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An overview of "omic" analytical methods applied in bioactive peptide studies

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ABSTRACT

Nowadays, there is an increased interest in health-promoting functional foods, whereby consumers hold higher expectations of health-promoting benefits beyond basic nutrition. Dietary proteins provide a rich source of bioactive peptides, which are hidden in a latent state within the native protein, requiring enzymatic proteolysis for their release. Bioactive peptides can be produced during in vivo gastrointestinal digestion and/or food processing. Lactic acid bacteria are among the most widely microorganisms used as starter cultures for the production of fermented foods, and through their proteolytic system, they contribute to the release of bioactive peptides from dietary proteins. *In vitro* and *in vivo* studies demonstrated several biological functions attributed to bioactive peptides, such as antimicrobial, immunomodulatory, enhancement of mineral absorption, antithrombotic, antihypertensive, opioid and antioxidant activities. The great complexity and the wide dynamic range of relative peptide abundance in these products severely challenge the capabilities of existing analytical methodologies. However, functional and comparative genomic studies as well as proteomic approaches provide a wealth of knowledge in the way in which these lactic acid bacteria can use food proteins releasing bioactive peptides.

1. Introduction

Proteins, carbohydrates and lipids are the three classes of macronutrients which provide the necessary energy to maintain body functions as well as the structural and functional integrity of the organisms. Nutritionally, the quality of proteins depends on the amino acid composition, the absence of co-existing anti-nutrients that can limit protein digestibility and absorptive ability. Nowadays, there is an increased interest in health-promoting functional foods, whereby consumers hold higher expectations of health-promoting benefits beyond basic nutrition. Dietary proteins provide a rich source of bioactive peptides, which are hidden in a latent state within the native protein, requiring enzymatic proteolysis for their release. Biologically active or functional peptides are food derived peptides that exert, beyond their nutritional value, a physiological, hormone-like effect in the body (Hebert, Saavedra, & Ferranti, 2010). Bioactive peptides derive from a wide range of animal and vegetable proteins; such as bovine and human milk, fish, egg, meat, soybean, rice, sunflower and cereals (Bouzerzour et al., 2012; Escudero, Aristoy, Nishimura, Arihara, & Toldra, 2012; Kussmann & Van Bladeren, 2011; Senevirathne & Kim, 2012).

Food proteins are selected as sources of bioactive peptides according to different criteria (Udenigwe & Aluko, 2012): (i) food industry by-products in order to reduce environmental contamination and manufacture value-added products, for example whey proteins; (ii) proteins containing specific amino acid sequences for a biological activity of interest; and (iii) a QSAR-based *in silico* approach was recently proposed for the prediction of food protein than can release bioactive peptides (Gu et al., 2012; Udenigwe & Aluko, 2012).

2. Production of bioactive peptides

Bioactive peptides are encrypted in the primary structure of animal and plant proteins but they can be released by proteolysis *in vitro*, *in vivo* or a combination of both (Fig. 1, Hebert et al., 2010). The *in vivo* release of bioactive peptides involves the gastrointestinal digestion (with digestive enzymes such as pepsin, trypsin, chymotrypsin and peptidases from the intestinal brush border membranes) as well as enzymes derived from the human microbiota. On the other hand, the *in vitro* production of bioactive peptides includes the enzymatic hydrolysis of the food protein by endogenous enzymes present in the food matrix as well as proteolysis occurring during food processing or ripening by action of starter cultures or by enzymes isolated from proteolytic microorganisms (e.g., *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, Espeche Turbay, de Moreno de LeBlanc, Perdigon, Savoy

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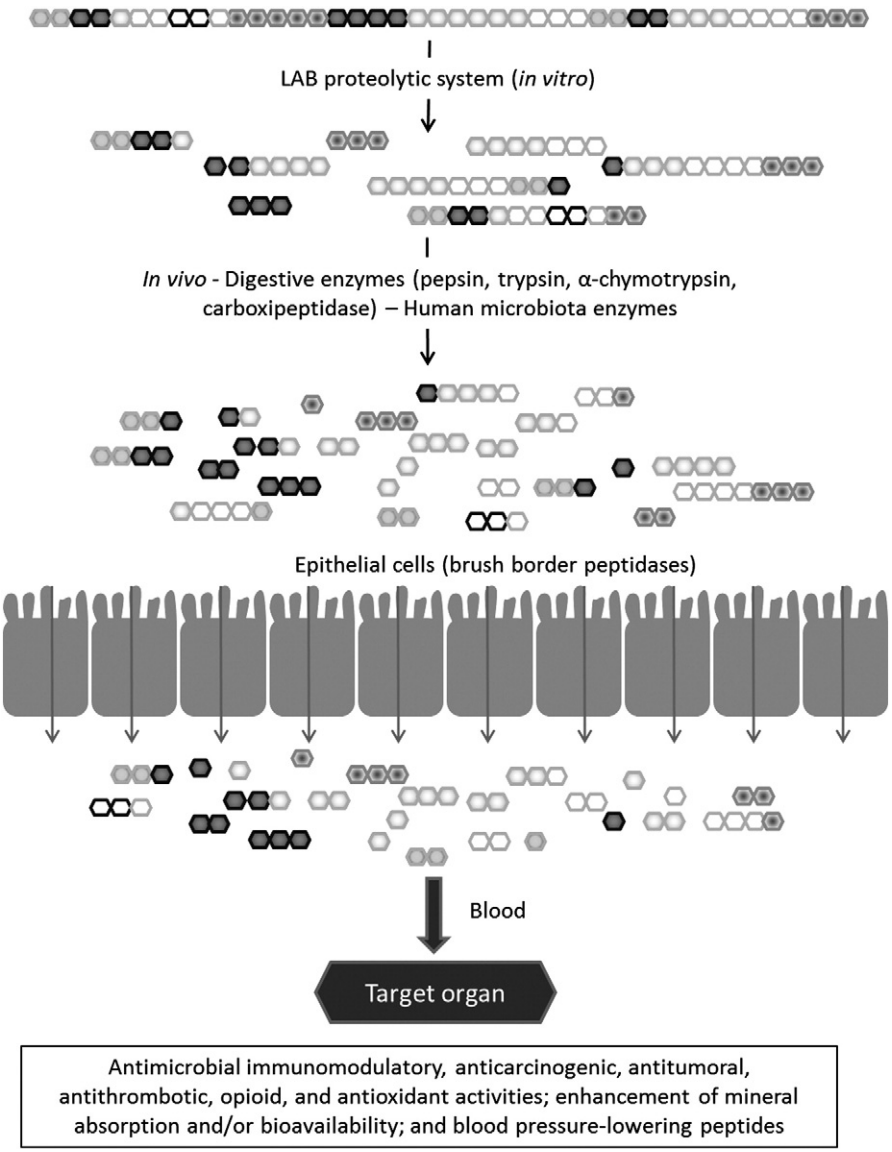

Fig. 1. Hydrolysis of food proteins by the proteolytic system of the lactic acid bacteria or by several digestive enzymes during the digestion process, which results in the release of bioactive peptides.

de Giori, & Hebert, 2012; Hebert et al., 2010). Therefore, certain lactic acid bacteria (LAB), mainly the strains belonging to the genera *Lactobacillus*, are currently marketed as health-promoting cultures or probiotics (Kleerebezem et al., 2010; Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). Microbial fermentation is one of the major processes to generate bioactive peptides, mainly in the dairy industry where the lactobacilli cell envelope-associates proteinases (Prt) release peptides during milk fermentation (Espeche Turbay et al., 2012; Hebert et al., 2008, 2010).

3. Main sources and target proteins involved in the production of bioactive peptides

Cryptic bioactive peptides have been isolated and characterized from a variety of sources: soybean, sunflower, corn, wheat, barley, rice, meat, fish, milk and its derivatives such as yogurt and cheese (Table 1, Bouzerzour et al., 2012; Escudero et al., 2012; Kussmann & Van Bladeren, 2011; Senevirathne & Kim, 2012). Depending on their amino acid sequence, bioactive peptides can exhibit diverse activities by binding to a specific receptor in the gastrointestinal tract or in target organs and tissues after absorption into the bloodstream (Fig. 1, Table 1). Antimicrobial, immunomodulatory, anticarcinogenic, antitumoral, antithrombotic, opioid, and antioxidant activities; enhancement of mineral absorption and/or bioavailability; and blood pressure-lowering effect are some of the biological activities attributed to food-derived peptides (Hebert et al., 2010). Furthermore, some peptides could play a role in prevention and treatment of metabolic syndrome via different mechanisms, for instance by decreasing body mass, regulation of blood pressure, insulinemia and cholesterolemia levels (Ricci-Cabello, Herrera, & Artacho, 2012). An exhaustive description of food bioactive peptides currently known is outside the scopes of this review, which is instead aimed to give an overview of their main sources, in conjunction with the most representative *in vitro* and *in vivo* activities and of *omic* approaches for their characterization from food matrices.

3.1. Bioactive peptides: main food sources

A number of bioactive peptides were shown to be released from soy protein by means of microbial, gastric and pancreatic enzyme digestion of soybean proteins in the last decades (Kwon, Daily, Kim, &

Table 1
Examples of bioactive peptides released from proteins derived from several food sources.

Food source	Treatment (enzymes/microorganisms)	Primary structure or peptide fragment	Activities displayed by bioactive peptides	References
Soybean				
	Pepsin, pancreatin	Peptides from 1 to 13 kDa	Antioxidant, improving muscle glucose uptake	Roblet et al. (2012)
	Endo-type protease from microorganism	KA VK	Reduced TG synthesis	Inoue et al. (2011)
		SY	Reduced TG synthesis Reduction of apoB secretion	
	Neutral and alkaline protease from *Bacillus amyloliquefaciens*	Peptides from 200 to 3000 Da	Stimulation of LDL-R transcription	Cho et al. (2007)
	Trypsin Pepsin	LPYP IAVPGDVA	HMG-CoA reductase inhibitor	Pak, Koo, Kasymova, & Kwon (2005)
	Pepsin and pancreatin	SP-Sepharose fractions	ACE inhibitor Reactive oxygen species scavengers	Farzamirad & Aluko (2008)
De-fatted soy protein	Thermolase	XMLPSYSPY	Anticancer	Galvez, Chen, Macasieb, & de Lumen (2001); Kim et al. (2000)
Rice				
	Alcalase and simulated gastrointestinal juices	EQRPR	Anticancer	Kannan et al. (2010)
Rice soluble protein	Tryptic hydrolysis	GYPMYPLPR	Antimicrobial and ileum-contracting activity	Takahashi et al. (1994)
Corn				
	Trypsin, thermolysin, GC 106	<1 kDa peptides	ACE inhibitor	Parris et al. (2008)
Defatted corn protein	Flavourzyme		Bile acid binding capacity	Kongo-Dia-Moukala et al. (2011)
Wheat				
	Acid protease	IAP	ACE inhibitor	Motoi & Kodama (2003)
	Proteolysis by lactobacillus strains	MAPAAVAAAEAGSK, DNIPIVIR	Antioxidant activity	Coda et al. (2012)
Meat				
Porcine myosin	Thermolysin	MNPPK, ITTNP	ACE inhibitor	Arihara et al. (2001)
	Peptic digestion	VKKVLGNP	ACE-inhibitor	Katayama et al. (2007)
Titin	Pepsin and pancreatic proteases	KAPVA	ACE-inhibitor	Escudero et al. (2010)
Sarcoplasmic beef proteins	Synthetic peptides	GFHI, DFHING, FHG, GLSDGEWQ	Antimicrobial and anti-cancer	Jang et al. (2008)
Fish and other aquatic organisms				
Conger eel	Tryptic hydrolysate	LGLNGDDVN	Antioxidants	Ranathunga et al. (2006)
Rotifer	Pepsin	LLGPGLTNHA DLGLGLPGAH	Antioxidants	Byun et al. (2009)
Tuna	Pepsin	VKAGFAWTANQQLS	Antioxidants	Je et al. (2007)
	Pepsin	GDLGKTTTVSNWSPPKYKDTP	ACE-inhibitor	Lee et al. (2010)
Oyster		LLEYSL/I	Antiviral (anti-HIV)	Lee & Maruyama (1998)
Whey proteins				
Lactoferrin	Pepsin	Lactoferricin	Antimicrobial	Oo et al. (2010)
α-Lactalbumin	Trypsin and chymotrypsin	EQLTK, GYGGVSLPEWVCTTF ALCSEK, CKDDQNPH ISCDKF	Bactericidal peptides	Pellegrini et al. (1999)
β-lactoglobulin	Enzymatic hydrolysis	IPA	Inhibitor of dipeptidyl peptidase-4	Tulipano et al. (2011)
Milk whey	Hydrolysis by *L. helveticus* LBK-16H	IPP, VPP	Osteoblast proliferation	Narva, Halleen, Vaananen, & Korpela (2004)
Caseins				
β-casein	Pepsin	4 to around 100 amino acids No sequence available	Phosphopeptides	Schmelzer et al. (2007)
	Pepsin or trypsin and chymotrypsin	Neocasomorphin-6 (YPVEPF)	Opiod properties	Jinsmaa & Yoshikawa (1999)
	Enterococcus faecalis CECT 5727	LHLPLP LVYPFPGPIPNSLPQNIPP	Ace inhibitors	Quiros et al. (2006)
α_{s1}-casein	Pepsin, trypsin, chymotrypsin, and elastase	PQEVLNENLLR, VAPFPEVFGK	MAP-kinase signaling cascade activation, cholesterol 7α-hydroxylase regulation	Nass et al. (2008)
Caseins (α_{s1}-casein, α_{s2}-casein, β-casein)	Hydrolysis by *L. helveticus* PR4	LVYPFPGPIPNSLPQNIPP, FVAPFPEVFGKEKVNELSKDIGSE, LGTQYTDAPSFSDIPNPIGSENSEK, LVYPFPGPIPNSLPQNIPP, FVAPFPEVFGKEKVNELSKDIGSE, LVYPFPGPIPNSLPQNIPP	Ace inhibitors Antimicrobial	Minervini et al. (2003)
Sodium caseinate	*L. acidophilus* ATCC 4356 and *Lc. lactis* subsp. *lactis* GR5	3 kDa-ultrafiltered peptide fraction No sequence available	Immunomodulatory activity	Stuknyte, De Noni, Guglielmetti, Minuzzo, & Mora (2011)
β-Casein	*L. delbrueckii* subsp. *lactis* CRL 581	No sequence available	Anti-inflammatory activity (colitis)	Espeche Turbay et al. (2012)

Park, 2010). For instance, the sequential treatment with pepsin and pancreatin resulted in the generation of well-defined peptides ranging from approximately 13 kDa to less than 1 kDa, displaying important activities as antioxidants as well as in improving muscle glucose uptake (Roblet et al., 2012). Inoue et al. (2011) digested soy protein with endo-type protease from *Bacillus* sp., and two dipeptides that lowered triacylglycerols (Lys-Ala, Val-Lys) and, Ser-Tyr, that also reduces apoB secretion in a hepatoma cell line, were characterized (Inoue et al., 2011). Larger soy peptides displayed hypocholesterolemic activity, although by different mechanisms. In this regard, Cho, Juillerat, and Lee (2007) described peptides ranging from 200 to 3000 Da that stimulate LDL receptor transcription thus inducing a reduction in the levels of circulating LDL particles. Pak, Koo, Kwon, and Yun (2012) recently identified two soy bioactive peptides Leu-Pro-Tyr-Pro and Ile-Ala-Val-Pro-Gly-Asp-Val-Ala, which inhibited the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) that catalyzes the rate-limiting step of cholesterol synthesis. Based on these sequences, they designed twelve peptides, one of which displayed 14,500-fold increase inhibitory activity as compared to the natural bioactive peptides (Pak et al., 2012). Other small bioactive peptides from soy were characterized as ACE inhibitors and reactive-oxygen species scavengers (Farzamirad & Aluko, 2008). In addition, the nonapeptide obtained by thermolase hydrolysis of de-fatted soy protein, X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr, was shown to arrest mouse monocyte macrophage cell line in G2/M phases (Kim et al., 2000). Up-to-date, evidence suggests that promising bioactive peptides can be found elsewhere. In this regard, the pentapeptide Glu-Gln-Arg-Pro-Arg, isolated from heat stabilized de-fatted rice bran displayed anti-cancer property (Kannan, Hettiarachchy, Lay, & Liyanage, 2010). Another interesting example is the nonapeptide oryzatensin, Gly-Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg, obtained by tryptic hydrolysis of rice soluble protein which enhances the antimicrobial activity of leukocytes and it also displayed an ileum-contracting activity (Takahashi, Moriguchi, Yoshikawa, & Sasaki, 1994). Corn and wheat are other plant sources of bioactive peptides. In fact, ACE inhibitors as well as hypocholesterolemic peptides were isolated from corn (Kongo-Dia-Moukala, Nsor-Atindana, & Zhang, 2011; Parris, Moreau, Johnston, Dickey, & Aluko, 2008). On the other hand, Ile-Ala-Pro tripeptide was isolated from wheat gliadin, exhibiting a powerful ACE inhibitory activity (Motoi & Kodama, 2003). Interestingly, sourdough fermentation of several cereal flours using *Lactobacillus* strains presented antioxidant activity due to the release of bioactive peptides from gliadins ranging from eight to fifty-seven amino acids (Coda, Rizzello, Pinto, & Gobbetti, 2012).

In meat, short peptides commonly increase their concentrations during post-mortem storage even at low temperatures (Nishimura, Rhue, Okitani, & Kato, 1988). However, there is scarce information on the formation of beneficial bioactive peptides in such conditions, where ACE-inhibition is the most common activity found. For instance, two pentapeptides released from the heavy chain of porcine myosin by thermolysin were tested *in vivo* showing that they were very effective in lowering blood pressure in animal model of spontaneously hypertensive rats (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001). On the other hand, pepsin also generated important ACE-inhibitors from the light chain of porcine myosin, such as the octapeptide Val-Lys-Lys-Val-Leu-Gly-Asn-Pro, which was also effective in the same animal model (Katayama et al., 2007). Troponin and collagen are also important contributors to the bioactive peptide production (Katayama et al., 2008; Saiga et al., 2008). The ACE-inhibitor pentapeptide Lys-Ala-Pro-Val-Ala was released from titin after treatment of porcine muscle with a sequential digestion of pepsin and pancreatic proteases (Escudero, Sentandreu, Arihara, & Toldra, 2010). In addition, antioxidant, antimicrobial and anti-cancer peptides derived from meat were also reported in the literature (Jang, Jo, Kang, & Lee, 2008; Li, Chen, Wang, Ji, & Wu, 2007; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011).

Fish and other aquatic organisms are also sources of bioactive peptides, including antioxidants such as the anionic nonapeptide Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn from conger eel (Ranathunga, Rajapakse, & Kim, 2006) and two decapeptides from rotifer (Byun, Lee, Park, Jeon, & Kim, 2009). Antioxidant peptides were also reported from fishes like tuna (Je, Qian, Byun, & Kim, 2007). In addition, pepsin digestion of tuna frame protein released a 21-mer cationic peptide that acted as ACE-inhibitor (Lee, Qian, & Kim, 2010). Anti-coagulant (Jung & Kim, 2009), anti-obesity (Cudennec, Ravallec-Plé, Courois, & Fouchereau-Peron, 2008), anticancer (Naqash & Nazeer, 2012), an anti-HIV activities were also reported. In the latter case, two hexapeptides from oyster, Leu-Leu-Glu-Tyr-Ser-Leu/Ile, proved to be highly effective at blocking HIV protease (Lee & Maruyama, 1998).

Bioactive peptides can also be released by hydrolysis with digestive enzymes from milk and dairy products. These peptides can be released from α-, β-, and κ-caseins and whey proteins such as α-lactalbumin, β-lactoglobulin, lactoferrin and immunoglobulins (Belem, Gibbs, & Lee, 1999; Fitzgerald & Murray, 2006). Lactoferricin, a 25 amino acid peptide derived from the digestion of the lactoferrin by pepsin, displayed an outstanding antimicrobial activity (Oo, Cole, Garthwaite, Willcox, & Zhu, 2010). Pepsin, trypsin or chymotrypsin digestion of α-lactalbumin results in the production of peptides with both, immunomodulatory and antimicrobial activities against bacteria, viruses and fungi (Pellegrini, 2003; Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999). Another interesting whey-derived bioactive compound is the tripeptide Ile-Pro-Ala, released from β-lactoglobulin hydrolysis, which may act as inhibitor of dipeptidyl peptidase-4, reducing glucose levels and stimulate insulin (Tulipano, Sibilia, Caroli, & Cocchi, 2011). In the same trend, other reports point out the possible role of whey peptides in reducing type II diabetes as well as obesity (Jakubowicz & Froy, 2012).

Two peptides derived from α_{s1}-casein, released by enzymatic treatment, were found to be involved in the regulation of the cholesterol 7α-hydroxylase expression i.e. the key enzyme of bile synthesis (Nass et al., 2008). κ-casein in turn was shown to release at least four potent ACE-inhibitors peptides from 3 to 11 amino acid length (Gomez-Ruiz, Ramos, & Recio, 2007).

3.2. Release of bioactive peptides by microorganisms

In milk, LAB undoubtedly have the greatest impact in releasing bioactive peptides. As milk contains only small amounts of amino acids and short peptides, LAB depend on a complex proteolytic system to obtain essential amino acids from caseins during growth in milk. This specialized proteolytic system comprises three major components (Fig. 2): (i) a cell envelope-associated proteinase (Prt) which is involved in the first step of casein degradation; (ii) peptide transport systems to allow uptake of the resulting peptides, and (iii) several intracellular peptidases that degrade peptides into shorter peptides and amino acids (Hebert et al., 2008; Lamarque et al., 2011; Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). This proteolytic system is essential for bacterial growth, it also contributes to the development of flavor and texture of fermented products and it can promote human health through the release of bioactive peptides during milk fermentation (Hebert et al., 2008, 2010; Savijoki, Ingmer, & Varmanen, 2006). Thus, certain Prts can release bioactive health-beneficial peptides during milk fermentation (Hebert et al., 2008, 2010; Savijoki et al., 2006). In fact, several immunomodulatory, hypocholesterolemic, antimicrobial, mineral-binding, opiod, and antihypertensive bioactive peptides have been isolated from fermented dairy products (Fitzgerald & Murray, 2006; Hayes et al., 2007). However, the antihypertensive activity is the main studied bioactivity produced during milk fermentation (Fitzgerald & Murray, 2006). ACE-inhibitory peptides from dairy origin usually contain up to 10 amino acids, being the strain selection one of the main factors that affects the release of bioactive peptides in dairy fermentations (Hayes

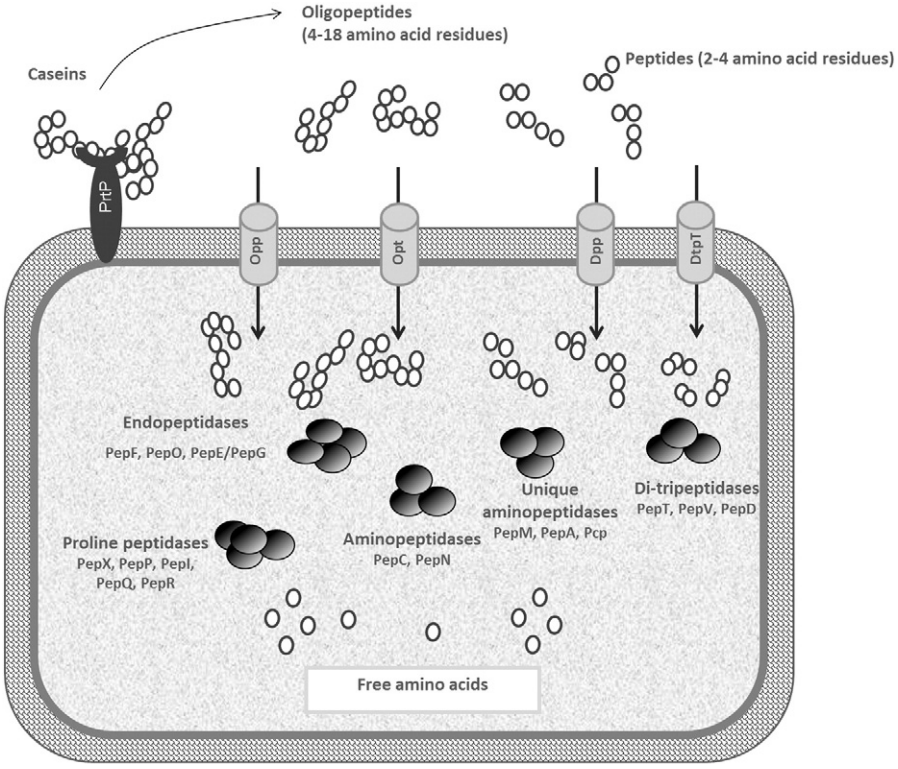

Fig. 2. Schematic representation of the proteolytic system of lactic acid bacteria. (data from Kunji et al., 1996; Savijoki et al., 2006; Lamarque et al., 2011; Liu et al., 2010). A cell-envelope associated proteinase (Prt), Opp oligopeptide permease, the Opt transporter, DtpT the ion-linked transporter for di- and tripeptides, and Dpp the ABC transporter for peptides, the intracellular endopeptidases (PepO, PepF, PepE/PepG), general aminopeptidases (PepN, PepC), unique aminopeptidases (PepM, PepA, Pcp), proline peptidases (PepX, PepP, PepI, PepQ, PepR), tripeptidase (PepT), and dipeptidases (PepD and PepV) are indicated.

et al., 2007). ACE-inhibitory peptides have been mainly isolated from dairy products fermented with *L. helveticus* (Hayes et al., 2007). Two potent ACE-inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro derived from β-casein, were isolated from milk fermented with *L. helveticus* CP790 (Takano, 2002) and sour milk fermented with *L. helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995). These peptides were also identified in a β-casein hydrolysate generated by *L. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al., 2008). Two commercial antihypertensive milk products containing Ile-Pro-Pro and Val-Pro-Pro, Ameal S™ and Evolus™, fermented with *L. helveticus* are available in the market (Fitzgerald & Murray, 2006). In addition, milks fermented with different strains of *Enterococcus faecalis*, *L. helveticus*, *Lactobacillus rhamnosus*, *Lactobacillus animalis*, *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Lactococcus* (*Lc.*) *lactis* subsp. *cremoris* have shown to present ACE inhibitory activity (Fitzgerald & Murray, 2006; Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Hayes et al., 2007; Miguel et al., 2005; Yamamoto, Maeno, & Takano, 1999). ACE inhibitory peptides may also be released during fermentation of whey proteins by *Kluyveromyces marxiams* var *marciams* (Belem et al., 1999).

ACE inhibitors are thought to be competitive substrates for ACE. Although there is no apparent consensus on the peptide sequence for the expression of ACE inhibitory activity, some common structural properties of ACE-inhibitory peptides are worth noting (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). The ACE binding is strongly influenced by the C-terminal tripeptide sequence of the substrate; ACE appears to prefer substrates or competitive inhibitors containing hydrophobic amino acids including aromatic amino acids such as Trp, Tyr, and Phe, or the amino acid Pro located at the three C-terminal positions. In addition, the positive charge from Arg and/or Lys residues may increase the inhibitory activity (Pripp, Isaksson, Stepaniak, & Sorhaug, 2004).

Theoretically, any food protein can be used as a potential source of bioactive peptides. Several bioactive peptides are produced *in vitro* by enzymatic proteolysis or fermentation. A combination of enzymatic hydrolysis by gastrointestinal digestion and fermentation of food proteins with proteolytic starter cultures has been demonstrated to be effective in producing short functional peptides (Hebert et al., 2010; Korhonen & Pihlanto, 2007). The choice of proteolytic enzymes or microorganisms used for food processing has a crucial impact on the composition of the released peptides (Panchaud, Affolter, & Kussmann, 2012). In this sense, the most important application of LAB is their use as starter cultures in the manufacturing processes of various fermented food, mainly because they contribute to raw-material preservation due to acidification, but also because of their contribution to the development of flavor and texture of fermented products (Canchaya, Claesson, Fitzgerald, van Sinderen, & O'Toole, 2006; Kleerebezem et al., 2010). The natural habitat of lactobacilli includes dairy, meat and vegetal material fermentations to the gastrointestinal and genital tracts of humans and animals (Kleerebezem et al., 2010; Satokari et al., 2003; Vaughan et al., 2002). Therefore, any existing food proteins could be used as possible substrate for generating bioactive peptides.

4. Genomics as a tool for the prediction of proteolytic potential of LAB

Beginning with the genome of *Lactobacillus plantarum* WCFS1 in 2003 (Kleerebezem et al., 2003), currently public databases contain 42 complete *Lactobacillus* genomes, while 105 more *Lactobacillus* genome sequencing projects containing scaffolds or contigs are available *on-line* (http://www.ncbi.nlm.nih.gov/genome/browse/). The availability of microbial genomes and extensive comparative analyses of LAB genomes have allowed to reveal the genomic features

that contribute to the ecological adaptability and examine the evolution of the species and their phenotypic diversities (Broadbent et al., 2012; Cai, Thompson, Budinich, Broadbent, & Steele, 2009; Canchaya et al., 2006; Kleerebezem et al., 2010; Liu et al., 2010; Makarova et al., 2006). Comparative analysis of several LAB genomes showed that a combination of gene gain and gene loss occurred during the evolution of LAB with different ecological habitats (Broadbent et al., 2012; Cai et al., 2009; Canchaya et al., 2006; Kleerebezem et al., 2010; Liu et al., 2010; Makarova et al., 2006). Niche-specific genomic adaptations are reflected within the genomes (Cai et al., 2009; Kleerebezem et al., 2010). Adaptation to the dairy niche has been associated with a trend toward metabolic simplification. Thus, representative dairy LAB, *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus*, contain many pseudogenes related to the utilization of several carbohydrates, reflecting their dedication to growth on lactose (Cai et al., 2009). In addition, their genome lost amino acid biosynthetic and cofactor biosynthetic genes while an increased in genes for peptide transport and proteolysis was observed, suggesting their adaptation to the protein rich dairy niche (Cai et al., 2009; Callanan et al., 2008; van de Guchte et al., 2006). Conversely, the lactobacilli commonly associated with the gastrointestinal tract display a great array for transport of a diverse group of carbohydrates and specific extracellular enzyme complexes that could be involved in complex carbohydrate degradation (Azcarate-Peril et al., 2008; Cai et al., 2009; Kleerebezem et al., 2010). These gastrointestinal lactobacilli encode other functions related with the gastric survival (e.g. genes encoding bile salt hydrolase) and with the intestinal mucosa interactions (e.g. mucus binding proteins).

Comparative genome analysis of lactobacilli revealed that amino acid biosynthetic pathways are deficient in different degrees in LAB, being related with the adaptation to a specific-niche. Thus, dairy LAB such as *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* have lost the majority of their amino acid biosynthetic genes (Cai et al., 2009; Callanan et al., 2008; van de Guchte et al., 2006). *L. acidophilus* and *Lactobacillus gasseri*, typical LAB found in the human gastrointestinal tract are auxotrophic for 14 and 17 amino acids, respectively (Azcarate-Peril et al., 2008; Cai et al., 2009). *L. plantarum* and *Lactobacillus casei*, ubiquitous microorganisms, possesses enzyme for biosynthesis of all amino acids except the branched-chain amino acids leucine, isoleucine, and valine (Cai et al., 2009).

Compensating for the inability of dairy-lactobacilli to synthesize most amino acids, these genomes contain several genes related to the proteolytic system that allows them to acquire amino acids from proteins present in their environment. This proteolytic enzyme system is vital to obtain essential amino acids and likely provides these lactobacilli with a selective advantage in protein-rich environments due to it is energetically favorable to obtain amino acids from environmental proteins than de novo synthesis (Cai et al., 2009; Hebert et al., 2008). As stated above, the proteolytic system of LAB consists of a cell envelope-associated proteinase, transport systems to allow uptake of the resulting peptides, and several intracellular peptidases, which degrade peptides to amino acids (Fig. 2, Hebert et al., 2008; Savijoki et al., 2006). The Prt is the key enzyme of this system as it is involved in the first step of casein degradation (Savijoki et al., 2006) and certain Prt can release bioactive health-beneficial peptides during food fermentation (Hebert et al., 2008, 2010; Savijoki et al., 2006).

Liu et al. (2010) performed a genome comparison of the proteolytic system of 22 LAB, including Prt, peptide transporters and peptidases. The 22 LAB genomes analyzed included *L. acidophilus* NCFM, *Lactobacillus johnsonii* NCC 533, *L. gasseri* ATCC 33323, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA365, *L. plantarum* WCFS1, *Lactobacillus brevis* ATCC 367, *Lactobacillus sakei* 23 K, *Lactobacillus salivarius* UCC118, *Oenococcus oeni* PSU1, *Pediococcus pentosaceus* ATCC 25745, *Leuconostoc mesenteroides* ATCC 8293, *L. casei* ATCC 334, *Lc. lactis* subsp. *lactis* IL1403, *Lc. lactis* subsp. *cremoris* MG1363, *Lc. lactis* subsp. *cremoris* SK11, *Streptococcus thermophilus* CNRZ1066, *St. thermophilus* LMG18311, *St. thermophilus* LMD9, *L. reuteri* F275, *L. helveticus* DPC 4571, and *L. rhamnosus* GG (Liu et al., 2010). The Prt enzyme, responsible for the primary hydrolysis of food proteins, was only found in a few LAB strains, such as on the chromosome of *L. acidophilus*, *L. johnsonii*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. rhamnosus* and *S. thermophilus* LMD9, as well as on the plasmid of *Lc. lactis* subsp. *cremoris* SK11 (Liu et al., 2010). With the exception of *L. rhamnosus* which presents two *prt* genes, the other mentioned strains possess a single *prt* gene (Liu et al., 2010). On the other hand, essential peptidases such as PepC, PepN, and PepM, proline peptidases PepX and PepQ, and endopeptidase PepO and dipeptidase PepV were found in all LAB genomes while members of both the PepE/PepG (endopeptidases) and PepI/PepR/PepL (proline peptidases) superfamilies are absent in lactococci and streptococci (Liu et al., 2010).

Data about the distribution of the proteolytic system genes can be used to predict the proteolytic potential of the LAB strains. For example, *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* have a very broad set of proteolytic enzymes including proteinase (Prt), aminopeptidases (PepC, PepN, PepM, PepA and Pcp), endopeptidases (PepO, PepF and PepG), dipeptidases (PepD and PepV), tripeptidase (PepT) as well as proline peptidases (PepX, PepI, PepR, PepP and PepQ). The presence of these enzymes agrees with the role of these bacteria in milk, *L. delbrueckii* subsp. *bulgaricus* serves as the proteolytic microorganism in yogurt and *L. helveticus* is a cheese starter culture that has been used to degrade bitter peptides in cheese. Vegetable origin LAB species such as *L. plantarum*, *O. oeni*, and *Leuc. mesenteroides* encode less proteolytic enzymes in their genomes, which is consistent with their habitat, a fiber-rich with less proteins (Liu et al., 2010). In addition, this comparative genomic approach allowed exploring the diversity of proteolytic system genes in various strains of *Lc. lactis*, confirming the proteolytic diversity between the two *Lc. lactis* subspecies, i.e. subsp. *lactis* and subsp. *cremoris*.

Genomic approaches may allow the prediction of proteolytic and flavor-forming potential of bacterial strains. In addition, this knowledge could be used to improve the functional properties of dairy and other fermented food products by supporting the strain selection process.

5. Application of "omic"-based analytical strategies for the characterization of bioactive peptides in food and related challenges

Several aspects are important for determining the biological activity of peptides including the enzymes used for hydrolysis, food processing conditions, and the size of the resulting peptides, which influences their absorption across the enterocytes and bioavailability in target tissues (Udenigwe & Aluko, 2012).

There are *in silico* and *in vitro* approaches aimed at discovering and identifying bioactive peptides in food matrix (Kussmann & Van Bladeren, 2011). The *in vitro* method consists of the following steps (1) selection of an appropriate food protein source; (2) enzymatic hydrolysis by selected enzymes, fermentation and/or gastrointestinal digestion; (iii) *in vitro* screening for the potential bioactivity properties; (iv) fractionation of the peptide mixture; (v) analysis of the peptide structure; and (vi) design of synthetic structural analogs or peptide mimetic to validate bioactivity *in vitro* and in vivo (Fig. 3).

The newest *omic* approaches combine cell biology, immunology, biochemistry, synthetic chemistry, and the use of combinatorial library with mass spectrometry to identify the patterns of peptide formation and the bioactivity of the peptides present in the sample. Typical protocol consists of sample purification, separation of proteins or peptides by 2D-PAGE or RP-HPLC, enzymatic digestion (in the case of intact proteins) and identification by MS and MS/MS techniques. Using this methodology, the peptides naturally present in human milk have been characterized (Picariello, Ferranti, Mamone,

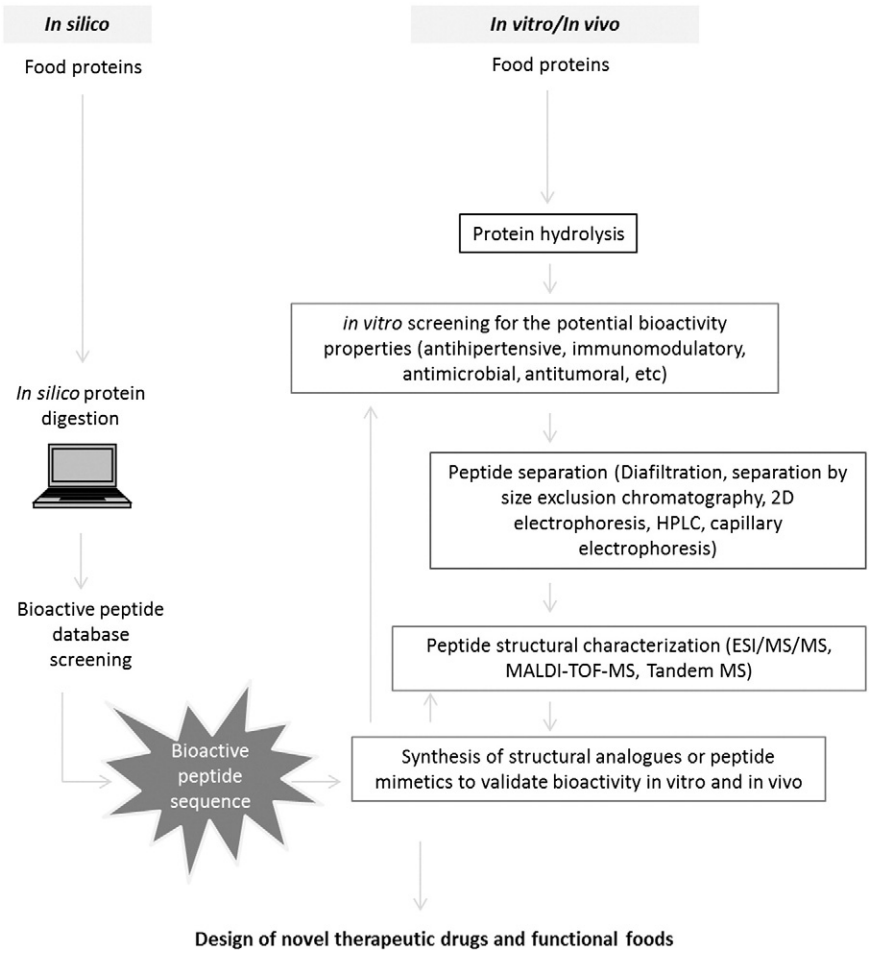

Fig. 3. Food bioactive peptide analysis workflow showing steps of production and purification integrated strategies for the structural and functional characterization.

Roepstorff, & Addeo, 2008), which allowed the detection of possible bioactive sequences. The formation of angiotensin-converting enzyme-inhibitory sequences was monitored in milk (Gomez-Ruiz et al., 2007) and during Manchego cheese ripening (Hernandez-Ledesma, Amigo, Ramos, & Recio, 2004) using multiple reaction monitoring (MRM) detection technique.

Also MALDI-TOF-MS has become an important analytical tool in the identification of peptides and evaluation of their role in biological processes. Unfortunately, this approach can be not exhaustive for the identification of complex mixture of peptides, which is in most cases what is found in a food. Peptides in mixture may have either hydrophobic or hydrophilic sections, in which case, the direct analysis of such a mixture by MALDI-TOF-MS does not allow desorbing of all components, due to the well-known suppression phenomena taking place in the analysis of complex peptide mixtures. In these cases, sequence coverage can be improved through the use of several matrices. Sinapinic acid (SA) is more suited for analysis of intact proteins and large peptide fragments, whereas α-cyano-4-hydroxycinnamic acid (HCCA) allows better detection of medium size peptides. More recent studies have proven the utility of using at least two matrices leading to a two-fold increase of the coverage of each protein. For instance, the use of HCCA in concert with the SA matrix has allowed to obtain a good coverage of hydrophilic proteins, and 2,5-dihydroxybenzoic acid (DHB) in concert with the SA matrix to obtain a good coverage of hydrophobic proteins (Gonnet, Lemaitre, Waksman, & Tortajada, 2003).

A further issue is the need for development of technologies to identify low molecular weight (LMW) peptides with bioactivity. The simplest and most used approach is again based on MALDI-TOF-MS analysis, but matrix suppression often prevents detection of LMW components, due in this case to matrix interference. A suitable method for analysis of small peptides in complex without extensive sample pre-treatment has been introduced recently (Nanostructure-Assisted Laser Desorption/Ionization—NALDI). This method is a matrix-free method and therefore provided better signal intensity for LMW components compared with MALDI using matrix. This method has been successfully applied to the study of bovine milk and colostrum (Nanostructure-Assisted Laser Desorption/Ionization (NALDI)) for analysis of peptides in milk and colostrum (Kütt, Malbe, & Stagsted, 2001).

6. *In vivo* and *in vitro omic* studies on production and metabolism of food peptides

Despite of the extensive knowledge of the effects that food-derived peptides exert *in vitro*, data about the ability of the active protein domains to be actually produced *in vivo* and to survive gastrointestinal digestion are still scarce and contradictory due to the extreme complexity of the peptide sets produced during digestion. Nevertheless, these aspects are essential for potential bioactive peptides to perform their specific functions. Most biological activity assays for milk-, cereal- or soy-derived peptides, have been carried out on peptide fractions produced in non-physiological environments, using purified enzyme and substrates, or enzyme extracts from LAB (Hartmann & Meisel, 2007; Hebert et al., 2008; Matar, Amiot, Savoie, & Goulet, 1996) and yeast (Roy, Watanabe, & Tamai, 1999). Peptides can also be quantified from biological fluids. For instance, blood levels of several ACE-inhibitor peptides were quantified in volunteers that have orally

taken up these peptides using LC-ESI-triple (van Platerink, Janssen, Horsten, & Haverkamp, 2006). Recently, Bouzerzour et al. (2012) exhaustively studied *in vivo*, the kinetics of hydrolysis of a complex matrix like infant formula in the different compartments of the gut (proximal jejunum, median jejunum and ileum) using the piglet as a model; where some bioactive peptides were identified by nano-liquid chromatography–MS/MS.

Picariello et al. (2012) analyzed the human milk proteome by using a gel-free shotgun proteomic analysis to overcome the limitations of the classical electrophoresis-based approach. Conventional 2DE for descriptive proteomics shows some limitations that are particularly critical for characterizing complex mixtures such as very low-abundance proteins, as well as those with extreme isoelectric points or molecular weight values which can escape detection on the gels. Briefly, the shotgun proteomic strategy consisted of the reduction of protein disulphide bridges and alkylation of cysteine residues, following by the trypsin digestion and dephosphorylation, this late step to prevent the missed detection of peptides due to phosphorylation. To enlarge the proteome coverage, the peptides are subjected to N-deglycosylation by PNGase F and analyzed separately. Finally, the complex mixture is analyzed by nanoflow-high performance liquid chromatography (HPLC)/Fourier Transform-Ion Cyclotron Resonance (FT-ICR) mass spectrometry (MS) (Picariello et al., 2012).

The characterization of food-derived peptides *in vivo* is complicated by the large occurrence of endogenous polypeptides but would be virtually faceable by using the available up-to-date strategies of high-resolution separation coupled to MS/MS targeting of specific sequences. By this approach, the κ-casein macro-peptide and the N-terminal fragments of α_{s1}-casein have been identified in human blood after ingestion of yogurt (Chabance et al., 1998). Similarly, peptides produced during *in vivo* digestion of milk cow caseins have been identified in rat.

The stability of peptides to gastric digestion also has toxicological implications, as it is one of the criteria used to assess the functional and immunogenic characteristics of food proteins. Therefore, the omic identification of food peptides surviving gastrointestinal digestion can provide a means to help in localizing bioactive peptides.

In order to identify the peptides generated from protein digestion, several model systems, reproducing the gastrointestinal digestion have been developed. Proteolytic systems simulating the physiological digestive enzyme pools including pepsin and chymotrypsin and pancreatin have been used (Hernández-Ledesma, Quirós, Amigo, & I., 2007). Agudelo et al. (2004) designed a pilot plant to perform the continuous removal of digestion products during in vitro proteolysis, in order to mimic the *in vivo* process and follow the fate of potentially bioactive peptides. A digestion model that mimics *in vivo* gastric–pancreatic intestinal processes has been also applied to identification of gluten immunogenic peptides (Mamone et al., 2004).

Identification of peptide sequences surviving digestion is only the first among required step to assess potential bioactivity *in vitro*. Peptides produced in the intestine must be absorbed before delivering to their final organ or tissue. The study of these processes requires development of efficient omic strategies which conjugate biochemical, cellular and MS tools. Transport studies across Caco-2 monolayers represent one of the most widely accepted models of *in vitro* permeation of drugs and peptides as they well correlate, at least under a qualitative standpoint, with intestinal absorption *in vivo* (Artursson, Palm, & Luthman, 2001). Combination of the Caco-2 monolayer adsorption with MALDI and ESI-LC–MS/MS analysis of the pool of peptides produced and translocated has allowed important progress in this research field. In one recent study, human Caco-2 cell line has been used as a model of the gut epithelium to predict the physiological behavior and the uptake of gluten peptides. The epithelial translocation of a canonical immunogenic peptide 33-mer by transcytosis was observed in Caco-2 cells (Schumann et al., 2008). Similarly, the permeability of toxic p31–43 and p31–49 across Caco-2 cell layer was also demonstrated (Lebreton et al., 2012; Rauhavirta et al., 2011). The same omic approach has been used to investigate how *in vitro* digestion contributes to the production of antioxidant peptides from soybean β-conglycinin and to monitor their uptake. The entire panel of peptides produced by digestion has been characterized by combining MALDI and HPLC–ESI–MS/MS investigation leading to identification of novel bioactive sequences and their precursor able to be produced and adsorbed (Amigo-Benavent et al., Molecular Nutrition and Food Research, 2013, in press).

In addition to this classical peptide discovery strategy, an *in silico* prediction and discovery of bioactive peptides approaches have been developed (Grigorov & van Bladeren, 2007; Panchaud et al., 2012). These *in silico* methods, named "reverse genome engineering" allow the prediction of bioactive peptides and their enzymatic release from a target protein diminishing the number of bioactivity tests developed (Grigorov & van Bladeren, 2007; Panchaud et al., 2012). Bioinformatics approaches together with the available protein and peptide databases offer the possibility to simulate the hydrolysis of a target animal of plant protein and predict the release of bioactive peptides. Several plant and animal genomes are available such as rice, maize, soy and bovine. On the other hand, some industrial and probiotic LAB are sequenced, being possible to predict the enzymatic hydrolysis of food proteins based on the specific enzyme activity of a selected LAB. Thus, nowadays, it is possible the *in silico* prediction of bioactive peptide produced by gastrointestinal digestion and/or food processing, providing a rapid and cost-effective strategy for novel bioactive peptide discovery.

7. Conclusions

The importance attributed to food proteins and bioactive peptides in nutrition and health is undeniable. Scientific evidence demonstrated that peptides derived from enzymatic food protein hydrolysates possess several activities such as antimicrobial, immunomodulatory, enhancement of mineral absorption, antithrombotic, antihypertensive, opioid and antioxidant activities, which have a positive impact in human health. These bioactive peptides can be produced during *in vivo* gastrointestinal digestion and/or food processing. Lactic acid bacteria are among the most widely microorganisms used as starter cultures for the production of fermented foods, and through their proteolytic system, they contribute to the release of bioactive peptides from dietary proteins. Therefore, microbial fermentation especially by LAB, acquires a major role, since the production of key bioactive peptides can be triggered as foods are being produced. In this sense, the genomic and proteomic analysis of new LAB strains capable of releasing milk-derived bioactive peptides constitute the basis for the development of novel and improved functional foods. Omic analysis will help to improve our knowledge on food-derived peptides identifying those bioactive peptides that have a beneficial effect on health.

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