Gastrointestinal digestion of food proteins under the effects of released bioactive peptides on digestive health

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Abbreviations

β -casomorphins (β -CMs); Bowman-Birk inhibitor (BBI); Dextran sulfate sodium (DSS); European Food Safety Authority (EFSA); Gastrointestinal tract (GIT); Kunitz-trypsin inhibitor (KTI); Reactive oxygen species (ROS)

Keywords

Bioactive peptides; Digestive health; Gastrointestinal digestion; Milk proteins; Soybean proteins

Abstract

The gastrointestinal tract represents a specialized interface between the organism and the external environment. Because of its direct contact with lumen substances, the modulation of digestive functions by dietary substances is supported by a growing body of evidence. Food-derived bioactive peptides have demonstrated a plethora of activities in the organism with increasing interest towards their impact over the digestive system and related physiological effects. This review updates the biological effects of food proteins, specifically milk and soybean proteins, associated to gastrointestinal health and highlights the study of digestion products and released peptides, the identification of the active form/s, and the evaluation of the mechanisms of action underlying their relationship with the digestive cells and receptors. The approach towards the modifications that food proteins and peptides undergo during gastrointestinal digestion and their bioavailability is a crucial step for current investigations on the field. The recent literature on the regulation of digestive functions by peptides has been mostly considered in terms of their influence on gastrointestinal motility and signaling, oxidative damage and inflammation, and malignant cellular proliferation. A final section regarding the actual challenges and future perspectives in this scientific topic is critically discussed.

1. Introduction

Several functions of the organism are regulated by endogenous proteins and peptides such as hormones, enzymatic mediators or neurotransmitters. During digestion of dietary proteins, a large number of peptides and amino acids are released. These food-derived peptides might share structural similarities with those endogenous peptides and hereby, exert a biological effect by interacting with the same receptors of the organism. Hence, bioactive peptides are defined as biologically active sequences derived from food proteins that are able to exert a positive effect due to its health-promoting properties. They are inactive within the parent protein but display the physiological effect once released by enzymatic hydrolysis during food processing and/or gastrointestinal digestion.^[1] The gastrointestinal tract (GIT) is in contact with several luminal compounds, such as dietary proteins, whereas it expresses a broad range of receptors and regulatory signals, and responds to the effect of orally ingested bioactive compounds. Hence, the modulation exerted by the diet over the physiological functions of the digestive system has been established as a key element for the maintenance and improvement of general health.^[2]

It results of particular interest understand the changes that gastrointestinal digestion induces over proteins by analyzing both protein degradation and peptide release in the GIT. Food peptides released during gastrointestinal digestion might exert local effects through interaction with digestive receptors and cells, or systemic effects, once they resist digestion, are transepithelially absorbed, metabolized, and reach target tissues/organs in an active form.^[3] To claim a cause-effect relationship between bioactive peptides and health, studies evaluating these aspects as well as the mechanisms of action involved and the identification of the active form/s are needed. Therefore, the interest over the interactions between food proteins and derived peptides and the components of the GIT need to be addressed by the scientific community. These studies point to a valuable information of physiological relevance both for the promotion of digestive health and the prevention of related chronic disorders, as well as for the formulation and design of peptide-based novel nutraceutical compounds. This review will

gather the current literature of food science studies addressing this topic but mainly focused on milk and soybean proteins as these foods are largely consumed worldwide, mostly milk that provides essential nutrients for the infant growth and development. Moreover, proteins from these two sources are representative examples of the most studied animal and vegetal proteins in this scientific field, with controversial results about the potential health effects attributed to soybean. We propose a double approach by exploring not only the modifications that food proteins and peptides undergo during gastrointestinal digestion, but also the mechanisms of action involved in the effects that bioactive peptides might exert in the digestive system.

2. Influence of protein digestion and sequences release on bioactivity of food-derived peptides

Bovine milk has approximately 3.0-3.5% content (weight:volume, w:v) of high-quality and highly digestible proteins. Considering their solubility at acidic conditions (pH 4.6, 20 °C), milk protein is mainly composed of casein (80%) and whey protein (20%), being classified in four different casein fractions (α_{s1} -, α_{s2} -, β -, and κ -casein) and three major whey proteins (α -lactalbumin, β -lactoglobulin, and bovine serum albumin), respectively.^[4] Soybean has a high protein content (35-45%, w:w). Soybean protein has a major component of storage globulins (70-83%), mostly consist of two subgroups termed as β -conglycinin and glycinin, in addition to a complex mixture of polypeptides such as lectins, lipoxygenases, α -amylases, protease inhibitors, and bioactive peptides.^[5,6]

Functionality of food proteins is influenced by their structural and physico-chemical properties. These properties have been demonstrated to have a key role on digestion, bioavailability and release of active sequences from both milk,^[7] and soybean proteins.^[8] Bioactive peptides derived from these food protein substrates have demonstrated multiple activities over the last years with increasing studies in the literature. Nevertheless, as evidenced by the literature search strategies summarized in **Supplementary Table S1**, studies regarding both the influence of digestive processes on these activities and the biological relevance of

peptides at this level have been less explored. Hence they account for a 10.1-20.8% of the total studies, considering those included in the research areas most related to the field.

2.1. Studies on digestion of milk proteins

Digestion of milk proteins has been evaluated in numerous studies mostly by using simulated models of gastrointestinal digestion in conjunction with analytical techniques of separation and identification based on HPLC-MS.^[9] These studies can have a “targeted” approach, being focused in specific sequences of particular interest because of their bioactive or allergenic potential,^[10,11] or either an “untargeted” approach, aiming to reveal a general analysis of the protein modifications and identify the total peptides released during digestion, i.e. the peptidomic profile. As an example, the latter strategy would be the case of the study of Jin *et al.*^[12] which identified the total peptides formed during fermentation and subsequent simulated gastrointestinal digestion of yoghurt. Similarly, the behavior of milk proteins subjected to *in vitro* digestion under physiological conditions followed by simulated transepithelial intestinal transport has been recently evaluated.^[13,14] Therefore, the aim of these studies was to track the fate of milk proteins in the GIT and, by incorporating cell absorption models as additional strategy, they approached the conditions of *in vitro* protocols to the real physiology of the GIT during *in vivo* digestion.

Few studies have evaluated the gastrointestinal digestion of milk proteins in the human setting. The first investigations already demonstrated that different milk proteins have diverse behavior during digestion. In humans, it was found that β -lactoglobulin and α -lactalbumin reach the upper jejunal compartment in an intact form in a 64 and 44%, respectively,^[15] whereas more than 60% of lactoferrin is not degraded in the stomach.^[16] More recently, a meta-analysis of controlled clinical trials by Bendtsen *et al.*^[17] has evidenced that “fast-digested” whey proteins have an acute effect on satiety by inducing the secretion of incretins glucagon-like peptide 1 and gastric inhibitory polypeptide, while the “slow-digested” casein fraction is more related with

long-term properties. Authors suggested that despite data were inconclusive and more studies are still needed, there is some evidence indicating that differences in, i) kinetics of protein hydrolysis, ii) amino acid composition, iii) rate of absorption and iv) protein/food texture, may have an impact on metabolic aspects related to hunger including the post-prandial use of protein nitrogen, the flux of peptides and amino acids towards bloodstream, and the secretion of hormones and enzymes.^[17]

The identification of peptides in the gastrointestinal lumen during digestion of milk proteins has been documented in animals,^[18–22] and humans,^[23–27]. However, data are still scarce and there is a high diversity between these investigations regarding species, ingested products, dairy matrix, gastrointestinal compartments evaluated and analytical techniques used.^[28] The development of proteomic and peptidomic MS-based analytical tools has allowed a better identification of sequences in complex samples such as *in vivo* gastrointestinal digests. Hence, the study of Boutrou *et al.*^[26] with human jejunum digests allowed the identification of a total of 146 and 356 peptides after the oral administration of whey proteins and caseins, respectively. Casein peptides derived mostly from β -casein (61.2%) and α_{s1} -casein (24.9%), while β -lactoglobulin, specifically in the regions f(40-58) and f(122-137), gave rise to the majority of whey peptides (72.0%). Interestingly, numerous sequences with previously described bioactive effects were found, such as 27 and 32 precursors from the anti-hypertensive peptides IPP and VPP, respectively, and 15 precursors from the opioid sequence YPFPGPI.^[26] More recently, analysis by MS/MS has also revealed the presence of milk peptides in the stool of infants, hence surviving the *in vivo* gastrointestinal digestion.^[29] Moreover, by *in silico* analysis, authors found that some of these peptides were homologous to known bioactive sequences with antimicrobial, DNA-stimulating, and antioxidant activities, hence, carrying protective effects that might act on intestinal cells and microbiota and positively influence the proper development of the infant gut.^[29]

In this context and with the aim of unifying the different investigations on *in vitro* methods of gastrointestinal digestion, the static protocol of Minekus *et al.*^[30] has been built by consensus of different research groups under the COST Action INFOGEST. Thus, this international network aims at overcoming the wide variation between the individual parameters previously used in those *in vitro* studies simulating the gastrointestinal digestion.^[31] Noteworthy, since its publication in 2014,^[30] this harmonized protocol has been applied in multiple investigations reaching over 1.000 citations in the last five years (2015-2019) from different research institutions worldwide (**Figure 1**). Regarding milk proteins, the consistency of this digestion method has been validated in comparison to pig^[22] and human^[27] digests. Hence, the peptidomes found after digestion of casein and whey proteins by the INFOGEST *in vitro* method and *in vivo* digests obtained from the human jejunum were recently compared.^[27] These authors found that both digests presented comparable peptide profiles (i.e. the correlation for the *in vivo/in vitro* comparison was similar to that found for the human inter-individual variability), hence suggesting that the harmonized *in vitro* protocol constitutes a good approximation to the physiological gastrointestinal digestion of milk proteins.

2.2. Studies on digestion of soybean proteins

The exploration of digestion and bioavailability of soybean proteins and derived peptides has been less documented, as compared to milk proteins (**Table S1**). The composition of soy proteins as well as the conformation of its secondary structure have been discovered to influence its resistance to *in vitro* proteolysis and digestibility.^[32] Concerning the release of peptides during soybean digestion, the product of β -conglycin gave rise to peptides with ability to activate the cholecystokinin receptor type-1, related to hunger physiology.^[33] Likewise, peptides from digests of germinated soybean recently showed inhibition of dipeptidyl peptidase-IV, α -amylase, and α -glucosidase enzymes, and thus may modulate the postprandial glycemic response.^[34] Amigo-Benavent *et al.*^[35] described peptides with antioxidant activity in

the digests of β -conglycin and glycosylated β -conglycin, some of them were not only resistant to gastrointestinal digestion but also transepithelially absorbed through Caco-2 monolayers. By using this cell model, it has been also confirmed that hydrolyzed soy proteins delivers an enhanced intestinal transport of dietary amino acids in comparison to intact protein.^[36] The global peptidome of *in vitro* digests of proteins from soybean seeds and drinks was analyzed by nano-LC-MS/MS.^[37] This study found a high degree of hydrolysis leading to the generation of 2430 sequences with lengths from 5 to 49 amino acids. Hence, the authors suggested that the presence of naturally occurring protease inhibitors in soybean proteins, such as Bowman-Birk (BBI) or Kunitz-trypsin (KTI) inhibitors, might justify the presence of those larger sequences after the simulated digestion.

Among soybean bioactive sequences, lunasin is considered one of the most investigated bioactive peptides encoded within the soybean 2S albumin.^[38] In relation with the aforementioned hypothesis about naturally present protease inhibitors in soybean proteins,^[37] a study simulating the gastrointestinal digestion confirmed the protective role exerted by the major isoinhibitor of the BBI family (IBB1) over synthetic lunasin digestion.^[39] Moreover, these authors recently showed that both lunasin and its digestion-derived fragment f(11-18) are absorbed through paracellular diffusion in Caco-2 cell monolayers.^[40] In addition to BBI, the presence of the inhibitor KTI in soybean products has been also linked to the *in vitro* stability of lunasin towards digestive enzymes.^[41] Noteworthy, lunasin has been detected in the plasma of healthy male volunteers after intake of soybean protein (50 g during 5 days), representing a 4.5% of intestinal absorption.^[42] However, studies have been mostly performed *in vitro* using different conditions and lunasin's preparations,^[38] hence, the study of the behaviour of natural lunasin in the human GIT as well as the implementation of technologies to detect and quantify lunasin content in human samples might reveal interesting findings for the current knowledge of the bioavailability and bioactivity of this peptide.

3. Bioactivity of peptides over digestive health

GIT is also considered as an endocrine organ which harbors a broad range of receptors and regulatory mediators connected to multiple signaling pathways. Besides the regulation exerted by internal factors, external compounds, such as those derived from gut microbiota or foods, might also play a modulatory role over digestive functions. Currently, more than 70% of “Food for Specified Health Uses –FoSHU–” products commercialized as nutraceuticals in Japan are related to the functions of digestive system.^[43] However, most of them include probiotic bacteria and prebiotic compounds such as dietetic fiber and non-digested oligosaccharides. Regarding food proteins, to date, the European Food Safety Authority (EFSA) has only claimed the effect of proteins over the maintenance of bone tissue, growth and muscles.^[44] With a focus on digestive health, EFSA has approved the use of fiber from wheat bran to increase the weight of fecal material and lower the gastrointestinal transit time,^[45] as well as that of live bacteria from yoghurt to improve the digestion of lactose.^[46] Interestingly, the gut has much far-reaching impact, more than food processing and nutrient uptake, on the overall good health of the body; immune tolerance, defense against infections, and signaling to the brain. With accumulating evidence from animal experiments and epidemiological studies, “gut health” is now regarded as a new objective, not only a target of treatment to widespread gastrointestinal disorders but also an approach to maintain status of well-being and resist illness.^[47,48]

Therefore, the scientific interest towards the effects of food proteins and peptides over the digestive system has been prompted in the last years,^[2,49] as these activities may provide modulatory effects over several physiological functions (**Figure 2**). Effects of bioactive peptides contributing to digestive health might be thus related to properties over intestinal absorption, secretion and metabolism of nutrients,^[50,51] gastrointestinal barrier,^[52] opioid receptors,^[53] satiety regulation,^[54] and mucosal immunity.^[55,56] Moreover, as discussed in the following sections of the present review, food-derived bioactive peptides might carry modulatory effects

over gastrointestinal motility and signaling, oxidation and inflammation status, and preventive actions against malignant cellular proliferation; all events closely linked to the onset, development and progression of several digestive disorders.

3.1. Opioid receptors and gastrointestinal motility and signaling

There are some reports about the regulatory properties of food proteins over the motility and signaling of the GIT. Most of the bioactive peptides demonstrated to date for this activity derive from milk proteins, in comparison with soybean or other plant proteins. The mechanisms of action have been mainly attributed to opioid agonist and antagonist effects.^[57] Indeed, the GIT is rich in opioid peptides and opioid receptors that are broadly distributed along its surface.^[58]

Milk casein was found to prolong the gastric emptying and gastrointestinal transit time in rats, when compared to whey proteins.^[59] These authors also found that pre-treatment of animals with the opioid antagonist naloxone was able to abolish this activity, which prompted the hypothesis that casein-derived opioid peptides might be the responsible factors. The first opioid peptides identified from dietary proteins were termed β -casomorphins (β -CMs), due to their presence in a β -casein hydrolyzate. Experiments in animal models have shown that β -CMs are able to bind to specific opioid receptors located in the intestinal epithelium.^[60] Thus, β -CMs have been demonstrated to decrease the amplitude and frequency of small intestinal contractions,^[61] delay gastric emptying, and inhibit the transit of intestinal content in several species.^[62] Moreover, the direct interaction between β -CMs and gut opioid receptors, preferentially the μ -opioid subtypes, have revealed digestive effects in a naloxone-antagonizable manner, such as antidiarrheal action and stimulation of insulin and somatostatin production.^[63] Because of these modulatory properties on gastrointestinal motility and secretion of gut regulatory signals, dietary opioid peptides have been proposed to exert a role in the control of food intake and satiety.^[64] In addition, novel milk peptides derived from α_{s1} -

casein f(143-149) have been recently identified with *ex-vivo* opioid agonistic activity in guinea pig ileum and, to a lower extent, in mouse vas deferens preparations,^[65] similar to what was previously described with β -CMs. These authors demonstrated opioid activity but also related mucin-stimulatory effects on intestinal HT29-MTX cells. This may provide additional protective effects on digestive health by acting over the mucosal barrier so, subsequently, it was further confirmed the *in vivo* potential of a casein hydrolyzate containing these opioid peptides to induce the production of mucus and mucin gene expression in the rat intestine after 8 weeks of oral administration.^[66]

Bovine κ -casein caseinomacropeptide and derived fragments have been suggested to influence the rat digestive function by decreasing gastrointestinal motility and increasing gastric and pancreatic hormones secretion.^[20] From this milk protein, the antagonist opioid peptide casoxin C was proved to evoke a biphasic contraction in longitudinal strips of guinea pig ileum through complement C3a receptors.^[67] Muscle contraction assays are widely used in the pharmaceutical industry to characterize entities with activity on gastrointestinal motility. However, its application into food research has been scarce. Recently, the study of Dalziel *et al.*^[68] developed an *ex-vivo* rat model system to evaluate the effect of dietary opioid peptides on intestinal transit by using the milk peptide β -CM-5, as an example of a bioactive peptide that alters the intestinal motility by a known opioid mechanism. These authors found that the isolated whole large intestine preparation preserve intact the enteric neural circuitry, being results consistent with those above-mentioned for milk casein peptides *in vivo*^[59] and, thus, providing a reliable model of intestinal propagation.^[68]

Casein-induced slowing of gastrointestinal transit has been found to vary depending on the degree of protein hydrolysis. Mihatsch *et al.*^[69] demonstrated that rats fed native casein had slower intestinal transit than those fed extensively hydrolyzed casein, being this effect only reverted by naloxone in the former animal group. Interestingly, it has been recently shown the

ability of a tryptic β -casein hydrolyzate to enhance the maturation of the rat primary enteric nervous system, main regulator involved in intestinal motility and barrier functions.^[70] Concerning the whey fraction of milk, it has been found in the isolated rat distal colon assay that whey proteins concentrates decreased motility, while increased frequency of contractions was found for whey proteins hydrolyzates.^[71] These authors hypothesized that differences between abundance of bioactive peptides in the dairy preparations might explain the opposite effects, as the maximum degree of protein hydrolysis was associated to the highest motility in the tissue. Likewise, hypothesis that whey proteins show faster gastrointestinal transit than casein, being this accentuated in the case of hydrolyzates, was further proved *in vivo* by using an aged rat model.^[72] Noteworthy, these studies were in agreement with accelerated gastrointestinal transit of milk and stools in preterm human infants administrated hydrolyzed whey as compared to non-hydrolyzed standard formula.^[73]

Similarly, experiments with soy proteins found that the intestinal transit of dogs was slowed in a load- and hydrolysis degree-dependent manner.^[74] Hence, intact soy proteins notably inhibited intestinal transit when compared to the hydrolyzed formula. Regarding soybean opioid peptides, fragments with 5 to 7 residues derived from β -conglycinin β -subunit, termed as soymorphins, have been also identified and demonstrated to exert anxiolytic-like activity.^[75] Moreover, oral administration of these peptides to mice abrogated food intake and small intestinal transit, acting over opioid μ_1 -receptor and 5-HT_{1A}, D₂, and GABA_B systems.^[76] Nevertheless, studies in the human setting on this bioactivity are still very limited and are therefore needed to ultimately propose these beneficial effects in the human intestinal well-being.

3.2. Oxidative damage and inflammation

Reactive oxygen species (ROS) are continuously generated in cells as physiological mediators, under a delicate balance between ROS production and neutralization. In pathological

states, ROS production might overcome the cellular capacity to neutralize reactive intermediates leading to oxidative stress state. Disruption of the steady-state homeostasis by redox signaling is recognized as underlying cause of long-term cellular damage, altered functionality and mucosal injury, with subsequent development of chronic disorders.^[77] Moreover, the digestive system is particularly prone to the attack of oxidizing entities derived from pro-oxidant compounds in diet, drugs, toxins or xenobiotics, coupled to those endogenous reactive species highly produced at the luminal surface.^[78] In turn, inflammation can stimulate ROS-producing systems at the GIT, inducing mucosal oxidative stress.^[79] Therefore, there is a large evidence about the involvement of redox imbalance and chronic inflammation on the development of multiple digestive disorders such as gastroesophageal reflux, Barrett's esophagus, esophageal adenocarcinoma and squamous cell cancer, gastritis, peptic ulcers, gastric adenocarcinoma, inflammatory bowel diseases, enteric infections, ischemic intestinal injury, and colorectal cancer, among others.^[80] Counteracting these oxidative-inflammatory imbalances, peptides derived from a variety of food sources including both animal and vegetable proteins might represent an interesting source of protective agents for digestive health as reviewed elsewhere.^[81,82]

Two studies have demonstrated bovine colostrum's ability to reduce the gut permeability increased by indometacin,^[83] and to reduce the symptom scores allowing clinical remission in patients with distal colitis.^[84] Colostrum is the first diet of mammalian neonates and its rich content on immunoglobulins, antimicrobial peptides, and growth factors may offer protective properties for gastrointestinal conditions.^[83] Antioxidant milk peptides have been mostly found in the whey fraction. Whey protein hydrolyzates exerted a protective effect on H₂O₂-induced rat PC12 cells acting over the superoxide dismutase and catalase enzymes, malondialdehyde, ROS levels, and against apoptosis.^[85] Hydrophobic nature of these hydrolyzates was associated to their antioxidant effects.^[81] *In vivo*, similar activities over antioxidant defenses were demonstrated by using rat models of diabetes,^[86] hepatotoxicity,^[87]

and D-galactose-induced ageing.^[88] Interestingly, human clinical trials have found that the intake of a cysteine-enriched whey isolate restored the hepatic function, plasmatic glutathione, and total antioxidant capacity in non-alcoholic steatosis patients,^[89] while that of hydrolyzed whey could reduce the muscle damage, and the lactate dehydrogenase and creatine kinase levels in volunteers under high physical effort.^[90]

Regarding the milk casein fraction, caseinphosphopeptides protected intestinal Caco-2 cells induced by H₂O₂ through maintenance of cellular viability and cell cycle progress, increase of glutathione and catalase activity, and down-regulation of lipid peroxidation.^[91] Using the same pro-oxidant stimuli, hydrolyzed casein by alcalase and gastrointestinal enzymes showed antioxidant properties in hepatic HepG2 cells.^[92] Although the authors highlighted the activity of the bioavailable fraction < 1000 Da, the responsible peptides were not reported. Similarly, the anti-inflammatory *in vivo* potential of a β -casein hydrolyzate by microbial protease was demonstrated in murine colitis via improvement of antioxidant defenses and tolerogenic IL-10, as well as IFN- γ decrease.^[93] Later on, a bioactive β -casein f(94-123) peptide, termed as β -casofensin, was found to protect against intestinal epithelial injury induced by both the drug indomethacin and neonatal maternal separation, improving the mucosal barrier status of treated rats.^[94,95] In addition, the anti-inflammatory properties of caseinomacropptide have been confirmed in some animal models of intestinal inflammation.^[82]

The short-term intake of soybean (2 g/kg body weight, daily during 7 days) has been found to increase the total antioxidant activity of healthy men and women, and diminish the advanced oxidation protein products in the female group.^[96] Considering only the protein fraction of soybean, Amigo-Benavent *et al.*^[35] found that deglycosylation of β -conglycinin improved the oxidative damage of H₂O₂-induced Caco-2 cells. Similarly, it has been recently found that soy protein concentrate not only protects Caco-2 cells from this disruptive agent, mitigating the cytotoxicity and the intracellular oxidative stress, but also exerts this protective

effect over gut barrier function in dextran sulfate sodium (DSS)-induced colitis in mice.^[97] The liver is susceptible to toxic insults delivered from gut compounds via portal vein. Hence, at hepatic level, oral supplementation with soybean protein ameliorated the progression of non-alcoholic steatohepatitis in rats via activation of catalase and superoxide dismutase enzymes, cytochrome P450 2E1 down-regulation, and improvement of insulin resistance.^[98]

Considering soybean peptides, Young *et al.*^[99] found that a soybean hydrolyzate enriched with di- and tri-peptides offered protective properties in porcine models of intestinal inflammation, while this effect was subsequently associated to the activity of glycine-peptide VPY through the epithelial transporter PepT1.^[100] Likewise, very recently, four peptides (VVFVDRL, VIYVVDLR, IYVVDLR, AND IYVFVR) from soy proteins hydrolyzed by alcalase were identified by nano-LC-ESI-MS/MS, showing radical-scavenging activities, and cytoprotective effects in H₂O₂-challenged Caco-2 cells through enhancement of cellular redox status and inhibition of ROS generation, lipid peroxidation and IL-8 production.^[101] Lunasin has shown promising properties by protection of cellular viability and enhancement of antioxidant defenses against chemical-induced oxidative stress and inflammation in macrophages, enterocytes, and hepatocytes models.^[102–106] Soybean BBI concentrate showed antioxidant activity and reduced ROS and pro-inflammatory cytokine levels in LPS-activated macrophages.^[107] In mice colitis, Utrilla *et al.*^[108] demonstrated the beneficial role of both soybean BBI and pea seed albumin extract enriched in BBI to counteract mice DSS-induced inflammation. Treated groups showed reduced microscopic histological damage in comparison with untreated mice. Moreover, the treatments ameliorated the colonic mRNA expression of different pro-inflammatory markers and restored bacterial counts, partially or totally, to values of healthy mice.^[108] A randomized, double blind, placebo-controlled trial was performed to evaluate the safety and efficacy of soybean BBI concentrate in patients affected of active ulcerative colitis.^[109] After 12 weeks of treatment (800 units/day), BBI showed safety in patients, and potential benefit over placebo (50.0% *versus* 29.0%) to induce clinical response and disease remission as assessed by the Sutherland disease activity index.

3.3. Chemoprevention in the digestive system

Although chemotherapy and radiation are the methods of choice for cancer management, both therapies have low therapeutic indices, and are expensive and highly toxic with many adverse effects. This fact has increased interest in searching new anticancer compounds as alternative to chemotherapy drugs, which can reduce the cost and side effects. *In vitro* and *in vivo* studies, and epidemiological evidence have revealed the preventive role that dietary compounds might play against cancer.^[110] Accordingly, chemoprevention is defined as “the use of natural and/or synthetic substances to block, reverse, or retard the process of carcinogenesis”. Among food compounds, phytochemicals from fruits, vegetables, spices and grains might rank as the predominant chemopreventive agents in the literature.^[111] In the last years, chemopreventive compounds have been also identified within dietary protein fractions.^[55,112] Bioactive peptides can act against proliferative diseases by apoptosis and autophagy induction, inhibition of cell cycle progress, block of signaling pathways, modulation of gene expression, inhibition of angiogenesis and metastasis, as well as immunomodulatory, anti-inflammatory and antioxidant effects, among other mechanisms.^[113,114]

Considering the interaction between protein digestion products and the gastrointestinal luminal surface, an increasing evidence over the potential chemopreventive effects exerted at this level has been suggested.^[115] **Table 1** provides a summary of those chemopreventive effects of milk and soybean proteins and peptides as demonstrated by cell cultures of the digestive system, including the cytotoxicity values and the mechanisms of action involved. The anti-tumor activity of milk products has been attributed to both whey proteins and caseins and their derived peptides,^[116] although it is highlighted the activity of whey protein lactoferrin and its pepsin enzymatic digestion-derived peptide lactoferricin. Moreover, protein-fatty acids complexes of whey lactoferrin with oleic acid^[117] as well as that of whey α -lactalbumin termed as “bovine alpha-lactalbumin made lethal to tumor cells –BAMLET–”^[118] have demonstrated cytotoxic

effects in some tumor cells. Similarly, peptide fractions derived from hydrolyzates of high oleic acid soybean lines (N98-4445A and S03-543CR) showed resistance to simulated gastrointestinal digestion and cell growth inhibition in colorectal (HCT-116), liver (HepG2) and lung (NCL-H1299) cancer cell lines.^[119] Considering soybean compounds, BBI, KTI, lunasin and lectins are the most prevalent agents against different types of cancer, including those affecting the hepatic and colonic tissues (**Table 1**).

Some of these effects demonstrated by cell cultures have been further translated into *in vivo* findings. Years ago, the effects of bovine lactoferrin were investigated in colon carcinogenesis azoxymethane-induced murine models. Lactoferrin was shown to inhibit the development of aberrant crypt foci formation, considered as putative precursor lesions for colon tumors, and to reduce both the incidence and number of adenocarcinomas of the large intestine in treated rats.^[120] Lactoferricin has been also shown to inhibit the metastatic and angiogenesis capacity of malignant cells towards hepatic and lung tissues in xenograft animal models.^[121] Indeed, the oral administration of this milk peptide induced chemopreventive, anti-metastatic, and immunomodulatory effects in the intestinal mucosa via induction of caspase-1, secretion of IL-18 and IFN- γ , and activation of T-cell and natural killer activity.^[122] In the last years, some human trials have been performed. A randomized controlled trial was conducted to evaluate whether a 1-year oral intake of bovine lactoferrin-containing tablets inhibits the growth of colorectal polyps.^[123] These authors found a statistically significant retardation of polyp growth in individuals 63 years old or younger ingesting 3 g lactoferrin daily over the course of 12 months. Moreover, in a recent double-blinded parallel randomized controlled clinical trial, bovine lactoferrin was used as an adjuvant therapy for metastatic colorectal cancer patients.^[124] In this study, the oral intake of lactoferrin (250 mg/day) was found to improve symptoms of cancer, such as anemia, and decrease chemotherapy adverse effects by protecting liver and kidney from toxicity.

Soybean lunasin showed *in vivo* inhibition of the metastasis of colorectal KM12L4 cancer cells towards hepatic tissue in mice.^[125,126] These studies found anti-cancer effects by reducing the proliferating cell nuclear antigen, related to progression of cell cycle, and induction of apoptosis over Bcl-2:Bax signaling. However, differences between oral (up to 20 mg/kg body weight) and intraperitoneal (4 mg/kg body weight) administrations hindered the interpretation of results and suggested the potential degradation of this peptide and consequent loss of activity during the passage through the GIT,^[125,126] as it has been evidenced in aforementioned studies simulating the peptide's gastrointestinal digestion. The anti-carcinogenic properties of soybean BBI have been extensively investigated, both in purified form and as a BBI concentrate. In animal models, BBI showed suppressive effects on dimethylhydrazine-treated mice when BBI concentrate was administered.^[127] Later on, similar results were found by Kennedy *et al.*^[128] reporting protective effects of BBI concentrate when ingested at low concentrations (10 mg/100 g diet) in this animal model. A decrease of the frequency and incidence rates of colorectal tumors was observed without affecting the animal growth neither the organ/tissue morphology. Moreover, either soybean or perennial horsegram BBIs, when administrated at a dose of 30 mg/kg during 12 weeks, exerted a protective role in the development of pre-neoplastic lesions in mice induced by intraperitoneal injections of dimethylhydrazine.^[129] These effects seem to be associated to the inhibition of both the lysosomal and proteasome-dependent proteolytic pathways. However, autoclaved BBI, in which the protease inhibitory activity was abolished, had no significant effects, suggesting that the innate ability of BBI to inhibit serine proteases is required for their chemopreventive properties.^[128,130]

4. Challenges and future prospects

Bioactive peptides have been recognized as an important category of functional food ingredients, which exert various bioactivities and possible health promoting impact in the digestive system. However, in spite of the rising evidence on these benefits, the use of food

bioactive peptides is still limited and several challenges must be addressed. Firstly, the high cost of existing processes for peptide production implores the need to apply systemic approaches that develop innovative production and purification strategies to obtain bioactive peptides at high yield.^[131] Moreover, researchers have found that peptides with bioactivity *in vitro* do not always correlate with *in vivo* activity. Indeed, the physiological effects of bioactive peptides largely depend on their ability to remain intact after digestive processes. Peptides that resist the digestion and arrive intact at the intestinal absorption site can have a local function or may be able to cross the epithelium, enter the bloodstream, reach the target organ, and have a systemic effect. Therefore, as much as it is in pharmacology research, investigating the digestion and bioavailability of bioactive peptides has become a new trend but also a main challenge in the current functional food field.^[132,133] As an alternative to animal/human models of digestion, the COST action INFOGEST elaborated a harmonized static digestion method based on physiologically relevant conditions,^[30] which has been recently updated.^[134] However, it still presents the limitations of static *in vitro* models as simplified methods of the intricate *in vivo* digestion process. Digestion models restricted only to simulated gastric and intestinal phases provide limited information about the bioavailability and bioaccessibility of peptides. Thus, combination of these *in vitro* protocols with cells absorption models, highlighting in the current literature the use of Caco-2 cell monolayers, results in faster and reliable methods for estimation of peptides bioavailability. Moreover, there is an urgent need to search for protective mechanisms to maintain the peptides in active forms until consumption or even to directly deliver them to the physiological targets with considerable activity in the organism. Currently, different strategies including chemical peptide modifications, permeation enhancers, enzyme inhibitors, cell-penetrating peptides, and polymeric carrier and encapsulation systems are being investigated.^[135] In addition, to assess the digestion kinetics and biostability of encapsulated peptides to ensure an improvement in the controlled release, timing as well as precise targeting is an important issue.^[136] On the other hand, to increase certainty about bioactive peptides, the

design of clinical trials has evidenced unique challenges that need to be carefully addressed.^[137] Regarding activity in the digestive system, bioactive peptides has showed promising results in the human setting. However, these studies are still very limited and encourage the scientific community to obtain more robust information about the definitive role of bioactive peptides in the GIT before their exploitation as novel functional compounds contributing to the digestive health.

Acknowledgements

This work has received financial support from the Ministry of Science and Innovation, Spain (MINECO, Spain) through project AGL2015-66886-R. S.F.T. is currently funded by the Instituto de Salud Carlos III (Sara Borrell fellowship CD17/00014). Authors kindly acknowledge Dr. Fernández-Fuertes for her excellent assistance on figure preparation.

Conflict of interest

Authors have declared no conflict of interest.

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Figure legends

Figure 1. Analysis of research documents quoting the harmonized *in vitro* digestion method of Minekus *et al.*^[30]. Citations and data were extracted from Web of Science (April 10th, 2020). (A) Number of times cited by year. (B) Percentage corresponding to the countries where the publications were conducted. The order of the legend is listed following a decreasing percentage. A total of 53 countries/regions displaying the lowest percentages were included in Others. 3 records did not contain data in the field being analyzed.

Figure 2. Schematic representation of the different physiological functions/levels of the organism where bioactive peptides might act through interaction with the digestive system.

755 **Table 1. Milk and soy proteins and peptides with chemopreventive activity in the digestive system as demonstrated by cell cultures.**

Source	Protein - Peptide	Cell line (tumor type)	Cytotoxicity, IC ₅₀	Mechanisms of action	Ref.
Milk	Lactoferrin	Colorectal carcinoma HCT-116	~ 5.0 mg/mL	* Antioxidant effects	[138]
	Lactoferricin	Oral carcinoma SAS	~ 1.0 mg/mL	* Caspase-3 and PARP pathways activation	[139]
				* ERK1/2 and JNK/SAPK pathways phosphorylation	
		Colorectal carcinoma C26	111.0 µM	* Cell membrane disruption	[140]
		Colorectal adenocarcinoma HT-29	~ 0.1 mg/mL	* Apoptosis induction	[141]
				* DNA fragmentation	
		Colorectal adenocarcinoma Caco-2	-	* S phase arrest	[142]
				* Cyclin E1 decline	
	Lactoferricin-OA	Colorectal adenocarcinoma HT-29	5.0 µM	* Apoptosis induction	[117]
		Hepatocellular carcinoma HepG2	4.9 µM	* Mitochondrial and cell death receptor pathways induction	
	α-lactoalbumin-OA	Colorectal adenocarcinoma HT-29	45.8 µM	* Apoptosis induction	[117]
		Hepatocellular carcinoma HepG2	50.2 µM	* Apoptosis induction	

	Seleno- β -lactoglobulin	Hepatocellular carcinoma HepG2	280.1 mg/mL (24h)	* Extrinsic apoptosis induction	[143]
			237.7 mg/mL (48h)	* Up-regulation of protein levels of p53, Fas and	
			192.5 mg/mL (72h)	FasL	
				* Activation of caspases 3 and 8, and cleaved-PARP	
				* ROS generation induction	
				* Cell cycle arrest in S phase	
		Hepatocellular carcinoma Hep3B	291.6 mg/mL (24h)	* Extrinsic apoptosis induction	
			296.7 mg/mL (48h)	* Activation of caspases 3 and 8, and cleaved-PARP	
			269.7 mg/mL (72h)	* ROS generation induction	
				* Cell cycle arrest in G0/G1 phase	
	Casein phosphopeptides	Colorectal adenocarcinoma HT-29	-	* Caspase-3/7 pathway induction	[144]
				* Voltage-gated calcium channels activation	
Soy	Lunasin	Colorectal adenocarcinoma HT-29	61.7 μ M	* G2/M phase block	[145]
				* p21 increment	

		<ul style="list-style-type: none"> * Mitochondrial apoptosis pathway induction. Bax, nCLU and caspase-3 increment. Bcl-2 decline * Synergetic effect with cisplatin 	
Colorectal carcinoma KM12L4	13.0 μ M	<ul style="list-style-type: none"> * G2/M phase arrest * p21 and p27 increment * Mitochondrial apoptosis pathway induction. Bax, nCLU, cytochrome c and caspases 3, 2 and 9 increment. Bcl-2 decline * Nuclear condensation and DNA fragmentation * FAK/ERK and NF-κB pathways activation * Integrin $\alpha_5\beta_1$ binding * Angiogenesis and metastasis modulation * Synergetic effect with cisplatin 	[125,146]
Colorectal carcinoma HCT-116	26.3 μ M	<ul style="list-style-type: none"> * FAK/ERK and NF-κB pathways inhibition 	
Colorectal carcinoma RKO	21.6 μ M	<ul style="list-style-type: none"> * FAK/ERK and NF-κB pathways inhibition 	

			* Cell migration inhibition	
	Colorectal adenocarcinoma HT-29	-	* Cell viability reduction	[40]
	Colorectal adenocarcinoma Caco-2			
	Gastric adenocarcinoma AGS			
	Colorectal carcinoma HCT-116	107.5 μ M (parental cells)	* Cell viability reduction	[147]
		161.0 μ M (tumorspheres)	* Inhibition of tumorsphere-forming activity	
			* Apoptosis induction	
			* Cell cycle arrest in G1 phase	
			* Activation of caspase-3, and cleaved-PARP	
			* p21 increment	
BBI	Hepatocellular carcinoma HepG2	140.0 μ M	-	[148]
	Colorectal adenocarcinoma HT-29	46.0 μ M (BBI)	* G0/G1 phase block	[149]
		48.3 μ M (IBB1)	* Protease-inhibitor-related activity	
		39.9 μ M (IBBD2)		
KTI	Hepatocellular carcinoma HepG2	> 25.0 μ M	* Cytokine induction	[150]

Lectin	Hepatocellular carcinoma HepG2	4.1 μ M	-	[151]
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OA: oleic acid, BBI: Bowman-Birk inhibitor, KTI: Kunitz trypsin inhibitor, IBB1 and IBBD2: Bowman-Birk iso-inhibitors, PARP: poly (ADP-ribose) polymerase, nCLU: nuclear clustering, ERK: extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinases, SAPK: stress-activated protein kinase, FAK: focal adhesion kinase, NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells, ROS: reactive oxygen species

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