based on the major m/z fragments. Unfermented samples were characterized mainly by aldehydes and alcohols such as hexanal, 1-hexanol, 1-heptanol, pyrrole, 1-penten-3-ol and (E)-2-nonenal, which sharply decreased with the fermentation. Microbial fermentation primarily drew the formation of esters such as acetic acid methyl ester, ethyl acetate and butyl acetate and benzyl acetate during the initial 24 h. Other aldehydes (decanal, benzaldehyde, 2methyl-propanal, 3-methyl butanal), ketones (2-butanone) and esters (isoamyl lactate) reached their maximum after 48 h fermentation. Extensive fermentation beyond the 48 h led to the increase in fatty acids, aldehydes and aromatic compounds with pungent, penetrating aroma, such as butanoic acid, octanoic acid, benzeneacetaldehyde, acetic acid, 2-methyl-butanal. Nonanoic acid was formed after 72 h. Other compounds such as nonanal, 2,3-butanedione, hexanoic and pentanoic acid were present in trace amounts in the unfermented samples but in constantly higher amounts in the fermented samples. To visualize the major changes in volatile compounds taking place during the fermentation, PCA and hierarchical clustering were used (Fig. 2 A, Supplementary Fig. 2). The PCA resulted in a clear separation of the samples based on their fermentation time. The unfermented samples (GL_t0) showed the furthest distance on PC1 with the samples fermented for 24 and 48 h (GL_t24, GL_t48). This was also the case for the hierarchical clustering, where unfermented samples showed the furthest Euclidean distance from all the other samples. Furthermore, 24 and 48 h (GL_t24, GL_t48) clustered closely on the PCA, and separated from the 72 and 96 h (GL_t72, GL_t96) on PC2 (Supplementary Fig. 2, A). Samples fermented for 72 and 96 h clustered in the top right part of the graph, separating from the 24 and 48 h fermented on PC2. 78% of variation could be explained by the first two principal components (PC), reaching almost 90% with PC3. To visualize the separation of the volatile compounds in relation to the fermentation time, loading plots were also included (Supplementary Fig. 2 B, D).

In yellow pea, 51 compounds could be identified (Table 1). The biological replicates showed considerable variation and were analysed by partial least squares-discriminant analysis (PLS-DA, Fig. 2B). A clear separation between unfermented (YP_t0) and fermented samples along component 1 could be observed. The 24 h (YP_t24) and 96 h (YP_t96) fermented samples clustered far from each other along component 2, while a less clear separation was observed for the 48 and 72 h (YP_t48, YP_t72) fermented samples. The 24 compounds drove the separation observed on the PLD-DA scores plot (VIP score > 1.0) are listed in Fig. 2C. Hierarchical clustering analysis, and a heatmap was used to visualize the major changes in volatile compounds taking place during the fermentation (Supplementary Fig. 3).

Similarly to green lentil, the unfermented samples showed high level of lipid oxidation and Strecker products, such as furan, 3-methyl and 3-mehtylbutanal. Linalool, also present at high concentration, was previously reported to accumulate in plants as defence mechanism from pathogen infection.[30] Microbial fermentation led to the formation of esters and fatty alcohols during the first 24 h, mainly ethyl acetate, 1-hexanol, 3-hexen-1-ol (Z) and pentanoic acid, 2-hydroxy-4-methyl-, ethyl ester. Extensive fermentation for 72 and 96 h led to the formation of aldehydic aroma compounds such as , benzeneacetaldehyde, 3-furaldehyde, benzaldehyde, hexanal, (Z)-2-heptenal. Methional, an important aroma compound derived from methionine degradation by *L. plantarum* or by light oxidation, also increased over time.[31,32]

L. plantarum is a facultative heterofermentative bacterium. In the presence of high glucose concentration and aerobic environment, the bacterium produces pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway. The EMP pathway employs NAD+ to produce energy; lactate dehydrogenase then converts pyruvate to lactic acid, with regeneration of NAD+. Pyruvate is also consumed via various other metabolic pathways, leading to mix acids formation.[33] During stationary phase, when the availability of glucose decreases, *L. plantarum* utilises the formerly produced lactic acid to form an equal quantity of acetic acid.[34] In fact, we observed acetic acid formation with prolonged fermentation time (Fig. 2A). Acetoin and 2,3-butanedione are produced via the butanediol fermentation pathway in *L. plantarum*, with α-acetolactate as intermediate.[35] Characteristic compounds contributing to the ripened aroma of lactic acid fermented foods derive from amino acid metabolism. The pathways involve multiple steps, initiated by transamination. Branched-chain amino acids (leucine, isoleucine, valine) metabolism leads to the formation of 2- and 3-methylbutanal, 2-methylpropanal, 3-methyl-1-butanol, 3-methylbu-tanoic acid and 2-methylpropanoic acid.[36–38] A pathway for phenylalanine conversion to benzaldehyde was previously described for *L. plantarum*. Phenylalanine was first enzymatically converted to phenylpyruvic acid, and finally to benzaldehyde via chemical oxidation.[39] Benzeneacetaldehyde and phenylethyl alcohol are also derived from phenylalanine metabolism.[40,41]

Altogether, unfermented green lentil and yellow pea samples were dominated by aldehydes and alcohols derived by lipid oxidation and Strecker products. Fermentation with *L. plantarum* concerned primarily carbohydrates and amino acids degradation, with major formation of esters and fatty alcohols in the initial 48 h and pungent aldehydes and aromatic compounds after 72 h. These were explained by the metabolic pathways previously described for *L. plantarum*.

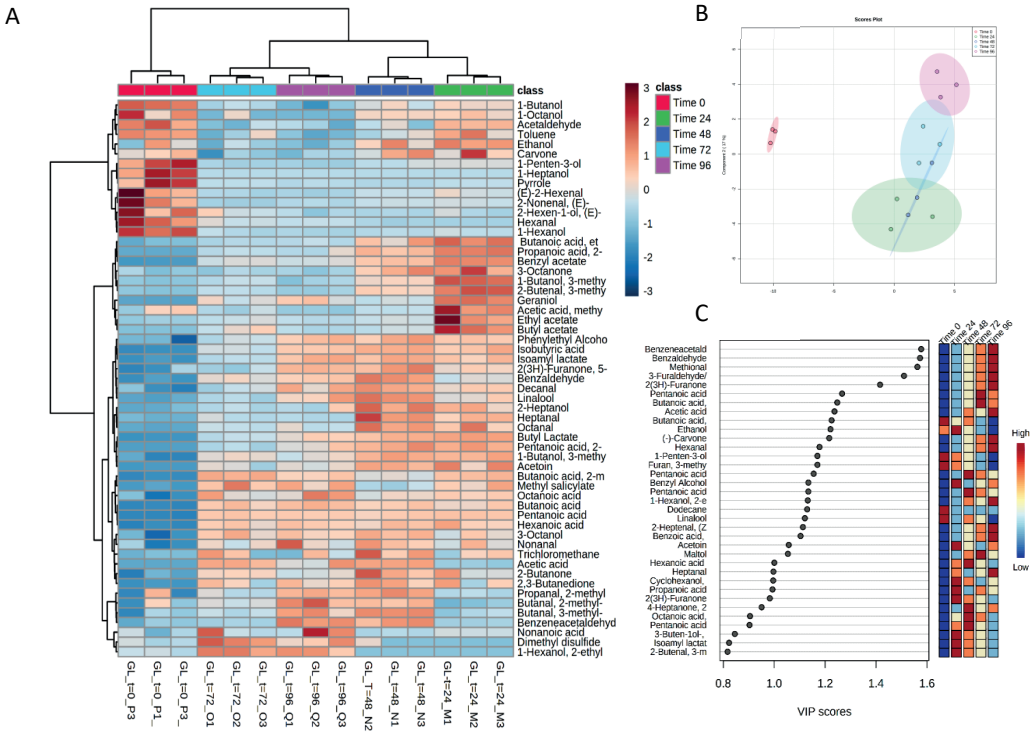

Figure 2. A: Hierarchical cluster dendrogram of volatile compounds detected by SPME-GC-MS during fermentation of green lentils and auto-scaled. The colour box indicates the abundance of each component; "class": extent of the fermentation (hours); "GL" = green lentils. B: PLS-DA analysis of volatile compounds during fermentation of yellow peas with L. plantarum detected by SPME-GC-MS and auto-scaled and (C) related VIP scores of the top 35 compounds for component 1. Every dot on the PLS-DA plot represent a biological replicate of the fermentation.

3.3 Beany flavour and aroma predictors

Of the volatile compounds identified, 40 in green lentil and 47 in yellow pea were previously described as aroma compounds (Table 1). The biplot in Fig. 3 shows the related clusters of aroma attributes resulting from PCA in green lentil and yellow pea, respectively. For green lentils (Fig. 3A), the aroma profile prior to fermentation appeared to be dominated by a green, beany flavour conferred by compounds 1 (acetaldehyde), 5 (hexanal), 23 (1-hexanol), 18 (1-penten-3-ol) and 13 ((E)-2-nonenal).[17,42,43]

Volatile fatty acids 1-hexanal and 1-hexanol derive from oxidation of unsaturated fatty acids linoleic and linolenic acid, catalysed by lipoxygenases.[17,44] Microbial fermentation with *L. plantarum* led to oxidation of 1-hexanal and (*E*)-2-hexanal and conversion to their corresponding volatile acids hexanoic acid and (*E*)-2-hexenoic acid, as previously reported for other *Lactobacillus* spp.[45](E)-2-nonenal is an unwanted aldehyde derived by fatty acid metabolism and present in the unfermented flour. Its conversion to nonanal was previously reported for other microorganisms and was observed in our study.[46] The substantial amount of acetaldehyde present in the unfermented flour likely originated as a result of the Strecker reaction, which could occur during grinding of the dry seeds.[47] Compounds 42 (toluene), 28 (1-heptanol) and 31 (1-octanol) further conferred off-flavours of pungent, skunky and aldehydic, respectively.[17,48] The aroma appeared to be improved at 24 to 48 h fermentation, with formation of compounds 45 (methyl ester-acetic acid), 50 (1-butanol, 3-methyl-, acetate), 7 (2-butenal,

3-methyl-), 49 (butyl acetate), 47 (butanoic acid, ethyl ester), 81 (geraniol), 19 (1-butanol, 3-methyl-) and 34 (phenylethyl alcohol) providing sweet, fruity aroma.[17,43,48–50] Additionally, typical products of lactic acid fermentation such as compounds 52 (propanoic acid, 2-hydroxy-, ethyl ester), and 63 (butanoic acid, 2-methyl-) conferred a buttery, cheesy flavour that is generally perceived as pleasant.[43,50] However, after 48 h, unpleasant compounds 64 (pentanoic acid), 66 (hexanoic acid), 12 (decanal) and 6 (heptanal) were also formed.[17,34,43,51,52] When fermentation was extended to 72 and 96 h, accumulation of unpleasant aroma compounds 2 (2-mehtyl-propanal), 4 (3-methyl-butanal), 78 (dimethyl disulfide), 44 (acetic acid), 11 (nonanal), 41 (butanoic acid), 68 (octanoic acid) and 69 (nonanoic acid) was observed.[17,34,43,48,50,52,53]

In contrast, the effect of fermentation on the acceptability of the aroma of yellow pea was not univocal (Fig. 3B). While important beany off-flavour compounds such as 18 (1-penten-3-ol) decreased with fermentation, others such as compounds 1 (acetaldehyde) and 23 (1-hexanol) were predominant in 24 and 48 h fermented samples.[17,42] Similar to green lentils, characteristic lactic acid aroma compounds were formed in the early stages of fermentation, such as 52 (propanoic acid, 2-hydroxy-, ethyl ester) and 76 (acetoin).[17,34] Extensive fermentation led to the accumulation of both pleasant and unpleasant aroma compounds.

In conclusion, microbial fermentation appeared to improve the aroma descriptors for green lentil flour in the initial 24 to 48 h, with decrease in compounds associated with the typical beany off-flavour and formation of compounds with sweet, fruity aroma descriptors. However, extensive fermentation to 72 - 96 h led to the accumulation of compounds with unpleasant aroma descriptors. In contrast, no clear pattern was observed for yellow pea flour.

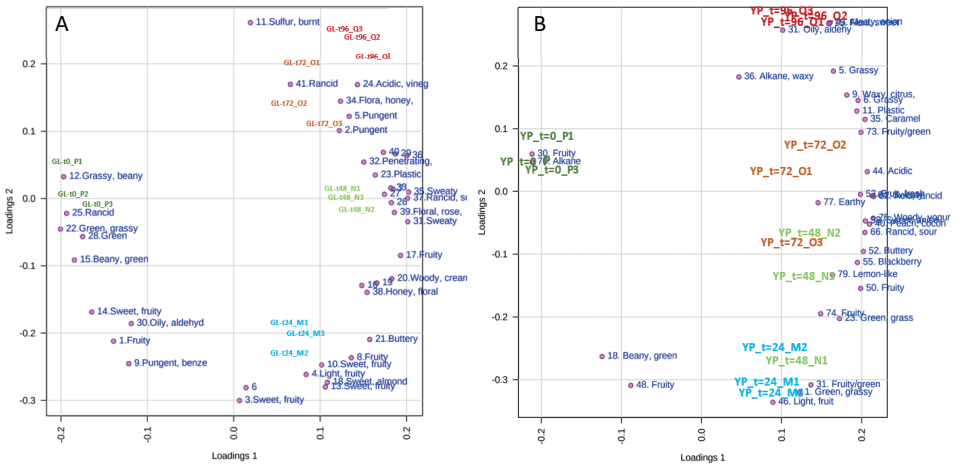

Figure 310. Biplot of aroma attributes of compounds identified by SPME-GC-MS during fermentation with L. plantarum of green lentils (A) and yellow peas (B).

3.4 RFOs analysis

Raffinose family oligosaccharides (raffinose, stachyose and verbascose) were quantified in digested green lentil and yellow pea fermented flours. Results are shown in Fig. 4. In both pulses, stachyose (Sta) was the dominant RFO, followed by verbascose (Ver) and raffinose (Raf). Digested green lentil flour had an initial content of 82.1, 40.9 and 13.8 µg/mL of Sta, Ver and Raf, respectively. Fermentation

for 24 h halved the initial RFO content, and the concentration continued decreasing over time. Ver was not detected after 72 h fermentation, while residual amounts of Sta and Raf were still detected at the end of the 96 h, although not in the quantifiable range of our standards. Digested yellow pea flour had an initial content of 74, 56.9 and 10.4 µg/mL of Sta, Ver and Raf, respectively. The degradation of Sta and Ver was faster than in the green lentil. In fact, in the first 24 h, Sta decreased by almost 90% and Ver was almost completely depleted. Sta was not detected after 72 h fermentation; residual amounts of Raf were still detected at the end of the 96 h, although not in the quantifiable range of the standards. For both pulses, Raf had a slower decrease over time, which can be explained by the fact that it has the smallest degree of polymerization among the RFOs. This implies that while some Raf molecules were metabolised by the bacterial enzymes, others were released by the hydrolysis of Sta and Ver. Interestingly, the speed of fermentation of Sta and Ver differed among the two pulses, being significantly faster in yellow pea compared to green lentils. This could be explained by the differences in macromolecular composition and matrix interactions of the two pulses. While showing similar protein and total carbohydrate content, green lentil has a higher phenolic content. Recent scientific evidence suggests the existence of chemical associations between polysaccharides and phenolic compounds in the food matrix.[54–56] Due to the weak nature of these chemical bindings, physiological and enzymatic changes happening during the gastric digestion or fermentation can disrupt some of these interactions.[54,57] Other complexes will instead remain intact, and decrease fibers and phenolic compounds bioaccessibility in the small intestine. Ferulic, sinapic, *p*-coumaric and caffeic acid were the major phenolics creating these chemical bounds with fibers in cereals.[58] In our previous study, we observed a significantly higher increase in the total phenolic content (TPC) in response to fermentation in green lentil compared to yellow pea flour.[21] Interestingly, one of the highest increase was observed for *p*-coumaric acid. Based on this, we could hypothesise that the higher TPC of green lentil implies that a greater amount of non-digestible carbohydrates is bound to them. The enzymatic activity of *L. plantarum* could therefore gradually disrupt these bindings, leading to the release of both the phenolic compounds and the fibers. This could explain on one hand the observed increase in TPC (and particularly *p*-coumaric acid), and on the other hand the slower fermentation of RFOs observed in green lentil compared to yellow pea flour (Supplementary Fig. 1).[21]

The digestion of RFO by *L. plantarum* requires the combined action of a raffinose transporter, an α-galactosidase and a sucrase.[59] The melA locus, containing the putative genes *galM*, *rafP*, *melA*, *lacM* and *lacL* appeared to be the primary involved in α-galactosidase metabolism.[19] The *melA* gene encodes for the α-galactosidase MelA, which hydrolyses terminal α-D-galactose residues from RFOs. The residual sucrose was then putatively metabolised by the sucrose-6-phosphate hydrolase encoded by the *ScrB* gene, which is present in the genome of our strain and was previously reported to be necessary for sucrose metabolism.[60,61] Since both enzymes are located in the cytoplasm of *L. plantarum*, a raffinose transporter is needed for the translocation of RFO in the cell. Silvestroni et al. (2002) suggested that this was primarily taking place via a putative raffinose transporter RafP, encoded by the gene *rafP* present in the melA locus.[19] Additionally, the lactose permease LacS encoded by the gene *lacS* was present in the genome of our strain, which also showed a certain affinity for raffinose transport, besides its primary substrate β-galactosidase and galactose.[59,60] Because of the intracellular localization, metabolism of RFOs is restricted by transport.[59] Moreover, oligosaccharide metabolism is affected by the type of carbohydrate and is suppressed by glucose.[19,59] When considering this, we can speculate that the faster RFOs consumption observed in yellow pea could be related to a higher availability of the oligosaccharides for enzymatic degradation by the bacteria, possibly due to a lower entrapment with phenolic compounds. The faster decline in CFU/mL observed when the bacteria was

growing on yellow pea rather than green lentil flour could therefore relate to the rapid initial bacterial growth (in the 24 h), causing a drastic change in carbohydrate content of the matrix and accumulation of inhibitory metabolites.

Taken together, the RFOs were substantially fermented by *L. plantarum* in both legumes during the initial 48 h. Sta and Ver were degraded faster in yellow pea compared to green lentil, possibly due to the difference in phenolic content of the two pulses, and were completely degraded within 72 h. In green lentil, Ver could not be detected after 72 h fermentation, while remaining amount of Sta persisted till the end of the fermentation. In both pulses, traces of Raf could still be detected after 96 h fermentation. The genetic makeup of *L. plantarum* ATCC8014 could explain the observed RFOs metabolism.

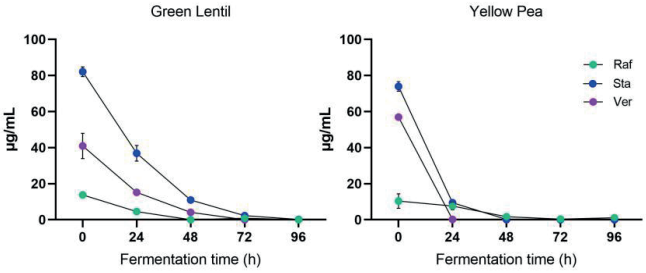

Figure 411.RFOs quantification in green lentil (A) and yellow pea (B) as a function of microbial fermentation time. Raf= raffinose; Sta= stachyose; Ver= verbascose. Each dot represents the mean value ± SD of quadruplicates (2 biological samples, each one digested twice with INFOGEST protocol).

3.6 Batch fermentation and short chain fatty acids production

In order to investigate the influence of the pre-treatment on the potential prebiotic functionality of the pulse flours, we performed *in vitro* batch fermentations with faecal inoculum obtained from two healthy donors. pH and gas production were recorded for quality control and results are reported in Supplementary Fig. 5. The pH showed a gradual decrease from an initial value of 6.5, and the 72 h pre-fermented flour led to a smaller decrease in pH when compared to the unfermented one. However, this corresponded to a slightly higher gas production of the pre-fermented flour samples compared to the unfermented. Colonic fermentation in samples supplemented with yellow pea flour led to a greater decrease in pH when compared to samples supplemented with green lentil flour.

Production of short chain fatty acids (SCFA) was measured after 0, 11 and 30 h of colonic fermentation. *In vitro* fermentation of legume flour led to a significant increase in total SCFA production (Fig. 5). Acetic acid, propionic acid and butyric acid were the most abundant, as previously observed during legume fermentation.[13,14,62] The ratio between the different SCFA showed little difference from the control sample, with the percentage of acetic acid decreasing in donor 1 and increasing in donor 2. On the contrary, the percentage of propionic acid increased in donor 1 and decreased in donor 2, when compared to their respective controls. When fermenting green lentil flour, butyric acid production was higher than in the control samples in both donors but decreased in the relative ratio. This result is in line with what was previously observed during *in vitro* colonic fermentation of a 50:50 lentil-chickpea flour, which led to an increase in acetic acid and propionic acid production, but decrease in butyrate.[12]

Differently, when yellow pea flour was used as substrate, a significant increase in butyric acid production was observed, which was more pronounced in donor 2. Previous studies on colonic fermentation of pea or pea fractions similarly reported an increase in butyrate production.[15,63] Butyrate production is favoured at a moderately acidic pH, which explains the increased production at the end of the batch fermentation (t=30 h). Propionate production was higher on the legume substrates compared to their respective controls but did not differ among legume samples. Isobutyric acid was present in lower amount but increased over time, especially on the yellow pea substrate.

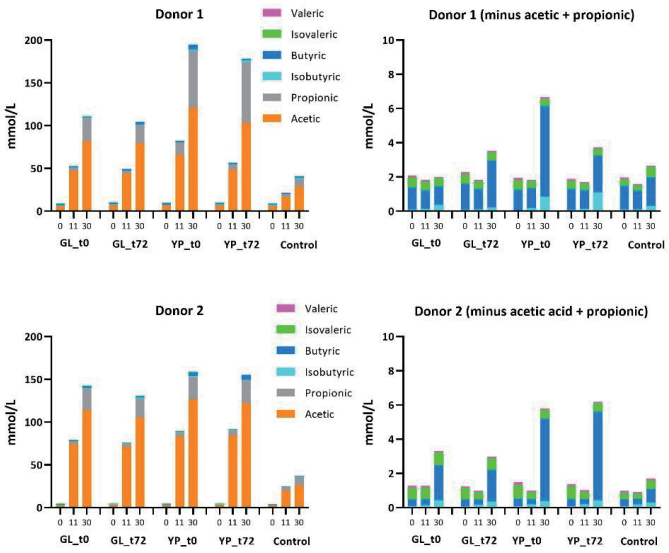

Figure 512. SCFA formation during colonic fermentation of green lentil and yellow pea fermented flour. Median values of three independent replicates.

3.5 Evaluation of prebiotic functionality of fermented green lentil flour in a continuous *ex-vivo* model of proximal microbiota fermentation

Based on the outcome of the volatile and RFOs analysis, we identified that fermentation with *L. plantarum* for up to 48 hours was sufficiently decreasing the RFOs content while improving the aroma profile of green lentil. The results from the batch fermentation further suggested that this pre-treatment was not compromising its beneficial activity on colonic fermentation, as measured by SCFA production. According to this, we selected the 48 h fermented green lentil sample as a promising candidate to test in the more complex *ex-vivo* system (NuGUT Research Platform) simulating the fermentation of the product by proximal colonic microbiota over a period of 3 days. The gut microbiota community was isolated from fecal samples of two healthy donors and immobilized in gellan-xanthan beads, used to inoculate the IR bioreactor. The population was allowed to stabilize over 15 days as previously discussed, then used to feed 3 sub-reactors. Test samples (lentil flour or 48h SSF lentil flour) were added every 8 hours at 1 % w/v concentration.

3.5.1 Microbial diversity

The alpha-diversity of the gut microbiota throughout the experiment and in response to the treatment with lentil or SSF lentil flour was assessed with Shannon and Chao1 indices of the identified OTUs (Fig. 6). The diversity was retained in all the samples, with a minor decrease in the control samples over

time. On the contrary, addition of lentil flour appeared to lead to a modest increase in alpha-diversity at all time points. This was the case also for the 48 h SSF lentil flour, which appeared to further improve the microbial diversity indices over prolonged fermentation time (48 to 72 h). Previous studies similarly showed an increase in fecal microbial alpha-diversity indexes in response to red lentil dietary intervention for three weeks in mice.[64]

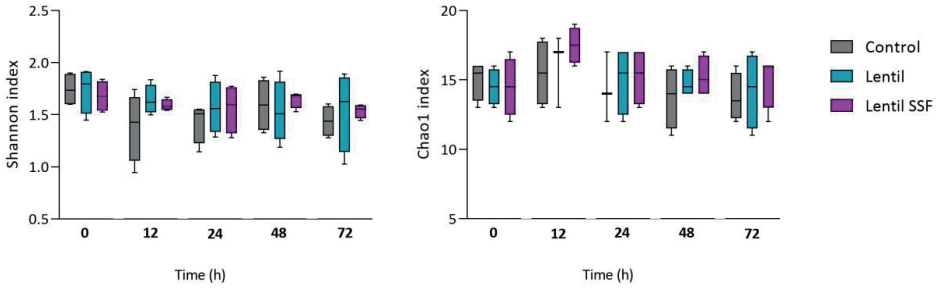

Figure 6. Alpha-diversity index and Kruskal-Wallis test. (A) Shannon diversity index. (B) Chao1 diversity index. Box & whiskers 5-95 percentile.

3.5.2. 16sRNA bacterial sequencing and SCFA production

Bacterial taxonomy, abundance and related short chain fatty acids production were analysed at time 0, 4, 8, 12, 24, 48, 72 h. The relative abundance of the sequenced microbiota population at phylum and family level is reported in Supplementary Table 1. At phylum level, both donors were characterized by Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria, but in different ratios. Over the course of the fermentation, the Actinobacteria and Bacteroidetes population decreased while the Firmicutes increased. At family levels, fermentation with both lentil flours led to an increase in Veillonellaceae, Lactobacillaceae, Ruminococcaceae and Lachnospiraceae (Fig. 7 A, B). Lactobacillaceae, Ruminococcaceae and Lachnospiraceae play a major role in hydrolysing starch and other sugars to provide butyrate and other SCFAs.[65,66] Moreover, Lachnospiraceae and Ruminococcaceae are highly specialized in digesting plant material by degrading hemicellulose and cellulose.[67] *Roseburia* spp., a member of the Lachnospiraceae family, has been described as one of the main butyrate-producing bacteria in this family. Particularly, the species *R. inulinivorans* activity in degrading starch, fucose, rhamnose, xylan, inulin and FOS has previously been described, with production of butyrate and propionate.[66] In our study, this species was profiled in the microbiota of both donors and showed a sharp increase in all samples until 48 h, while its relative abundance continued to increase until 72 h only in the lentil samples (both unfermented and fermented). Similarly, a significant increase in *R. inulinivorans* was previously described during colonic fermentation of lupin seeds and broad beans, and an increase in Lachnospiraceae family in rats fed pea fiber.[16,62] The Ruminococcaceae family increased more upon fermentation of SSF lentil flours compared to the unfermented one. Within the Veillonellaceae family, the species *Anaerosin glycerini* increased significantly only in the lentil samples (both unfermented and pre-fermented) in Donor 2, while species *Succinispira mobilis* showed the opposite trend (Supplementary Fig. 5). The relative abundance of Lactobacillus spp. also increased in the lentil samples (both SSF fermented and unfermented) only, in both donors, but was statistically significant only in Donor 2 (Supplementary Fig. 5, 6). A moderate increase in Bifidobacteriaceae could be observed after 48 h of fermentation of both unfermented and SSF lentil flour, compared to the control (Fig. 7A), with *B. breve* being the dominant species in all the

samples. *Bifidobacteria* are well-adapted to live and ferment oligosaccharide-rich environments and a relative increase was previously reported upon colonic fermentation of other legume flours.[59,68] The pre-digested lentil sample was used as a whole in this study, therefore simpler carbohydrates could still be present in it and could have favor the *Lactobacillus* populations over the *Bifidobacterium*. Lactobacilli are also among the proteolytic species in the colon, and the protein content of the lentil samples could have contributed to their increase.[69] This could explain the observed increase in Lactobacilli in both pre-fermented and unfermented lentil samples, despite the difference in RFOs profile. A limited increase in Enterobacteriaceae was also observed. A significant increase in Lactobacillus, Bifidobacterium and Enterobacteriaceae was previously observed during fermentation of cowpea and black beans.[14] The *Prevotella* genus was also previously reported to increase in response to consumption of legume-derived fractions (pea dietary fibers, bean soluble fraction, lupin seed, broad bean, chickpea-lentil flour), but did not increase in our study.[13,16,62,70,71] *Prevotella* is indeed a characteristic species of individuals adapted to long-term intake of carbohydrates and plant-based foods, in opposition to the Bacteroides, which characterizes protein and animal fat diets.[72] The absence of an increase in *Prevotella*, expected with plant food fermentation, could therefore be related to the lack of *Prevotella* spp. in the enterotype of the donors from our study.[73] Differently to what observed in the batch fermentation experiment, we did not observe a significant increase in SCFA in response to fermentation of the lentil flours. This can be related to the difference in the concentration of the lentil flour added for the experiment (1.2 % w/v in penicillium bottle of batch fermentation vs 1 % w/v in sub-reactor of NuGut), and to the intrinsic difference between the continuous fermentation system in which the waste products are continuously removed, and the closed batch fermentation system. Acetic and butyric acid were the most abundant, which differed from previous batch fermentation studies on pulses which showed higher production of acetic and propionic acid over butyric.[13,74] Higher concentrations of propionic acid were measured in the microbiota of donor 2, which can be related to its higher relative abundance of the butyrate-producing Lachnospiraceae family.

To conclude, both lentil flours showed a potential prebiotic effect, causing an increase in alpha-diversity of the gut microbiota, and the relative abundance of beneficial species such as *Lactobacillus* spp, *Bifidobacterium* spp., without increasing the abundance of harmful species. Differences in increase of the beneficial species could be observed between the two donors.

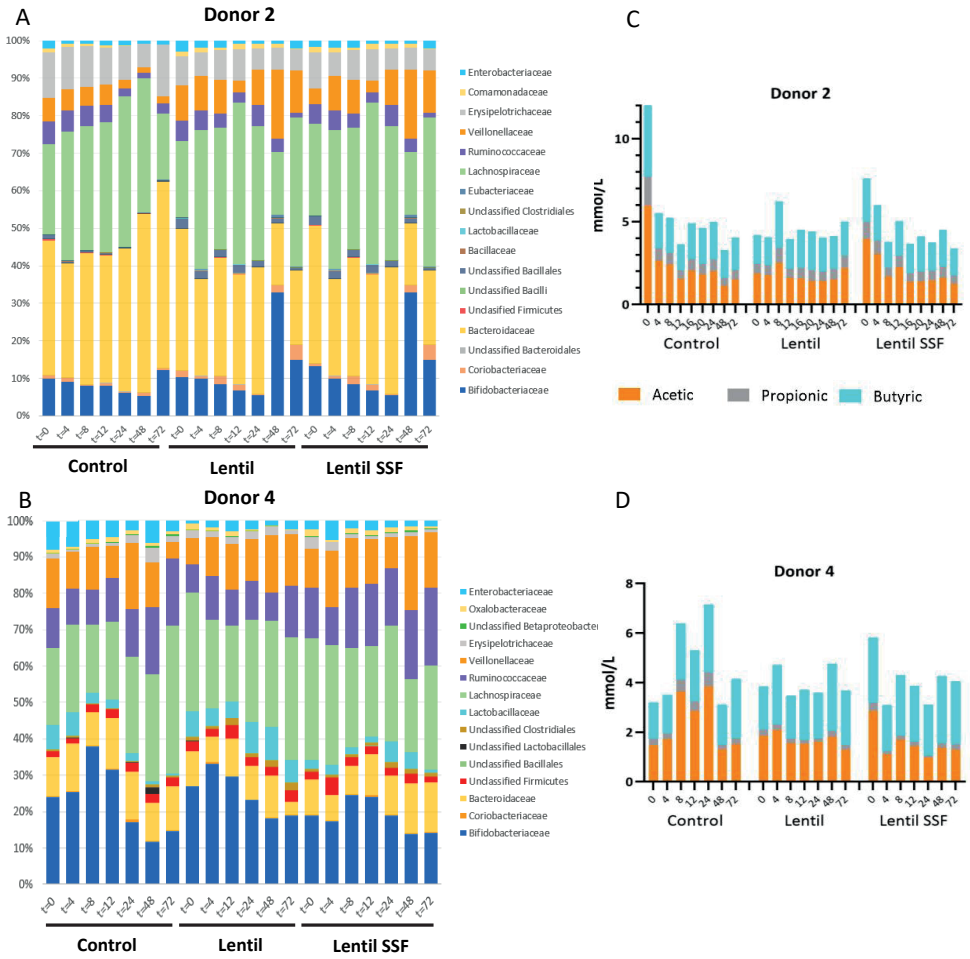

Figure 7. 16sRNA sequencing at family level (A, B) and related SCFA analysis (C, D).

Conclusions

Data here presented proved that a short fermentation of green lentil and yellow pea flour with *L. plantarum* was effective in depleting the flatulence forming oligosaccharides while retaining their beneficial functionality on colonic fermentation. The selective promotion of certain beneficial gut bacteria suggests that the fermented flours are a potential candidate for prebiotic designation. For green lentil, the bioprocessing additionally improved the aroma profile by significantly decreasing the volatile compounds conferring beany off-flavour. This bioprocessing offers a great opportunity for the development of healthy and sustainable novel foods, working towards the acceptance of pulse products in the daily diets of Western countries. A follow up human trial to assess the findings *in vivo* is recommended.

Conflicts of interest

The authors declare no conflicts of interest.

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Nº	Compound	GL / YP	Rt	Formula	MS Quantitation peaks	RI Calculated	RI Reference (NIST)	R Match	Previously identified sources	Aroma Description	Pathway Formation
	Aldehydes										
1	**Acetaldehyde**	GL, YP	2.20	C_2H_4O	44, 43, 42	N.D.	702 ± 12	GL:975 YP:989	Products fermented with L. plantarum (*C. mannii* cotyledons, wine, milk);[75–77] apple juices, spirits, wine, cider, cheese, yoghurt, ripened butter.[42]	Low levels: pleasant fruity aroma. High levels: pungent, green, grassy, apple-like off-flavour.	Carbohydrates metabolism
2	**2-Methyl-Propanal**	GL	3.08	C_4H_8O	43, 41, 72	811	819 ± 9	864	Milk, green thread tea, wheats, common grapes, other cereal products, and oxheart cabbages.[78]	Aldehydic, pungent, floral odor. Its odor is also described as that of wet cereal or straw.[43,53]	Amino acids metabolism;[79] Strecker aldehydes
3	**2-Methyl-Butanal**	GL	4.39	$C_5H_{10}O$	57, 41, 58	913	914 ± 8	880	Green lentil, yellow pea[80]	N.D.	Amino acids metabolism; Strecker aldehydes
4	**3-Methyl-Butanal**	GL, YP	4.45	$C_5H_{10}O$	44, 41, 43	918	918 ± 7	GL:909 YP:903	Green lentil, yellow pea, pea flour[18,80]	Apple-like, pungent, penetrating odour;[48] ethereal, aldehydic chocolate, peach or fatty odour;[43]	Amino acids (leucine) metabolism; Strecker aldehydes
5	**Hexanal**	GL YP	7.08	$C_6H_{12}O$	56, 44, 41	1099	1083 ± 8	GL:963 YP:952	Green lentil, yellow pea, pea flour;[18,80] products fermented with *L. plantarum* (cotyledon *C. mannii*, rice).[42,43]	Green pea, grass; marker of beany flavour in pulse flour.[17]	Fatty acids metabolism (fatty aldehyde)
6	**Heptanal**	GL, YP	8.79	$C_7H_{14}O$	70, 44, 57	1208	1184 ± 9	GL:864 YP:823	Green lentil, yellow pea, pea flour.[18,80]	Gives grassy, herbaceous aroma to fermented milk;[52] green, sweet.[51]	Fatty acids metabolism.[52]
7	**2-Butenal, 3-methyl-**	GL, YP	9.19	C_5H_8O	84, 55, 83	1235	1215 ± 13	GL:934 YP:896	A variety of foods (e.g. cereals, beans).[43]	Sweet, almond, and brown tasting compound.[43]	Lipid peroxidation or after oxidative stress.
8	**2-Hexenal, (E)-**	GL	9.42	$C_6H_{10}O$	41, 55, 69	1250	1216 ± 8	951	Pea flour.[18]	ND[18]	Linoleic acid degradation.[81]
9	**Octanal**	GL, YP	10.37	$C_8H_{16}O$	44, 43, 41	1314	1289 ± 9	GL:953 YP:947	Green lentil, yellow pea;[80] products fermented with *L. plantarum* (cotyledon *C. mannii*, rice).[35,77]	Fruit-like (green, orange, rose, coconut) odor and a waxy, citrus or fruity taste.[43]	Fatty acids metabolism (fatty aldehydes)

10	**2-Heptenal, (Z)-**	YP	11.04	$C_7H_{12}O$	43, 83, 41	1361	1322 ± 9	948	Yellow pea;[80,82] a variety of foods (oats, garden tomato, cucumbers).[43]	ND	
11	**Nonanal**	GL, YP	11.87	$C_9H_{18}O$	57, 41, 43	1421	1391 ± 8	934	Green lentil, pea flour;[17,18,80] products fermented with *L. plantarum* (rice).[35]	Plastic, citrus peel-like;[17] provides citrus, fatty aroma to milk fermented with *L. plantarum*.[52]	Fatty acids metabolism (fatty aldehydes)
12	**Decanal**	GL	13.29	$C_{10}H_{20}O$	43, 41, 57	1526	1498 ± 8	879	Products fermented with *L. plantarum* (rice);[35] legumes;[17] coriander, dill, and ginger, lime, sweet orange, lemon grass.[43]	Sweet, aldehydic, and citrus tasting.[43]	Fatty acids metabolism (fatty aldehydes)
13	**(E)-2-Nonenal**	GL	13.87	$C_9H_{16}O$	43, 41, 70	1571	1534 ± 10	915	Pea flour;[18] asparagus, carrots, corns, and lemons.[43]	Cardboard-like flavor (in beer aging); cucumber, fatty, and green.[43]	Fatty acids oxidation
14	**Benzaldehyde**	GL, YP	13.92	C_7H_6O	77, 105, 106	1575	1520 ± 14	GL:942	Green lentil;[80] products fermented with *L. plantarum*.[35,50,83]	Almond, sweet, woody.[17]	Amino acids (phenylalanine) metabolism
15	**Benzeneacetaldehyde**	GL, YP	15.35	C_8H_8O	91, 92, 120	1692	1640 ± 13	GL:938 YP: 952	Products fermented with *L. plantarum* (maize).[84]	Honey, sweat, fermented;[84] flora, honey, sweet.[17]	Amino acids metabolism; Strecker aldehydes
	Alcohols										
16	**Ethanol**	GL, YP	4.65	C_2H_6O	45, 46, 43	934	932 ± 8	GL:944 YP: 938	Products fermented with *L. plantarum* (cotyledon *C. mannii*, rice).[50,77]		Carbohydrate metabolism
17	**1-Butanol**	GL	8.16	$C_4H_{10}O$	56, 41, 43	1169	1142 ± 11	942	Green lentil, yellow pea;[80] highbush blueberry and bilberry, cabbage, wild rice, red rice, bamboo shoot, and lemon verbena.[43]	Sweet, balsam, and fruit tasting compound.[43]	Fatty acids metabolism (fatty alcohol)
18	**1-Penten-3-ol**	GL, YP	8.35	$C_5H_{10}O$	57, 41, 39	1180	1159 ± 10	GL:918	Green lentil, yellow pea, pea flour.[17,18,80]	Beany, green.[17]	Fatty acids (linoleic acid) metabolism
19	**1-Butanol, 3-methyl- (=Isoamyl alcohol)**	GL, YP	7.62	$C_7H_{14}O_2$	43, 70, 55	1134	1122 ± 7	GL:938 YP: 934	Products fermented with *L. plantarum* (cashew apple).[50]	Whiskey, fruity, banana;[17] whiskey, malt, burnt.[50]	Amino acids metabolism
20	**1-Pentanol**	GL, YP	9.75	$C_5H_{12}O$	42, 55, 41	1271	1250 ± 9	GL:863 YP:852	Pea flour, legumes;[17,18] products fermented with *L. plantarum* (cotyledon *C. mannii*).[77]	ND	Fatty acids metabolism (fatty alcohol)
21	**3-Buten-1-ol, 3-methyl- (=Isoprenol)**	YP	9.82	$C_5H_{10}O$	68, 67, 56	1276	1248±8	844	Herbs and spices and sweet cherries.[43]	Sweet and fruity tasting.[43]	

22	**2-Heptanol**	GL	10.68	$C_7H_{16}O$	45, 55, 69	1335	1320 ± 9	875	Yellow pea;[82] products fermented with *L. plantarum* (cotyledon *C. mannii*).[77]	ND	Fatty acids metabolism (fatty alcohol)
23	**1-Hexanol**	GL, YP	11.21	$C_6H_{14}O$	56, 43, 55	1373	1355 ± 7	GL:908 YP: 903	Green lentil, yellow pea, pea flour;[17,18,80] products fermented with *L. plantarum* (cotyledon *C. mannii*, horse gram sprouts).[50,77,83]	Resin, flower, green, lemon, grass, green; used as marker of beany flavour in legume flour.[17]	Fatty acids metabolism (fatty alcohol)
24	**Cyclohexanol, 2,3-dimethyl- (=1,2,3-Dimethylcyclohexanol)**	YP	11.40	$C_8H_{16}O$	43, 41, 67	1386	-	765		ND	
25	**3-Hexen-1-ol, (Z)-**	YP	11.68	$C_6H_{12}O$	67, 41, 82	1406	1382±9	925	Pea flour.[18]	ND	Lipid (α-linolenic acid) oxidation
26	**3-Octanol**	GL	11.71	$C_8H_{18}O$	59, 83, 55	1408	1393±5	914	Yellow pea.[17]	ND	Fatty acids metabolism (fatty alcohol)
27	**2-Hexen-1-ol, (E)-**	GL	11.95	$C_6H_{12}O$	57, 41, 82	1426	1405 ± 9	885	Legumes.[17]	ND	Fatty acids metabolism (fatty alcohol)
28	**1-Heptanol**	GL	12.59	C7H16O	43, 45, 70	1474	1453 ± 8	807	Yellow pea;[82] legumes.[17]	Skunky/ rancidity.[17]	
29	**2-Ethyl-1-Hexanol**	GL, YP	13.05	$C_8H_{18}O$	57, 41, 43	1508	1491 ± 5	GL:933 YP:942	Pea flour;[18] tea, cereals and cereal products, fats and oils, and alcoholic beverages.[43]	ND	Fatty acids metabolism (fatty alcohol)
30	**Linalool**	GL, YP	13.71	$C_{10}H_{18}O$	71, 74, 93	1559	1547 ± 7	GL:738 YP:696	Products fermented with *L. plantarum* (hors gram sprout);[83] beer.[85]	Fruity, hoppy aroma to beer.[85]	Could be produced by the plant as pathogen-induced antibacterial metabolite
31	**1-Octanol**	GL, YP	13.99	$C_8H_{18}O$	56, 69, 70	1580	1557 ± 8	GL:918 YP:863	Yellow pea.[82]	Oily, aldehydic odour.[17]	Fatty acids metabolism (fatty alcohol)
32	**1-Nonanol**	GL	15.19	$C_9H_{20}O$	56, 55, 41	1679	1660 ± 7	929	Green lentil, yellow pea.[80]	Rose-orange odour.[17]	Fatty acids metabolism (fatty alcohol)
33	**Benzyl alcohol**	GL, YP	17.96	C_7H_8O	79, 108, 77	1921	1870 ± 14	GL:955 YP:963	Legumes.[17]	ND	
34	**Phenylethyl Alcohol**	GL	18.44	$C_8H_{10}O$	91, 92, 60	1967	1929[17]	822	Legumes;[17] products fermented with *L. plantarum* (mango slurry).[49]	Rose aroma;[49] Floral, rose, honey.[17]	Amino acids (phenylalanine) metabolism
35	**Maltol**	YP	19.06	$C_6H_6O_3$	126, 71, 43	2040	1969±15	953	Germinated pulse seeds;[17] products fermented with *L. plantarum* (malt).[34]	Caramel.[17]	
	Aliphatic Hydrocarbons										

36	**Tetradecane**	YP	11.59	$C_{14}H_{30}$	71, 57, 43	1399	1400	873	Green lentil, yellow pea;[80] black walnuts, buckwheat, cucumber, allspice, green bell pepper.[43]	Mild, alkane, and waxy taste.[43]	
	Aromatic Compounds										
37	**Furan, 3-methyl-**	YP	4.13	C_5H_6O	82, 81, 53	893	853±2	918	Coffee, baby foods, biscuits, crackers, crispbread.[86]	ND	Lipid oxidation
38	**Pyrrole**	GL	13.57	C_4H_5N	67, 41, 39	1548	1514 ± 9	953	ND	ND	
39	**2(3H)-Furanone, 5-ethyldihydro- (= γ-Caprolactone)**	GL, YP	16.18	$C_6H_{10}O2$	85, 57, 56	1763	1694 ± 16	895	Legumes.[17]	ND	Fatty ester
40	**2(3H)-Furanone, dihydro-5-pentyl-**	YP	20.05	$C_9H_{16}O_2$	85, 43, 109	2179	2024 ± 15	GL:793 YP:895	Germinated pulse seeds.[17]	Peach, coconut.[17]	
41	**Butanoic acid**	GL	14.79	$C_4H_8O_2$	60, 73, 43	1645	1625 ± 12	872	Legumes;[17] products fermented with *L. plantarum* (mango slurry)[49]	Penetrating, rancid, butter-like odour.[17]	Lipid metabolism[40]
	Aromatic hydrocarbons										
42	**Toluene**	GL	6.49	C_7H_8	91, 92, 65	1062	1042 ± 11	941	Green lentil, yellow pea;[80] products fermented with *L. plantarum* (rice);[35]	Sweet, pungent, benzene-like odor.[48]	Amino acids metabolism
	Benzoyl Derivatives										
43	**Methional**	YP	12.89	$C_{10}H_{18}O_2$		1496	1458		Potato, tropical fruits, meat, cheese;[87] beer.[88]	Meaty, onion-like odor;[87] cooked/boiled potato odour.[89,90]	Fatty acids metabolism[89]
	Carboxylic Acids										
44	**Acetic acid**	GL, YP	12.47	$C_2H_4O_2$	43, 45, 60	1464	1449 ± 13	GL:963 YP:955	Products fermented with *L. plantarum* (oat, wheat, malt, barley. cotyledons *C.mannii*, vegetable juice, horse gram sprouts, rice).[50,77,83,91]	Acidic, vinegary.[34]	Carbohydrates metabolism (conversion of lactic acid)
	Esters										
45	**Acetic Acid Methyl Ester**	GL	3.27	$C_3H_6O_2$	43, 74, 42	826	828 ± 6	937	Apple, grape, banana, orange mint, and ginger.[43]	Sweet, fruity, cognac or rum-like aroma and a green, fruity taste.[43]	
46	**Ethyl Acetate**	GL, YP	4.00	$C_4H_8O_2$	43, 70, 45	883	888 ± 8	GL:942 YP:960	Green lentil, yellow pea;[80] products fermented with *L. plantarum* (cotyledon *C. mannii*, cashew apple, rice).[50,77]	Light and fruity odour, brandy notes, highly volatile;[92] pineapple flavour.[34]	Carbohydrates metabolism

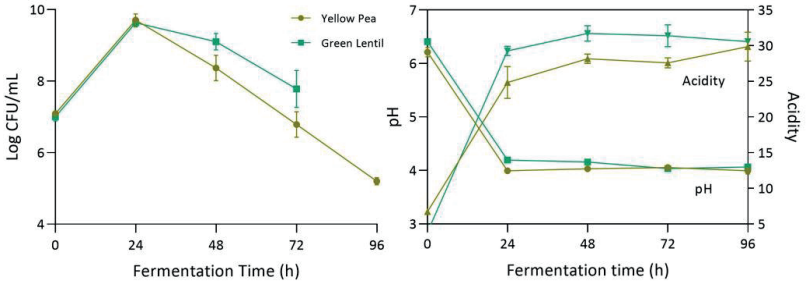
47	**Butanoic Acid, Ethyl Ester**	GL	6.31	$C_6H_{12}O_2$	43, 71, 42	1051	1035 ± 8	840	Many fruits (e.g. apple, apricot, banana, plum, tangerine, fig, citrus, passion fruit).[43]	Banana, pineapple odour.[48]	Fatty acids metabolism
48	**Butanoic acid, 3-methyl-, ethyl ester (= Ethyl Isovalerate)**	YP	6.79	$C_7H_{14}O_2$	88, 85, 57	1081	1068 ± 8	897	Products fermented with *L. plantarum* (cashew apple).[50]	Fruity.[50]	
49	**Butyl Acetate**	GL	6.87	$C_6H_{12}O_2$	43, 56, 41	1086	1074 ± 8	952	Many fruits; apples, especially of the Red Delicious variety.[43]	Fruity odour, sweet smell of banana.[43,48]	Carbohydrate metabolism
50	**1-Butanol, 3-methyl-, acetate (= Isoamyl acetate)**	GL	9.11	$C_5H_{12}O$	55, 73, 70	1229	1209 ± 9	859	Products fermented with *L. plantarum* (cotyledon *C. mannii*).[77]	Pear-like odour, fruity, banana, sweet, fragrant, powerful odor;[48]	
51	**Hexanoic acid, ethyl ester**	YP	9.45			1252	1233 ± 9	921	Goat cheese.[93]	Fruity/green apple.[93]	
52	**Propanoic acid, 2-hydroxy-, ethyl ester, (= lactic acid, ehtyl ester; actylol; ethyl lactate; solactol)**	GL, YP	11.16	$C_5H_{10}O_3$	45, 75, 43	1369	1347 ± 9	GL:929 YP:935	Products fermented with *L. plantarum* (cotyledon *C. mannii*);[77] cabbage, peas, pulses, vinegar, bread, roasted chicken, butter, blackberry, pineapple, raspberry and various wines and spirits.[43]	Mild, buttery, creamy, with hints of fruit and coconut odor.[43]	Carbohydrates metabolism
53	**Octanoic acid, ethyl ester (=ethyl octanoate)**	YP	12.34	$C_{10}H_{20}O_2$	41, 70, 69	1455	1435 ± 6	834	Products fermented with *L. plantarum* (cashew apple).[50]	Fruit, fresh[50]	
54	**Butyl lactate**	GL	13.55	$C_7H_{14}O_3$	45, 41, 57	1546			ND	ND	Carbohydrates metabolism
55	**Pentanoic acid, 2-hydroxy-4-methyl-, ethyl ester**	GL, YP	13.81	$C_8H_{16}O_3$	69, 87, 43	1566	1547 ± 1	GL:888 YP:906	Wine;[94] formed during *L. paracasei* fermentation.[95]	Fresh blackberry aroma.[94]	
56	**Pentanoic acid, 2-hydroxy-4-methyl-, methyl ester**	YP	13.60	$C_7H_{14}O_3$	69, 87, 43	1550	1513±6	853	ND	ND	
57	**Isoamyl lactate**	GL, YP	14.14	$C_8H_{16}O_3$	45, 43, 70	1592	1580 ± 20	GL:803 YP:857	ND	ND	
58	**Ethyl benzoate (Benzoic acid, ethyl ester)**	YP	15.60	$C_9H_{10}O_2$	105, 77, 122	1713	1658±11	893	Yellow pea;[82] germinated pulse seeds;[17] products fermented with *L. plantarum* (hors gram sprout);[83] various fruits (e.g. apple, banana), milk, butter, wines, black tea, bourbon vanilla, fruit brandies.[78]	Sweet, anise, and balsam tasting.[78]	
59	**Acetic acid, phenylmethyl ester (=benzyl acetate)**	GL	16.25	$C_9H_{10}O_2$	108, 91, 90	1770	1720 ± 22	900	Sweet basils, figs, fruits, pomes, tea, and alcoholic beverages.[43]	ND	
60	**Methyl salicylate**	GL	17.01	$C_8H_8O_3$	120, 105, 92	1837	1765 ± 21	861	Products fermented with *L. plantarum* (elderberry juice).[96]	Wintergreen minty, positive fruity notes.[96]	
	Fatty acids										

61	**Propanoic acid, 2-methyl- (=Isobutyric acid; isobutanoic acid)**	GL	14.04	$C_4H_8O_2$	43, 41, 73	1584	1570 ± 12	903	Products fermented with *L. plantarum* (barley, cotyledon *C. mannii*).[34,77]	Sweaty.[34]	Amino acids metabolism
62	**Isovaleric acid (Butanoic acid, 3-methyl-)**	YP	15.29	$C_5H_{10}O_2$	60, 43, 41	1687	1666 ± 11	858	Products fermented with *L. plantarum* (cashew apple).[50]	Sweat, acid, rancid.[50] cheese, dairy, sour, pungent, fruity, stinky, ripe fatty and fruity notes.	
63	**Butanoic acid, 2-methyl-**	GL	15.30	$C_5H_{10}O_2$	74, 60, 41	1688	1662 ± 8	816	Products fermented with *L. plantarum* (cashew apple).[50]	Cheese, sweat.[50]	Amino acids metabolism
64	**Pentanoic acid (=valeric acid)**	YP	16.10	$C_5H_{10}O_2$	60, 73, 41	1757	1733 ± 13	GL:894 YP:923	Products fermented with *L. plantarum* (barley).[34]	Sweaty.[34]	
65	**Pentanoic acid, 3-methyl-**	YP	16.71	$C_6H_{12}O_2$	60, 41, 87	1810	1782 ± 2	906	ND	ND	
66	**Hexanoic acid**	GL, YP	17.29	$C_6H_{12}O_2$	60, 73, 41	1863	1846 ± 12	GL:959 YP:962	Products fermented with *L. plantarum* (cotyledon C. *mannii*, horse gram sprouts).[77,83]	Sickening, sweaty, rancid, sour, sharp, pungent.[17]	Fatty acids metabolism (Fatty carboxylic acid)
67	**(E)-2-Hexenoic acid**	GL	18.71	$C_8H_{16}O_2$	73, 42, 68	1988	1967 ± 11	921	Alcoholic beverages, fruits, tea, and fats and oils.[43]	ND	
68	**Octanoic acid (=Caprylic acid)**	GL	19.86	$C_8H_{16}O_2$	60, 73, 43	2156	2060 ± 15	925	Products fermented with *L. plantarum* (oat, horse gram sprouts, mango pulp).[34,49,83]	Fatty acid, dry, sweaty.[17]	
69	**Nonanoic acid**	GL	21.62	$C_9H_{18}O_2$	60, 73, 57	2280	2171 ± 17	911	Products fermented with *L. plantarum (*oat, barley, Cotyledon *C. mannii*).[34,77]	Sweet, butter-like;[34] unpleasant, rancid odor.[97]	
	Hydrocarbons										
70	**Dodecane**	YP	8.55	$C_{12}H_{26}$	57, 43, 71	1193	-	925	Yellow pes;[82] products fermented with *L. plantarum* (maize);[84] black walnuts, butter, lamb, cocoa, dill, wild strawberry, peas, tea and papaya.[43]	Alkane.[84]	
	Ketones										
71	**2-Butanone**	GL	4.21	C_4H_8O	43, 72, 57	899	907 ± 11	911	Green lentil, yellow pea, pea flour.[18,80]	ND	Fatty acids metabolism (Fatty ketones)
72	**2,3-Butanedione**	GL	5.40	$C_4H_6O_2$	43, 86, 42	992	979 ± 10	928	Products fermented with *L. plantarum* (vegetable juice, rice).[91]	Butter flavour	Carbohydrates metabolism
73	**4-Heptanone, 2,6-dimethyl- (= diisobutyl ketone)**	YP	8.50	$C_9H_{18}O$	85, 57, 43	1190	1191 ± 16	822	ND	Banana, fruity, and green tasting compound.[43]	

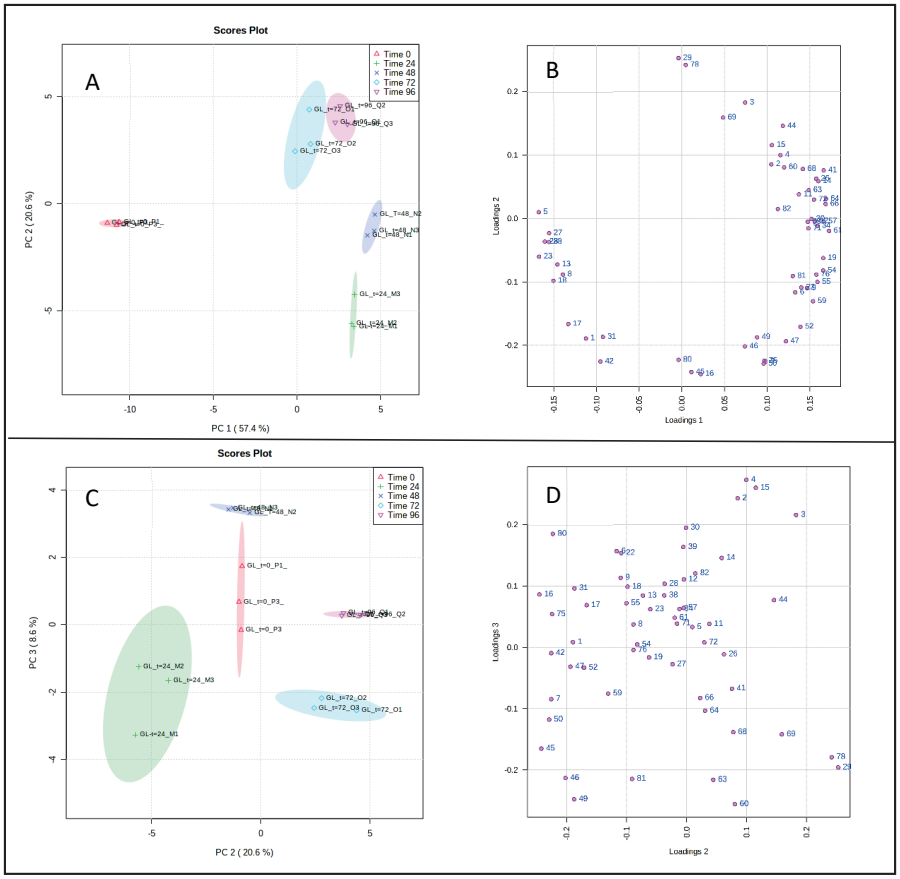
74	**2-Heptanone**	YP	8.76	$C_7H_{14}O$	43, 58, 59	1206	1182 ± 8	882	Green lentil, yellow pea.[17,80]	Blue-cheesy, banana-like, fruity or cinnamon odor; cheesy, cocount-like taste.[43]	
75	**3-Octanone**	GL	9.83	$C_8H_{16}O$	43, 57, 72	1278	1253 ± 11	902	Legumes; [17] products fermented with *L. plantarum* (cherry, pineapple, carrot, tomato juice).[98]	Mushroom, fruity.[99]	
76	**Acetoin**	GL, YP	10.55	$C_4H_8O_2$	43, 45, 88	1324	1284 ± 12	GL:876 YP:885	Products fermented with *L. plantarum* (wheat, barley, malt, cotyledons *C. mannii*, vegetable juice, cashew apple, horse gram sprouts).[34,50,77,83,91]	Woody, yogurt odour, creamy;[17] floral, wet;[34] butter, cream.	Carbohydrates (pyruvate) fermentation
	Pyrazines										
77	**Pyrazine, 2-methoxy-3-(2-methylpropyl)-**	YP	13.64	$C_9H_{14}N_2O$	124, 88, 94	1550	1517 ± 7	869	Pepper, grape.[43]	Earthy.[43]	
	Sulphur Compounds										
78	**Disulfide, dimethyl**	GL	7.00	$C_2H_6S_2$	94, 79, 45	1094	1077 ± 8	903	Green lentil, yellow pea;[80,82] coffee beans (increases during storage).[100]	Sulphur, onion, garlic, burnt rubber.[100]	Amino acids (methionine) metabolism[79]
	Terpenes and Terpenoids										
79	**Limonene**	YP	8.94	$C_{10}H_{16}$	79, 68, 67	1218	1200 ± 7	849	Green lentil, yellow pea.[80]	Pleasant lemon-like odor.[80]	
80	**Carvone**	GL, YP	16.47	$C_{10}H_{14}O$	82, 93, 54	1789	1740 ± 12	GL:917 YP:917	ND	ND	
81	**Geraniol**	GL	17.47	$C_{10}H_{18}O$	69, 41, 68	1879	1847 ± 10	916	Products fermented with *L. plantarum* (hors gram sprout, tomato juice, cherry juice).[83,101]	Honey, tea, floral.[17]	
	Others										
82	**Trichloromethane (=chloroform)**	GL	6.07	$CHCl_3$	83, 85, 47	1036	1022 +/- 6	906	Green lentil, yellow pea.[80,82]	ND	Could derive from the chlorinated drinking water added.[102]

Table 4. Volatile compounds identified by SPME-GC-MS in fermented green lentil and yellow pea flours. GL: green lentil; YP: yellow pea. RI calculated: retention index calculated from a C7 to C40 saturated alkanes standard. RI reference: retention index obtained from NIST 14 library. R match: reverse match factor obtained from NIST 14 library.

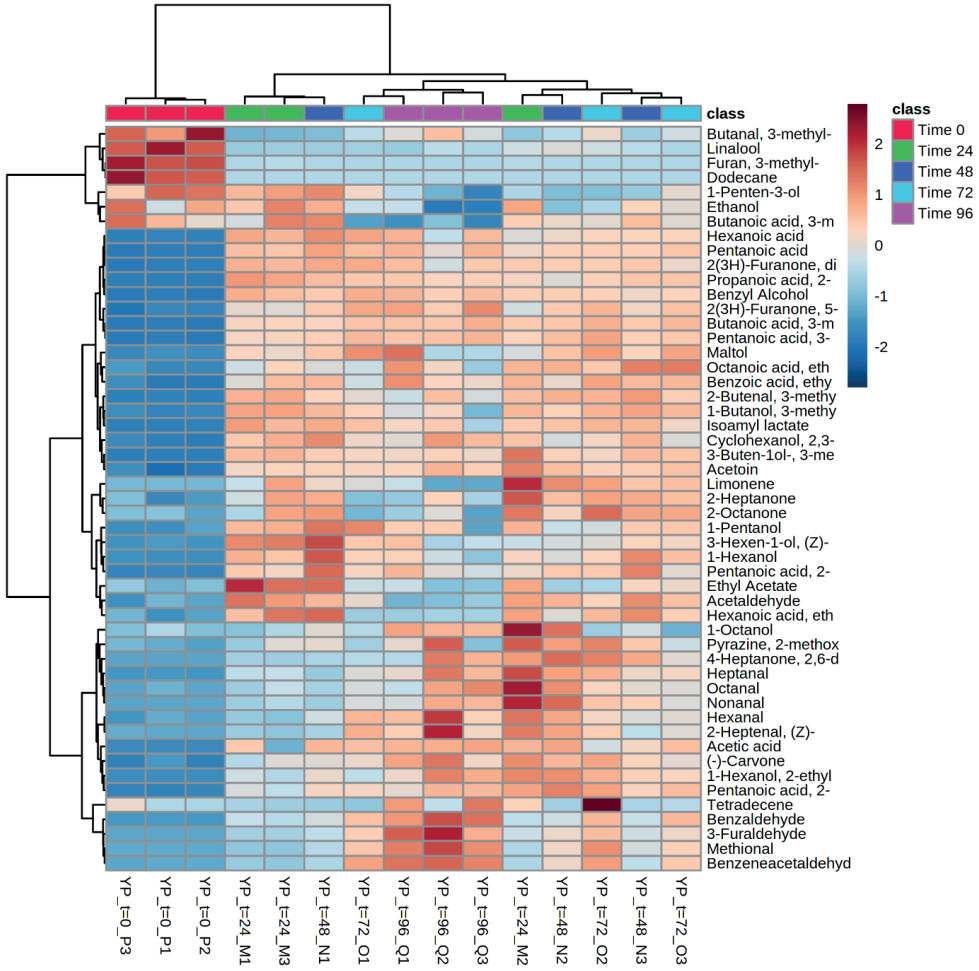
Supplementary Material



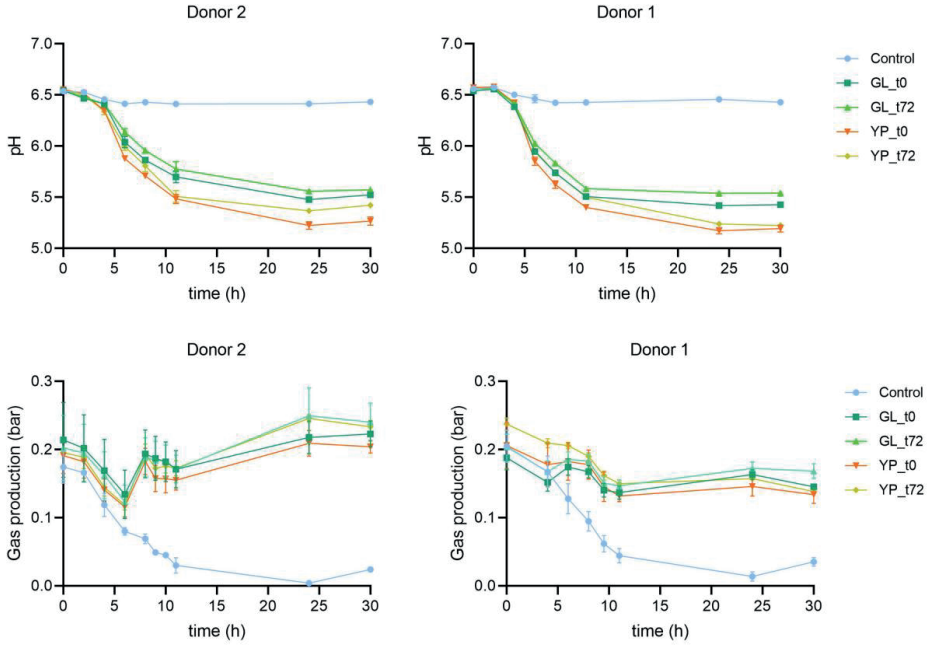
Supplementary Figure 13. Efficacy of the solid state-fermentation of green lentil flour with Lactobacillus plantarum. Results are shown as ± STD of three independent replicates.



Supplementary Figure 14. PCA of volatile compounds identified during fermentation of green lentils detected by SPME-GC-MS and auto-scaled. A, C: score plots. B, D: loading plots. Peak numbers are explained in Table 1.



Supplementary Figure 3. and hierarchical clustering analysis of volatile compounds during fermentation of yellow peas with L. plantarum detected by SPME-GC-MS. Auto-scaled data. The colour box indicates the abundance of each component; "class": extent of the fermentation (hours); "YP" = yellow peas.



Supplementary Figure 4. pH and gas production during batch fermentation. Each dot represents the average value ± SD of four replicates.

Treatment		Control (Donor 2)							Lentil (Donor 2)							Lentil SSF (Donor 2)						
Time		t=0	t=4	t=8	t=12	t=24	t=48	t=72	t=0	t=4	t=8	t=12	t=24	t=48	t=72	t=0	t=4	t=8	t=12	t=24	t=48	t=72
Phylum	Family																					
Actinobacteria		10.7	10.0	8.3	8.7	6.43	6.11	12.62	12.1	10.6	10.5	11.2	5.6	34.7	18.8	13.9	15.5	12.2	9.0	7.6	9.0	10.6
	Bifidobacteriaceae	9.93	9.16	8.09	7.99	6.24	5.28	12.18	10.25	9.86	8.42	10.31	5.47	32.99	14.84	13.28	13.67	11.05	8.13	6.76	8.39	9.60
	Coriobacteriaceae	0.76	0.85	0.16	0.68	0.19	0.83	0.44	1.81	0.74	2.07	0.92	0.17	1.70	3.99	0.59	1.85	1.18	0.88	0.82	0.59	1.01
	unclassified Actinobacteria	0.16	0.00	0.00	0.01	0.00	0.00	0.00	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00
Bacteroidetes		36.0	30.7	35.2	34.0	38.11	47.70	49.71	37.8	25.9	31.6	21.1	33.9	16.5	19.8	36.9	30.3	34.6	34.6	41.8	19.7	18.0
	Bacteroidaceae	35.83	30.62	35.10	33.90	37.98	47.67	49.63	37.76	25.83	31.55	21.04	33.84	16.50	19.78	36.87	30.30	34.50	34.43	41.73	19.52	17.95
	Marinilabiaceae	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00
	Unclassified Bacteroidales	0.14	0.04	0.06	0.10	0.06	0.01	0.04	0.01	0.05	0.09	0.05	0.04	0.04	0.05	0.02	0.02	0.10	0.11	0.03	0.13	0.05
Firmicutes		50.1	57.6	55.1	55.4	53.98	45.37	36.69	46.1	60.4	55.3	66.9	58.4	46.9	59.1	46.2	50.5	50.4	53.3	48.8	68.9	66.5
	Unclassified Bacilli	0.06	0.01	0.01	0.03	0.01	0.03	0.01	0.00	0.01	0.02	0.01	0.01	0.03	0.02	0.00	0.01	0.06	0.05	0.00	0.07	0.01
	Unclassified Bacillales	0.98	0.41	0.51	0.37	0.20	0.10	0.08	2.7	2.10	1.82	0.83	1.72	1.31	0.58	2.38	3.45	2.58	2.62	1.55	1.16	0.88
	Bacillaceae	0.00	0.06	0.01	0.00	0.01	0.14	0.01	0.00	0.14	0.03	0.00	0.04	0.14	0.04	0.01	0.10	0.01	0.00	0.03	0.12	0.07
	Unclassified Lactobacillales	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.01	0.01	0.11	0.12
	Lactobacillaceae	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.27	0.13	0.08	0.03	0.02	0.09	0.02	0.02	0.01	0.01	0.02	0.12	0.27
	Unclassified Clostridiales	0.04	0.01	0.01	0.13	0.01	0.00	0.00	0.01	0.00	0.07	0.00	0.01	0.03	0.01	0.01	0.01	0.09	0.10	0.00	0.07	0.03
	Eubacteriaceae	0.18	0.35	0.13	0.13	0.10	0.16	0.39	0.07	0.08	0.09	0.02	0.06	0.81	1.00	0.10	0.10	0.08	0.09	0.10	1.00	1.76
	Lachnospiraceae	24.21	34.19	33.12	34.76	40.13	35.78	17.73	20.32	37.05	32.32	53.07	35.87	16.73	39.66	24.48	27.80	29.68	33.80	30.25	22.06	26.19
	Ruminococcaceae	5.93	5.76	5.37	4.48	2.26	1.38	2.57	5.53	5.19	3.85	0.69	5.60	3.61	1.31	5.36	6.77	5.47	6.31	5.29	3.47	6.45
	Veillonellaceae	6.37	5.65	5.09	5.46	2.14	1.43	1.94	9.31	9.15	9.03	6.83	9.41	18.39	11.20	4.10	4.57	3.72	3.63	6.43	29.50	18.65

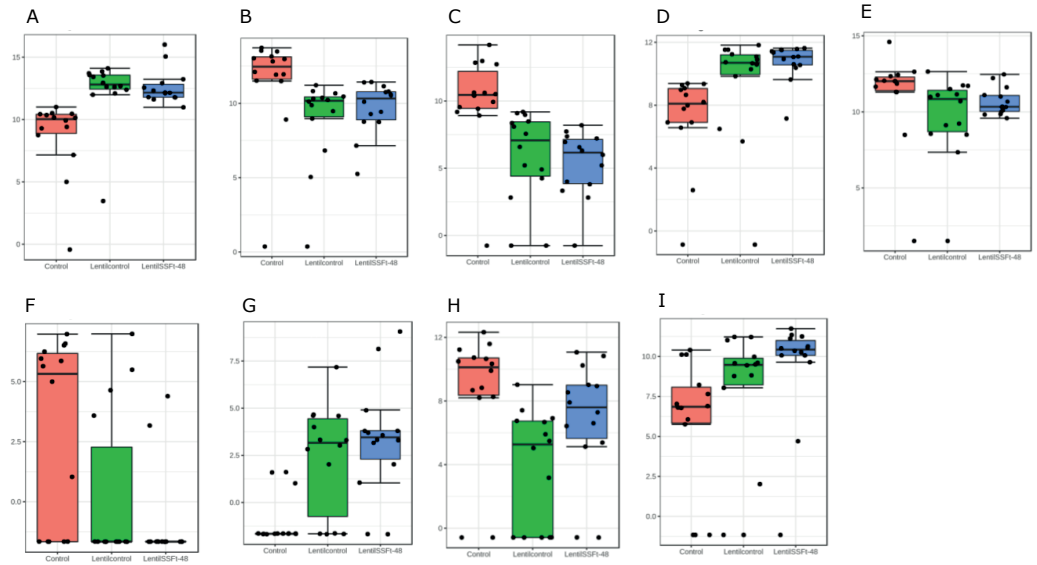
	Erysipelotrichaceae	11.99	11.17	10.75	9.79	9.09	6.36	13.87	7.80	6.35	7.86	5.29	5.57	5.79	5.63	9.73	7.63	8.42	6.42	5.05	10.99	11.93
Proteobacteria		3.2	1.7	1.5	1.9	1.53	0.82	0.98	4.1	3.1	2.5	0.8	2.1	1.8	2.3	3.0	3.7	2.7	3.1	1.9	2.4	4.9
	Unclassified Betaproteobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
	Unclassified Burkholderiales	0.04	0.03	0.02	0.05	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Comamonadaceae	1.13	0.79	0.62	0.60	0.08	0.03	0.19	1.15	1.23	0.70	0.22	1.18	1.02	0.19	1.33	2.55	1.75	2.18	1.00	0.47	0.79
	Oxalobacteraceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	Enterobacteriaceae	2.05	0.88	0.89	1.29	0.62	0.76	0.93	2.95	1.89	1.82	0.59	0.91	0.81	2.09	1.69	1.12	1.00	0.93	0.89	1.95	4.07

Supplementary Table 5. Microbial profile at phylum and family level (% relative abundance) for Donor 2.. Counts lower than 0.01 % were not included.

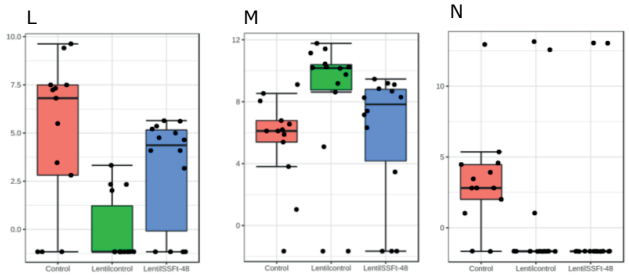
Treatment		**Control (Donor 4)**							**Lentil (Donor 4)**						**Lentil SSF (Donor 4)**						
Time		t=0	t=4	t=8	t=12	t=24	t=48	t=72	t=0	t=4	t=12	t=24	t=48	t=72	t=0	t=4	t=8	t=12	t=24	t=48	t=72
Phylum	Family																				
Actinobacteria		23.9	25.6	37.9	31.7	17.8	11.8	14.7	27.0	33.7	29.7	23.3	18.2	19.0	19.1	22.1	24.5	24.5	18.9	14.0	14.4
	Bifidobacteriaceae	23.94	25.35	37.87	31.60	17.04	11.76	14.66	27.01	33.16	29.73	23.27	18.21	18.95	19.04	22.12	24.48	24.07	18.89	13.95	14.25
	Coriobacteriaceae	0.00	0.27	0.03	0.05	0.73	0.07	0.01	0.00	0.55	0.00	0.00	0.01	0.01	0.02	0.01	0.00	0.46	0.01	0.00	0.11
Bacteroidetes		11.1	13.1	9.4	14.1	13.1	10.6	1.3	9.6	6.8	10.4	9.4	11.7	3.9	9.7	9.6	8.1	11.1	10.9	13.8	13.7
	Bacteroidaceae	11.08	13.13	9.42	14.06	13.11	10.55	12.30	9.57	6.79	10.42	9.40	11.65	3.77	9.67	9.57	8.12	11.10	10.89	13.82	13.60
	Marinilabiaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Porphyromonadaceae	0.01	0.01	0.01	0.01	0.03	0.03	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Unclassified Bacteroidales	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.00	0.00	0.02	0.01	0.01	0.08	0.01	0.00	0.01	0.01	0.00	0.00	0.07
	Unclassified Bacteroidetes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.02
Firmicutes		55.8	53.2	46.1	48.0	65.0	70.2	68.7	60.7	56.5	55.1	64.3	68.5	74.4	66.6	64.5	63.5	59.9	66.7	69.0	69.4
	Unclassified Firmicutes	1.48	1.40	2.00	2.29	2.54	2.51	2.36	2.77	2.17	3.52	2.34	2.40	3.02	2.17	5.88	2.35	2.31	2.21	2.56	1.65
	Unclassified Bacilli	0.07	0.00	0.00	0.04	0.23	0.05	0.01	0.00	0.00	0.02	0.00	0.00	0.02	0.02	0.03	0.00	0.12	0.02	0.00	0.00

	Unclassified Bacillales	0.00	0.00	0.01	0.01	0.00	0.01	0.00	0.02	0.01	0.05	0.02	0.01	0.17	0.02	0.00	0.07	0.08	0.00	0.02	0.04
	Bacillaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
	Paenibacillaceae	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Unclassified Lactobacillales	0.03	0.05	0.01	0.01	0.05	1.84	0.10	0.04	0.03	0.04	0.07	0.04	0.01	0.02	0.03	0.02	0.01	0.08	0.05	0.00
	Carnobacteriaceae	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Lactobacillaceae	6.60	6.48	2.72	2.30	2.28	0.66	0.42	6.78	4.79	4.60	8.53	9.10	6.05	2.44	1.84	1.74	1.69	5.66	4.42	0.85
	Unclassified Clostridiales	0.65	0.56	0.45	0.42	0.28	0.83	0.54	1.25	0.87	1.94	0.91	1.88	2.08	0.88	0.48	0.78	0.82	1.56	1.47	0.89
	Eubacteriaceae	0.02	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Lachnospiraceae	21.08	24.10	18.87	21.24	26.47	29.40	40.57	32.63	24.36	20.62	28.21	29.23	33.84	33.41	31.40	27.46	24.84	31.72	20.07	28.76
	Ruminococcaceae	10.93	10.01	9.69	12.22	12.84	18.37	18.54	7.93	11.99	9.94	10.52	7.64	14.06	13.80	13.26	16.54	17.06	15.85	19.11	21.32
	Veillonellaceae	13.68	9.91	11.75	8.82	18.13	12.38	4.65	7.23	10.64	12.74	11.61	15.83	14.03	10.73	10.01	13.61	12.24	8.46	20.17	15.16
	Erysipelotrichaceae	1.21	0.61	0.63	0.60	2.22	4.12	1.46	2.04	1.60	1.64	2.10	2.31	1.11	3.07	1.52	0.94	0.71	1.08	1.14	0.68
Proteobacteria		9.2	8.1	6.5	6.3	4.0	7.4	4.3	2.7	3.0	4.7	3.0	1.7	2.8	4.7	3.8	3.9	4.5	3.6	3.2	2.6
	Unclassified Betaproteobacteria	0.09	0.06	0.19	0.26	0.22	0.29	0.51	0.15	0.08	0.27	0.10	0.05	0.00	0.23	0.00	0.28	0.32	0.38	0.50	0.40
	Unclassified Burkholderiales	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.06	0.00	0.01	0.00
	Comamonadaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
	Oxalobacteraceae	0.99	0.82	1.16	1.50	1.22	0.97	0.81	1.87	1.09	1.45	0.38	0.20	0.44	2.03	0.41	1.30	1.34	1.28	1.12	0.56
	Rhodocyclaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	Unclassified Gammaproteobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Enterobacteriaceae	8.12	7.21	5.18	4.54	2.60	6.12	3.01	0.72	1.87	2.97	2.52	1.42	2.31	2.39	3.44	2.28	2.75	1.90	1.58	1.58

Supplementary Table 6. Microbial profile at phylum and family level (% relative abundance) for Donor 4. Counts lower than 0.01 % were not included.



Supplementary Figure 5. Family and bacterial species significantly different between control and fermented samples (all time points, time 0 excluded) in Donor 2 after Mann-Whitney/Kruskal-Wallis test (p-value cut-off 0.05). Values were log transformed. A: Anaerosin glycerini (Veillonellaceae); B:Clostridium clostridioforme (Lachnospiraceae); C: Succinispira mobilis (Veillonellaceae); D: Bacillales; E: Erysipelotrichaceae ; F: Clostridium spp. (Erysipelotrichaceae); G: Lactobacillus spp. (Lactobacillaceae); H: Clostridium spp. (Lachnospiraceae); I: Comamonadaceae;



Supplementary Figure 6. Bacterial species significantly different between control and fermented samples (all time points, tim excluded) in Donor 4 after Mann-Whitney/Kruskal-Wallis test (p-value cut-off 0.05). Values were log transformed. Ruminococcaceae; M: Catenibacterium mitsuokai (Catenibacterium); N: Anaerorhabdus furcosa (Erysipelotrichaceae).

CHAPTER 6

General discussion and future directions

1. Main findings and objectives

Noncommunicable diseases are on the rise, accounting for 71 % of the yearly deaths globally. Unhealthy diets leading to metabolic syndrome, characterized by raised blood pressure, hyperglycemia, overweight or hyperlipidemia, are one of the strongest and modifiable risk factors.[1] It therefore becomes imperative to rethink food consumption as a strategy to prevent the diseases. Pulses are an excellent food for this transition, being a low fat and low glycemic index food rich in dietary fibers and proteins. Recent research further highlighted the presence of bioactive molecules in pulse seeds that could help to improve glucose regulation similarly to drugs. However, the low digestibility of the plant food matrix by humans and limited appeal of pulses hinder their nutritional and functional benefits, and their adoption in daily diets in Western countries. This thesis addressed three major issues related to pulses: (i) acceptability in terms of aroma and flatulence factors, (ii) digestibility of the plant seed structures to access nutrients and bioactive compounds, and (iii) use of sustainable bioprocessing approaches to address the first two issues. We explored the use of germination and fermentation as a natural approach to minimally process pulse seeds while improving digestibility, acceptability and enhance the bioactivity. The findings of this thesis can be used to design novel fermented functional foods to improve glucose regulation, and more broadly to enhance the adoption of pulse foods in daily diets.

Table 7. Summary of the objectives and main findings from the thesis.

	Objective	Main findings
Chapter 2	To assess the impact of bioprocessing (germination, microbial fermentation with *L. plantarum*) alone or in combination with physical (grinding) and thermal processing on (i) the bioaccessibility and profile of proteins and phenolic compounds in five common pulses (chickpea, fava bean, green lentil, kidney bean, yellow pea), (ii) the seed microstructure, (iii) the *in vitro* inhibition of α-glucosidase and DPP-IV.	• Germination and fermentation have diverse efficacy on different pulses. Likewise, the implications for bioactivity are pulse-treatment dependent. • Germination increased protein digestibility in all tested pulses and improved its bioaccessibility in chickpeas and yellow peas. It also led to putative *de novo* synthesis of flavonoids and phenolic acids. • Fermentation did not affect protein digestibility, but it improved its bioaccessibility in kidney bean, green lentil and yellow pea. It also led to putative bioconversion and deglycosylation of flavonoids and phenolic acids. • At a microstructural level, both bioprocessing led to a depletion of starch and protein granules. Differently, germination caused thickening of the cell wall, while fermentation was able to break down the cell wall at certain sites. • Two promising bioprocessing were significantly improving the bioactivity: (i) germinating chickpeas for 3 days, in regard to its significant increase in α-glucosidase inhibition, (ii) fermentation of green lentil flour, in regard to its significantly improved DPP-IV inhibitory activity.

Chapter 3	To explore the improved DPP-IV inhibitory activity of green lentil flour upon fermentation with *L. plantarum* in a human intestinal Caco-2 cell monolayer model, and the implications for glucose transport.	• Exposure of the intestinal cells to pre-fermented green lentil digesta increased the inhibition of cellular DPP-IV by 27 % compared to the untreated green lentil digesta. • The DPP-IV inhibitory activity of the basolateral fluids was also improved, but generally low. • A correlation was observed between increase in fermentation time and bioactivity. • There was dose-response when 100 and 200, but not 500 mg/ml of digesta were tested. • Glucose absorption and uptake from Caco-2 cells was not affected.
Chapter 4	To combine peptidomics techniques and chemometrics analysis to identify and characterize the bioaccessible peptides transported across the Caco-2 intestinal absorptive model.	• A total of 461 bioaccessible peptides were identified in unfermented green lentil samples, and 558 in microbially fermented samples. • Different fermentation times clustered separately on a PCA, as they had unique sets of peptides. • About 14 % of the bioaccessible peptides were absorbed by intestinal Caco-2 cells. • 63 peptides (43 % of the total number of absorbed peptides) were only present, or where significantly higher, in the BL fluids compared to the bioaccessible fraction.
Chapter 5	To explore the use of lactic acid bacteria fermentation as a tool to improve acceptability (decrease flatulence factors and improve aroma profile) while retaining the beneficial activity of green lentil on colonic fermentation.	• Fermentation tremendously decreased the content of flatulence-forming raffinose family oligosaccharides in green lentil and yellow pea digested flours, although at different speeds. • A 24 to 48 h fermentation significantly decreased the content of common off-flavor volatile compounds associated with green lentils, while increasing the content of volatile compounds associated with pleasant aroma descriptors. • Extending the fermentation to 72 - 96 h led to accumulation of unwanted volatile compounds. • The implications of fermentation on the aroma was less clear for yellow pea. • Colonic fermentation of unfermented and fermented lentil flour led to increase in alpha-diversity indices, and in beneficial microbial species such as *Bifidobacterium*, *Lactobacillus* and *Roseburia*.

2. Digestion and Absorption: key components of food and nutrition studies

As illustrated in Chapter 1, the digestive enzymes secreted along the human gastrointestinal tract have a profound impact on structure and function of bioactive components from food. Additionally, when the bioactivity of interest is exerted on a different location from the luminal side of the intestine, the

bioactive compounds will further have to withstand the activity of the intestinal epithelial enzymes, cross the intestinal barrier, and be transported in their bioactive form to the target location. While this will likely affect the initial structure and functionality of the compounds, still most of the studies concerning DPP-IV and α-glucosidase inhibitory potential of food-derived components barely consider all of these steps.

2.1 Bioactive peptides

As shown in supplementary Table 2 in Chapter 1, most of the recently published studies concerning the five pulses of interest in this thesis record the bioactivity on undigested pulse-components. Among the studies involving protein fractions, few included an *in vitro* digestion step, which often increased the observed bioactivity. However, when protein digesta were further exposed to the activity of brush border enzymes, the bioactivity was generally negatively affected. Measuring the bioactivity at the target site, such as the enterocytes or the blood circulation in the case of DPP-IV, led to significantly lower values. This was also the case in our study, where the initial DPP-IV inhibitory activity of 67 % reported in Chapter 3 for the 72 h fermented green lentil flour was minimally affected by incubation with the Caco-2 cell monolayer (apical fluids), but substantially decreased in the transported fluids. In fact, the brush border contains many proteases and peptidases, which could inevitably digest most of the peptides to amino acids. Conversely, a fraction of peptides is transported through the monolayer in its intact form and can therefore potentially enter the blood circulation and exert its functionality at target sites in the body. This was observed to be below 2 % for individual DPP-IV inhibitory peptides, but nevertheless still sufficient to confer a certain bioactivity.[2–4] In our study, about 24 % of the bioaccessible peptides were transported across the intestinal monolayer (Chapter 4), and a DPP-IV inhibitory activity of 7 % was retained till the basolateral side of the enterocytes (Chapter 3). Moreover, in chapter 4, we identified 63 peptides that were exclusively present in the basolateral fraction, on top of the 83 peptides absorbed from the bioaccessible fraction. This means that 31 % of the peptides present in the basolateral fraction were not constituent of the bioaccessible fraction and were formed by hydrolysis from brush border enzymes. As these peptides can also exert a certain bioactivity, it becomes evident the key role of digestion and adsorption when investigating the bioactivity of food-derived components.

2.2 Protein digestibility

From a nutritional point of view, it is also critical to investigate protein digestibility in the contest of gastrointestinal digestion. This was evident in Chapter 2, where the conclusions regarding digestibility of protein (measured as degree of hydrolysis) were very different when considering undigested or digested samples (Table 1). For instance, germination of kidney beans appeared to progressively increase the degree of hydrolysis of proteins in the undigested samples, while opposite trend was observed in the same samples after simulated gastrointestinal digestion.

2.3 Role of polyphenols

What was previously described also applies for phenolic compounds, which were the second bioactive components investigated in the thesis. Also in this case, almost all the studies reported in literature concerning the bioactivity of the five investigated pulses did not involve a gastrointestinal digestion step (Chapter 1, Supplementary Table 2). However, when profiling the phenolic compounds in green lentil and yellow pea before and after *in vitro* digestion (Chapter 2, Table 2), profound changes were observed. Digestion can, in fact, profoundly affect the content and bioactivity of phenolic compounds. All digestive stages can have implications on this, but since phenolic compounds are more reactive

around neutral pH, the intestinal stage is believed to lead to the highest phenolic reactivity, affecting their structure and related biological functionality.[5] As a consequence, digestion can hinder as well as enhance the bioaccessibility and bioavailability of phenolics. For instance, the breakdown of the food matrix by digestive enzymes and motion can improve the phenolic release from the bolus / chyme. Accordingly, we observed a significant increase, often doubling, in total phenolic content between the undigested and *in vitro* digested samples (Chapter 2, Table 1). However, breakage of the plant cell wall upon digestion also allows to phenolic compounds to create complexes with dietary fibers, therefore decreasing their bioavailability, and increasing the amount of phenolics reaching the large intestine. Conversely, proteolytic digestive enzymes can break down the polyphenols-protein bonds, thus increasing bioaccessibility of both classes of compounds.[5] Hence, it is difficult to predict the bioaccessibility of a phenolic compound prior digestion, since food matrix and composition can greatly alter their release/binding to other components during digestion. In our study, for instance, we noticed contrasting results concerning the bioaccessibility of catechin glucoside, which increased in pre-germinated samples, but decreased in the pre-fermented ones. Overall, the bioaccessibility of flavonoids and hydroxycinnamic acids was lower than that of phenolic acids, as previously observed in other plant sources (broccoli, corn, bean) and fruit beverage.[6–8]

In the small intestine, absorption of phenolics is low. This was reported to be below 1 % for *in vitro* digested *p*-coumaric acid, epicatechin, rutin and quercetin- and cyanidin-glycosides, below 6 % for *in vitro* digested gallic acid, chlorogenic acid, luteolin and naringenin.[9,10] The fraction absorbed by the intestinal cells was significantly lower when the phenolic extract was not digested, with most of the phenolics being completely unable to cross the barrier.[9] Conversely, the antioxidant activity measured in the study was not retained in the absorbed fraction.[9] This strongly indicates the importance of digestion on the assessment of absorption and bioactivity of bioactive food components. This was indeed the case in Chapter 3, where the total phenolic content was 37 % lower in the basolateral fluids compared to the digesta, and the related bioactivity dropped. For flavonoids, it was suggested that aglycones can be transported across the intestinal cells via passive diffusion, while glycosides are transported via active transport, or are hydrolyzed by brush border enzymes and absorbed by passive diffusion.[11] Lower absorption in the small intestine results in a higher amount of phenolic compounds, and phenolic-fibers complexes reaching the colon. The formation of polyphenols-fibers complexes can, at the same time, facilitate the transport of these bioactive components to the colon. Here, about 40 % of the polyphenols could be metabolized by commensal bacteria, contributing to gut health and with the catabolites potentially being re-absorbed by intestinal cells.[12] While this was not the focus of the thesis, we can assume that the fermentation with *Lactobacillus plantarum*, affecting pH and acidity of the green lentil and yellow pea flours, likely affected the formation or dissolution of phenolics-fibers complexes, contributing to the differences in colonic fermentation observed in Chapter 5.

2.4 Indigestible carbohydrates

Likewise, indigestible carbohydrates should be *in vitro* digested before being analyzed for colonic digestion. Digestion can in fact increase or decrease the amount of dietary fibers-polyphenols complexes in the food. Components of the plant foods such as cellulose, hemicellulose, resistant starch, pectin and β-glucans can in fact form bonds with polyphenols under various conditions. Ionic strength, pH and temperature are major factors involved in the formation or dissolution of these bonds.[12] To take into account these factors, in Chapter 5 and 6 we pre-digested green lentil and yellow pea flours before assessing their colonic fermentation. In the case of RFOs, Luzardo-Ocampo et al. noticed that the ratio between Raf, Sta and Ver changed upon *in vitro* digestion in corn-bean chips,

with Raf being initially absent, but the most abundant after digestion.[7] On the contrary, Caicedo-Lopez et al. reported a decrease in Raf and increase in Sta when digesting *Moringa oleifera* leaves.[13] Food matrix, digestion methodology and sample preparation could explain the differences between the two studies. Indeed, the corn-bean chips were cooked before digestion, which could have caused thermal hydrolysis of RFOs with higher degree of polymerization to lower ones, such as Raf.[7,14] Nevertheless, this shows the importance of including a digestion step in food studies, since differences in matrix and sample preparation can greatly affect the behavior of individual components.

Altogether, this thesis provided further evidence supporting the importance of digestion and absorption in studies focusing on bioactivity of food components. The physicochemical changes occurring during gastrointestinal digestion deeply affect the proportion and structure of bioaccessible components, profoundly affecting their bioactivity. The inclusion of these steps and the development of more advanced and accurate models to simulate the upper digestion and absorptive process are imperative to progress in the field.

3. Bioprocessing as an effective and reproducible tool to modulate bioactivity and organoleptic properties of pulse-foods

Bioprocessing encompasses a wide range of biological processes using living sources or their components, such as microbial fermentation or enzymatic digestion, to form value-added products.[15] In this thesis, we investigated the use of germination and microbial fermentation for modulating the bioactive profiles of pulses and improving bioactivity and acceptability. In Chapter 2, we observed that the effect of each bioprocess greatly differed among the different pulses. For instance, the germination rate varied among pulses, reaching 100 % in green lentils and only 65 % in chickpeas. Nevertheless, when the substrate was suitable for the bioprocessing, such as yellow pea for both germination and fermentation, we observed good replicability. This was not only in terms of response to the bioprocessing, but also in terms of the nutritional implications of the bioprocess.

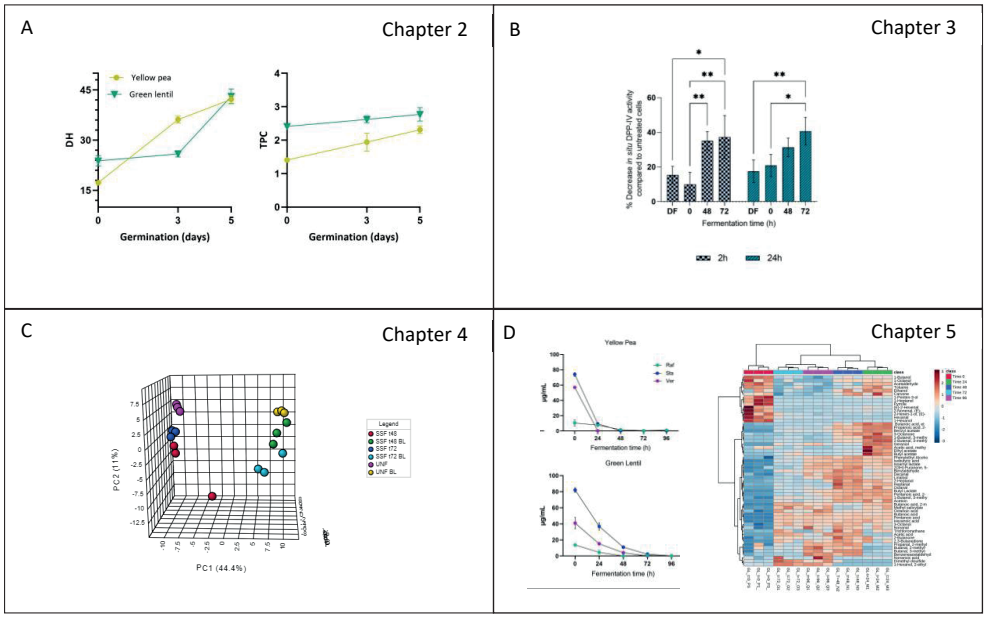


Figure 1. Replicability of experimental results obtained from bioprocessing. A: protein degree of hydrolysis (DH) and total phenolic content (TPC) upon germination of yellow pea and green lentil. B: in situ DPP-IV inhibitory upon exposure to fermented and digested green lentil flour. C: PCA of bioaccessible and intestinally absorbed peptides obtained from fermented green lentil flour. D: raffinose family oligosaccharides (RFOs) in fermented green lentil and yellow pea, and volatile profile of green lentil. A, C and D show values obtained by three independent biological replicates.

Microbial fermentation proved to be a solid and reliable transformation process for pulses, looking the biochemical and physiological outcomes. The digestibility of proteins and total phenolic content (Fig. 1.A), and the DPP-IV and α-glucosidase inhibitory activities were consistent across replicates. Interestingly, the same consistency was observed in the peptides profiled in Chapter 5. Here, three independent biological replicates were exposed to the Caco-2 cell monolayers. As observed in Fig. 1.C, the replicates were clustering together on the PCA plot, as they were characterized by similar peptides profiles. To the best of our knowledge, this is the first study in which the replicability of the peptidic profile obtained upon fermentation was investigated. As we reported in Chapter 1, the vast majority of pulse-derived bioactivity investigations have been performed on pulse extracts such as phenolic or protein isolates. These are often fractionated or hydrolyzed with commercial enzymes in order to improve the bioactivity. This approach is often chosen because the absence of food matrix and use of enzymes can provide better standardization of the process and results. However, this has significant implications in terms of gastrointestinal stability, as previously discussed in paragraph 2.1. Moreover, the use of enzymes is costly and this approach requires extensive processing of the raw ingredients, with possible implications for sustainability and nutrition.[16] On the contrary, we proposed a simple bioprocessing approach that involves the use of the whole seed without extracting any of the components. The use of microbial fermentation in the food industry is still mainly related to traditional dairy and soybean products, while still minimally explored in bioactivity studies. In Chapter 3, we showed that this application is also possible, and provide consistent results (Fig. 1.B). We further explored the use of fermentation for modifying volatile compounds and reduce raffinose family

oligosaccharides in pulses. Also in this case, we used three independent biological replicates and we observed good replicability of the results (Fig. 1.D). Altogether, this thesis provides evidence for the use of germination and microbial fermentation as a tool for modulating the nutritional (phenolic and peptides profile, α-glucosidase and DPP-IV inhibitory activity) as well as the acceptability (aroma attributes from volatile compounds, content of raffinose family oligosaccharides) characteristics of pulse seeds. The bioprocesses showed good replicability in terms of efficacy and of the biochemical modifications derived by the process.

4. The microbial machinery and the added value of fermentation

Microorganisms harbor a great variety of metabolic pathways that can be activated according to the nutritional sources available in their environment. Therefore, a wide range of enzymes can be expressed by microbes according to their genome and the substrate for fermentation. In this thesis, the lactic acid bacteria *Lactobacillus plantarum* (Orla-Jensen) Bergey et al. ATCC8014 was used. This commercial strain has been fully sequenced and its genome spaces 3,170,206 bp, potentially encoding 1284 different proteins. *L. plantarum* is a major component of the microbiota naturally present on legume seeds, suggesting an adaptation to this biological niche.[17] With this rationale, we selected this bacteria for performing solid-state fermentation of pulse seeds. As observed in Chapter 2, this strain was indeed able to grow and ferment all the five types of pulses investigated. More in detail, fermentation had specific implications for the biological activities, nutritional profile and acceptability of the pulse seeds, which could be ascribed to its genome. The implications were specific for each pulse type and the combined pretreatment. As we focused on green lentil in most of the thesis, we use this pulse as a model to discuss the observed implications of solid-state fermentation with *L. plantarum*. The putative enzymes and pathways involved in the fermentation of green lentil in our study are illustrated in Fig. 2.

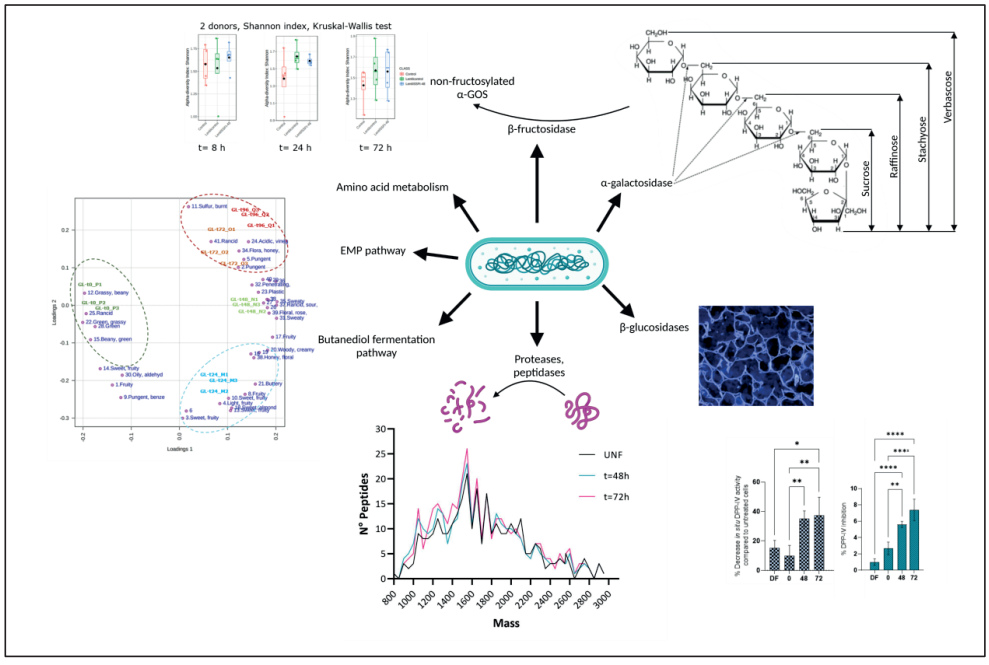


Figure 15. Putative enzymes secreted by L. plantarum ATCC8014 responsible for the observed outcomes of this thesis.

In Chapter 2, we observed that fermentation of whole pre-soaked seeds for 48 h was sufficient to break down certain sites of the cell wall of lentils and peas (Chapter 2, Fig. 3). Cellulases, hemicellulases and endoproteases were previously identified in *L. plantarum*, which allowed for its biotransformation of plant biomass.[18] For instance, the genome of our strain encoded for two β-glucosidases (*gmuD*, *bglA*), which possibly played a role in the cell wall degradation.[19] Furthermore, the fermentation led to a decrease in kaempferol tetraglycoside and kaempferol triglycoside, and increase in kaempferol glycoside. Biotransformation of flavonoids, including deglycosylation, was previously described for lactic acid bacteria, with β-glucosidases also involved in this processes.[20] As previously mentioned, aglycones can be absorbed more easily by enterocytes compared to their glycosylated forms, which is valued when bioactivities such as DPP-IV inhibition are targeted. *L. plantarum* ATCC8014 genome also encodes a wide range of proteases and peptidases. It is therefore not surprising that 47 additional bioaccessible peptides were identified in the 72 h fermented green lentil compared to the unfermented samples (Chapter 4). The absorbed peptides and phenolics likely led to the improved *in situ* and basolateral DPP-IV inhibitory activity observed in Chapter 3. Fermentation also significantly affected volatiles composition and raffinose family oligosaccharides, with important implications for the acceptability of the product (Chapter 5). The melA locus, containing the putative genes *galM*, *rafP*, *melA*, *lacM* and *lacL*, present in the genome of our strain, was putatively responsible for α-galactosidase metabolism and related fermentation of RFOs.[21] A putative sucrose-6-phosphate hydrolase (*ScrB* gene), raffinose transporter RafP (*rafP* gene) and lactose permease LacS (*lacS* gene) likely completed the metabolic pathway by translocating raffinose and degrading sucrose at a last stage.[22] At the same time, the Embden-Meyerhof-Parnas (EMP) pathway, butanediol fermentation pathway, amino acid metabolism (e.g., phenylalanine, leucine, isoleucine, valine) were implicated in the formation of volatile compounds with characteristic aroma attributes. Moreover, β-fructosidase possibly converted part of RFOs to their corresponding non-fructosylated α-galacto-oligosaccharides, which could explain the retained bifidogenic effect (Chapter 5) and increased microbial diversity observed upon colonic fermentation (Chapter 6). From a sensory perspective, a 24 to 48-h fermentation appeared to significantly improve the aroma descriptors of green lentils, as major compounds associated with the beany off-flavors greatly decreased, while pleasant attributes typical of lactic acid fermentation were formed. However, prolonged fermentation to 72 to 96 h led to accumulation of volatile compounds with strong unpleasant aroma attribute. Therefore, the timing of the fermentation is a very important parameter to consider. Moreover, fermenting for 48 h dramatically decreased the content of RFOs while retaining the bifidogenic activity (Chapter 5). Continuous colonic fermentation of the green lentils for 3 days led to an increase in alpha-diversity and beneficial microbes, such as Lactobacillus spp., suggesting a potential for prebiotic denotation of the product (Chapter 6). The additional fermentation of the flour with *L. plantarum* for 48 h led to an even higher alpha-diversity index at colonic level, making this product interesting from a nutritional, functional and organoleptic point of view.

Therefore, we provided evidence for the utilization of microbial fermentation of pulse seeds with *L. plantarum*. The right match of microorganism and substrate, and an appropriate fermentation time are of primary importance for achieving the targeted outcomes.

5. Methodological aspects and limitations

5.1 Static *in vitro* digestion model

As previously mentioned (paragraph 2.1), gastrointestinal digestion has a profound effect on the physicochemical structure in which bioactive compounds reach the sites of absorption and bioactivity. Great variety exists in the approaches reported in literature for simulating this step, from simplified models in which only the enzymes are applied, to complex systems in which motion is included that resembles peristaltic movements. The INFOGEST model was used in our study. This consensus method was developed as a tool to attempt to standardize *in vitro* digestion of foodstuff among laboratories. Particular attention is placed on the enzyme activity and salt concentrations of the oral, gastric and intestinal fluids, which were established from human *in vivo* data. Moreover, the physiological relevance of the model was recently validated for protein rich-foods against *in vivo* data from pig digestion, with good correlation in terms of protein digestion at the end of the gastric and intestinal phases.[23] As pulses are protein-rich foods, we therefore expect good correlation of our findings with *in vivo* application. The simple set up and detailed description of the method allows for standardization and applicability in different laboratories set-ups. Indeed, good replicability was observed among the samples digested in our study. In Chapter 5, we duplicated the *in vitro* digestion for every biological (fermented) sample, in order to test whether batch variabilities would affect oligosaccharides composition. Interestingly, the method showed high-level replicability, with almost identical replicates. Moreover, good replicability was observed among digested biological replicates in terms of volatile compounds (Chapter 5) and peptides (Chapter 4) composition, implying that the *in vitro* digestion step did not add variability to the results. INFOGEST is a static model with motion applied by shaking or rotating the samples during the incubation. This ensures constant interaction of the enzymes with the substrate but is of considerably lower intensity compared to the peristaltic movements *in vivo*. This is a major limitation of this system when performing digestion of solid food, with implications on the degree of disintegration and particles size of the bolus and chyme. Dynamic models have been recently developed to address this issue, however due to their complexity and specificity, they require validation for the different food categories, which is still missing in many cases.[24] In our study, we bypassed this limitation by grinding the samples to flour prior to digestion. Mechanical damage of pulse seed cotyledons was observed to cause disruption of the cell wall and increase digestibility of starch.[25] Therefore, adding a grinding step prior to digestion could affect the results of studies focusing on the digestion of intact pulses. However, as we suggest the application of bioprocessed pulses as functional flour ingredient, this did not affect the outcome of our study. The discontinuity between compartments and in enzymes inclusion and pH change is a second major limitation of this model, which could have implication on food structure and the gradual accessibility of nutrients.[24] To address this issue, recent research is looking at semi-dynamic *in vitro* models based on the INFOGEST method[26], but this model still needs to be validated and was therefore not chosen in our study.

5.2 Polarized Caco-2 cell monolayer to assess transport of nutrients and bioactive compounds, and DPP-IV inhibitory activity

Another aspect that is not considered by the INFOGEST model is the activity of intestinal brush border enzymes, which can have a profound effect particularly on peptides and phenolics absorption and on the related bioactivity. Since a major aspect of our study focuses on the bioactivity of pulse-derived bioactive components, and particularly peptides, we addressed this issue by combining the INFOGEST *in vitro* gastrointestinal digestion to an intestinal cellular model. We chose to use Caco-2 cells because of their validated absorptive capacity[27] and documented use in bioactivity studies, particularly for food-derived bioactive peptides and phenolics.[2,9,28] While originally isolated from colonic carcinoma

tissue, this cell line spontaneously differentiates into a small intestine phenotype after 15-21 days of cultivation from confluence. Growing the Caco-2 cells on filter supports allows for better functional differentiation and polarization of the monolayer, with typical microvilli on the apical side and tight junctions formation.[29] This further allows the creation of a distinct basolateral chamber where the transported molecules can be collected for characterization. The expression of major nutrient transporters for sugars, amino acids, di- and tripeptides, and vitamins characteristic of the enterocytes was previously demonstrated in Caco-2 cells.[30] Particularly, a minimum of 21 days were reported as needed to have an homogeneous expression of peptides transporter and an overall stable apical uptake capacity[31], which is the time we chose for our transport and peptidomics studies. Moreover, the Caco-2 cell line was previously reported to express dipeptidyl-peptidase-IV, and successfully used to assess *in situ* DPP-IV inhibitory activity of food-derived inhibitors.[32] This was also the case in our study, where we measured an initial DPP-IV activity of 1500 U/mg protein in cells extracted from our monolayer (Chapter 3). Nonetheless, the Caco-2 cell line carries some limitations. Being originally cancer cells, Caco-2 cells harbor parallel patterns of gene expression of enterocytes and human colon cancer when undifferentiated.[33] However, they express morphological and functional traits of mature small intestinal enterocytes when fully differentiated (~ 21 days).[34] Therefore, it is important to apply proper growing conditions and time to ensure enterocytic phenotype. Moreover, enterocytes are only one of the cell types present in human intestinal epithelium. Importantly, goblet cells, responsible for mucin production, are not included in this model. The mucus layer can affect nutrients absorption, therefore we cannot immediately extrapolate our results to the *in vivo* scenario. However, the model has extensive and validated use for drug absorption. A good correlation between *in situ* DPP-IV inhibition in Caco-2 cells and ex *vivo* human serum was previously observed for sitagliptin and legume-derived peptides.[35] Similarly, we expect that our bioprocessing would show the same trend of inhibition (Chapter 3) in *ex vivo* or *in vivo* models, although possibly at a different intensity. Mice models have also being used to address this issue as DPP-IV is also expressed in murine epithelial cells harboring similar functionality.[36] However, major differences exist in the gastrointestinal system of humans and mice concerning both the anatomy and food transit time.[37–39] This can have important implications on the digestion and biochemical structure in which the bioactive components from food reach the intestinal epithelium. Therefore, animal models would not be the most suitable models for food and nutrition research. Differently, innovative approaches such as the use of intestinal organoids or the intestine on-a-chip have the potential to provide physiological human models for intestinal absorption that could closely resemble the *in vivo* response.[40]

5.3 Simulation of the colonic fermentation

As described in chapter 1, various *in vitro* and *ex-vivo* models have been developed in recent years for simulating the colonic fermentation by commensal bacteria. In this thesis, we used two of these models: a simple batch fermentation (Chapter 5), and a highly complex continuous fermentation system (NuGUT, Chapter 6). In both cases, the inoculum was derived from human faeces; however, in the second model the isolated bacteria were then immobilized on gellan/xanthan beads. This allows a gradual and sustained release of the microbes over stabilization time, with validated retention of the microbial diversity of the original inoculum.[41] The two systems allowed us to answer different research questions. In Chapter 5, we aimed at comparing the implication of pre-fermentation with *L. plantarum* of yellow pea and green lentil flour on the colonic fermentation, with particular focus on the SCFA production. As we wanted to compare various pre-treatments time and from different legumes, a batch fermentation system was considered optimal, allowing cost-effective and quicker screening. In

chapter 6, we wanted to have an in depth understanding of the implications of the pre-treatment of an individual sample that showed interesting results in terms of bioactivity, aroma and decreased flatulence factors. In this case, the main interest was in the microbial profile and change in abundances of specific species in response to the treatment over time, therefore a more complex system was required. In fact, as showed from the results of Chapter 6, the microbial diversity, counts and ratio between species were retained over time. This would have been unlikely with a batch system, as the accumulation of metabolites, change in pH and depletion of media would likely affect the microbial profile over time. However, the use of highly complex systems such as the NuGUT comes at the expense of a limited possibility for biological replicates, due to the elevated experimental time and costs. Moreover, physiological parameters such as motility and absorption from the colonic epithelium are missing in both systems. Another important limitation of our approach was the lack of absorption step from the small intestine to the large intestine. This is a limitation not yet solved by many of these *in vitro* models, however many laboratories use dialysis to address this issue. Still, dialysis is purely based on size, while it is known that size is only one of the determinant of intestinal transport, while a large part of the absorption is regulated by transport systems that selectively transport substrates based on other parameters such as charge, polarity and hydrophobicity. Though, by using the digested samples as they are, a higher amount of simple carbohydrates was likely present compared to the *in vivo* situation. Because of this, the prebiotic activity suggested in Chapter 6 for the lentil flour cannot be extrapolated as it is, and further investigation is needed to validate this observation. Nevertheless, the use of unfermented flour as a control of the solid-state fermented flour allowed us to drive reliable conclusions regarding the implications of the pre-treatment, which was the main objective of our study (Chapter 6).

6. Implications of findings and future directions

6.1 An holistic approach to assess bioactivity

The approach used in this thesis differs from the majority of the studies on bioactivity reported in literature as we chose to always retain the complete composition of the pulse seed throughout the investigations on bioactivity, intestinal transport, aroma and colonic fermentation. On the contrary, common studies on bioactivity and transport are generally designed to test individual food components at time, such as phenolic extract or protein isolate, in order to unequivocally assign a certain behavior to a specific compound. This certainly provides interesting information regarding the potential bioactive components of foods. However, as previously discussed in the thesis, the food matrix plays a tremendous role in defining interactions between macro and micronutrients and their subsequent digestion and absorption. We suggest that it is therefore not possible to draw conclusions about the bioactivity of a food product when the bioactive components are analyzed individually. With this rationale, this thesis suggests a novel holistic approach to study food-derived bioactivity. The contribution of the individual components can still be evaluated in a second stage but should be in the context of a standardized food matrix. We further suggest that the bioactivity is likely to result from a contribution of multiple bioactive components rather than from a unique constituent. While with this approach it is certainly more difficult to draw conclusions regarding the molecular mechanisms of the bioactivity, we strongly believe that a closer correlation to the *in vivo* bioactivity can be expected.

6.2 Inclusion of the fermented flours to functional food products and *in vivo* assessment

As mentioned in paragraph 3, this thesis provided evidence for the use of bioprocessing as a valuable tool for improving bioactivity, aroma profile and flatulence factors of pulse seeds in a reproducible

way. The value of each bioprocess was specific for each pulse seed, with the results from Chapter 2 highlighting some of these relationships. Particularly, we observed that solid-state fermentation of green lentil flour significantly improved the DPP-IV inhibitory activity. This was further validated in the Caco-2 cellular model (Chapter 3), offering the *in vitro* evidence needed for moving to a clinical trial. As a follow up step, we therefore suggest the inclusion of lentil flours as main ingredients of food products to be tested in humans for validation of the bioactivity on postprandial glycemic control, incretin effect and insulin release. Moreover, another bioprocess showed a remarkable activity *in vitro*, which was the germination of chickpea seeds for 3 days. The significantly improved bioactivity in this case, that is α-glucosidase inhibition. Further investigation of this bioprocessing in cellular, tissue or *in vivo* models is suggested.

6.3 Computational bioprocessing: computational selecting enzyme-functions through genome databases

An important component of the thesis was to evaluate the use of microbial fermentation in place of other industrial processes, to break down the cell wall structure and biotransform the potential bioactive components. In fact, as described in section 4 of this chapter, each microbe carries its own specific set of metabolic pathways that can be activated according to the external stimuli. This is defined by the set of genes and putative enzymes enclosed in the chromosome and plasmids. As whole genome sequencing became more and more accessible over time, thousands of microbial genomes became available in databases such as the Integrated Microbial Genomes (IMG) and the Kyoto Encyclopedia of Genes and Genomes (KEGG).[42,43] Similarly to the approach we used for the commercial strain *L. plantarum* ATCC8014, we envision the opportunity to use the information from these databases in order to select potential unexplored strains to carry out specific functionalities. While the activation of specific pathways depends on the substrate and the environmental conditions, good guesses can be made based on the encoded number of specific genes as well as the source of isolation of the bacterial strain.

With a growing interest in minimally processed and "natural" foods such as the microbially fermented ones, we believe that there is a great and still unexplored potential in using the sequenced bacteria for developing novel plant-based fermented foods providing additional health benefits to the consumers, and decreasing the environmental footprint derived by extensive industrial processing.

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APPENDIX

Thesis Summary

Summary

Noncommunicable diseases are on the rise, with unhealthy diets being one of the major contributors. Food consumption should therefore be rethought as a strategy to prevent the diseases. Pulses are an excellent candidate for this transition, being a rich source of macro and micro-nutrients, fibers and bioactive compounds, with low fat content. However, the low digestibility of the plant food matrix by humans hinders their nutritional and functional benefits. Alongside, the consumer choice of pulse products remains low in Western counties. Off-flavours attributes such as "beany, green, earthy" are commonly associated with green lentil and yellow pea consumption. Formation of flatus, primarily associated with the high content of raffinose family oligosaccharides (RFOs), is a second major problem. To promote inclusion of pulse products in daily diets, these limitations have to be addressed. In this thesis, we explored the use of natural processing methods, namely germination and bacterial fermentation, to address the major issues involving acceptability and digestibility of pulse seeds, and enhancement of the bioactivity towards improving glucose regulation in humans.

Digestibility and bioactivity were the focus of **chapter 2**. DPP-IV and α-glucosidase inhibitors have been observed in a wide variety of plant-derived foods isolates such as purified protein hydrolysates and phenolic extracts. However, little attention has been placed on the interaction of these bioactive components within the seed matrix, and the related bioaccessibility issues. In this chapter, we investigated the implications of germination and microbial fermentation on seed microstructure and cell wall integrity, on the bioaccessibility of peptides and phenolics, and the *in vitro* DPP-IV and α-glucosidase inhibitory activity. We identified a potential in germinating chickpeas for 3 days, in regard to its significant increase in α-glucosidase inhibition. We also identified a potential in fermenting green lentil flour, in regard to its significantly improved DPP-IV inhibitory activity.

Following up on the findings from chapter 2, in **chapter 3** we investigated the effect of fermentation of green lentil with *L. plantarum* on circulating and cell-bound (*in situ*) DPP-IV inhibitory activity in polarized Caco-2 cells. We observed a dose-dependent inhibition, which however was peaking within the exposure of 500 mg/mL of digested product. Increasing fermentation time from 48 to 72 hours led to an increase in bioactivity. The inhibition was more pronounced *in situ* than on the circulating DPP-IV. We further tested if the bioactivity would extend to the glucose transport enzymes, by measuring the transport of glucose across the cell monolayer. This was not affected, confirming that the beneficial effect of the bioprocessing was centred on DPP-IV inhibition, therefore confirming the observation from chapter 2.

An important component potentially involved in the DPP-IV inhibitory activity of pulse-foods are bioactive peptides. Recent studies have emerged, investigating the bioaccessibility and intestinal transport of purified bioactive peptides, however comprehensive studies investigating the proteome of pulse foods and related potential functionalities are still scarce. In **chapter 4**, we assessed the impact of gastrointestinal digestion and *L. plantarum* fermentation on the formation of bioaccessible peptides. We further evaluated the transport of such peptides across a Caco-2 cell monolayer. Overall, 16.1 – 24.5 % of the bioaccessible peptides were transported. Interestingly, we observed a size and weight effect on the intestinal transport, as peptides larger than 2000 Da or 22 amino acids were not transported. 63 peptides were formed upon brush border enzymatic activity, and were more negatively charged and less hydrophobic compared to the other peptides identified in the study. Additionally, *L. plantarum* fermentation led to the formation of more hydrophobic peptides, suggesting higher bioactivity. The amino acids located in P_1, P_2, P_3 and C-terminal of some of the hydrophobic peptides formed by *L. plantarum* fermentation also matched the ones predicting high

potential for DPP-IV inhibition. We speculate a role of these peptides in the improved DPP-IV inhibitory bioactivity of fermented lentil samples observed in chapter 3, although further studies are needed to confirm this observation.

Lastly, this thesis aimed at addressing two important factors currently limiting consumer choice for pulse foods, which are flavour and intestinal discomfort. These issues were addressed in **chapter 5**, where we profiled the volatiles present and/or formed during microbial fermentation. We then profiled the major RFOs, to investigate whether it was possible to decrease them while retaining the related potential prebiotic functionality. As a result, we observed that fermentation of the two pulses with *L. plantarum* was effective in depleting the RFOs, without affecting the short-chain fatty acids profile. The bioprocessing additionally improved the aroma profile of green lentil, while the effect on yellow pea was less clear. The 48 h fermentation of green lentil flour therefore emerged as promising treatment to lower RFOs content and improve aroma. This was tested in an *ex-vivo* system simulating the fermentation of the product by colonic microbiota over a period of 3 days, to see if the potential prebiotic effect was also retained. An increase in beneficial commensal species was observed in both unfermented and fermented green lentil samples compared to the control, suggesting a potential prebiotic designation for both products. *L. plantarum* pre-fermentation was not compromising the functionality of the lentil flour on the gut microflora.

To conclude, in **chapter 6** we discussed the results as a whole, looking at the major findings of the thesis and their implication for the development of novel pulse-based foods with enhanced functionality and improved acceptability. We further discussed the methods used for addressing these issues and their limitations. Finally, we looked at future directions set by this work, and the recommendations for future research on the topic.

APPENDIX

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APPENDIX

About the author

About the author

Elisa Di Stefano was born on 28 April 1991 in Moncalieri, Italy. She obtained her BSc degree in Food Technology from the University of Turin (Italy) in 2014, with a thesis entitled “The use of probiotic microorganisms as functional components in the food industry”.

She then moved to The Netherlands for following the MSc program in Food Fermentation and Biotechnology and a MSc minor in Physical Interactions in Food at Wageningen University, completed in 2017. For her MSc. thesis, she worked on investigating the plasmids-encoded functionalities in dairy strains *Lactococcus lactis* subs. *diacetylactis* FM03 and *Leuconostoc mesenteroides* FM06 at the Laboratory of Food Microbiology. As an internship, she spent 6 months at the Canadian Centre for Human Microbiome and Probiotic Research (Canada), where she joined Prof. Reid lab working on the development of a novel fermented food product functioning as delivery vehicle for probiotic strain *Lactobacillus rhamnosus* GR-1, using locally available and affordable grains in Uganda. This was in collaboration with the Yoba For Life foundation.

In May 2017, Elisa started her PhD project in collaboration between the Food Quality and Design group at Wageningen University and the School of Nutrition Sciences at the University of Ottawa (Canada). The results of her research are presented in this thesis.

Overview of completed training activities

Discipline specific activities

Courses

- Intestinal Microbiome of Humans and Animals, VLAG, Wageningen, NL, 2019
- Big Data Analysis in Life Science, VLAG, Wageningen, NL, 2019
- Metabolomics Data Processing and Data Analysis, Birmingham University, Online, 2020
- Chemometrics, VLAG, Wageningen, NL, 2020
- VLAG Online Series, VLAG, Online, 2020
- Energy Metabolism and Body Composition in Nutrition and Health Research, VLAG, Online, 2021

Conferences

- AOCS Annual Meeting & Expo, AOCS, Minneapolis, USA, 2018[1],[2]
- CIFST Annual Meeting, CIFST, Niagara on the Lake, CA, 2018[2]
- Food and Function Symposium, University of Ottawa, Ottawa, CA, 2018[1]
- AOCS Annual Meeting & Expo, AOCS, St. Louis, USA, 2019[2]
- 33rd EFFoST International Conference, EFFoST/IUFoST/Elsevier, Rotterdam, NL, 2019[1]

General courses

- Presenting with Impact, VLAG, Wageningen, NL, 2019
- Critical Thinking and Argumentation, WGS, Wageningen, NL, 2020
- Career Perspectives, WGS, Wageningen, NL, 2020
- Introduction to R, VLAG, Wageningen, NL, 2020
- Applied Statistics, WGS, Wageningen, NL, 2020
- Adobe InDesign – from Dissertation Layout to Poster Design, WGS, Online, 2021
- Scientific Artwork, Data Visualization and Infographics with Adobe Illustrator, WGS, Online, 2021

Other activities

- Randomized Clinical Studies (MSc course), WUR, Online, 2018
- Preparation of Research Proposal, FQD, NL, 2018
- Weekly lab meetings, School of Nutrition, UOttawa, CA, 2017-2019
- Meetings and colloquia, FQD, Wageningen, NL, 2019-2021

[1]oral presentation

[2]poster presentation

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences

WGS: Wageningen Graduate School

WUR: Wageningen University & Research

FQD: Food Quality and Design group

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