Food-derived peptides with hypocholesterolemic activity: Production, transepithelial transport and cellular mechanisms

Jianqiang Li, Carlotta Bollati, Lorenza d'Adduzio, Melissa Fanzaga, Ivan Cruz-Chamorro, Anna Arnoldi, Cesare R. Sirtori, Carmen Lammi

PII: S0924-2244(23)00394-1

DOI: https://doi.org/10.1016/j.tifs.2023.104279

Reference: TIFS 104279

To appear in: *Trends in Food Science & Technology*

Received Date: 19 July 2023

Revised Date: 31 October 2023

Accepted Date: 23 November 2023

Please cite this article as: Li, J., Bollati, C., d'Adduzio, L., Fanzaga, M., Cruz-Chamorro, I., Arnoldi, A., Sirtori, C.R., Lammi, C., Food-derived peptides with hypocholesterolemic activity: Production, transepithelial transport and cellular mechanisms, *Trends in Food Science & Technology* (2023), doi: https://doi.org/10.1016/j.tifs.2023.104279.

Food-derived peptides with hypocholesterolemic activity: production, transepithelial transport and cellular mechanisms

Jianqiang Li[1], Carlotta Bollati[1], Lorenza d'Adduzio[1], Melissa Fanzaga[1], Ivan Cruz-Chamorro[2,3], Anna Arnoldi[1], Cesare R. Sirtori[4], Carmen Lammi[1]*

[1.] Deptartment of Pharmaceutical Sciences, Università degli Studi di Milano, Milano, Italy;

[2.] Departamento de Bioquímica Médica y Biología Molecular e Inmunología, Universidad de Sevilla, 41009, Seville, Spain;

[3.] Instituto de Biomedicina de Sevilla, IBiS/Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla;

[4.] Dyslipidemia Center, ASST Grande Ospedale Metropolitano Niguarda, Milano, Italy.

Abstract

Background

In recent years, food-derived peptides have gained much attention for their potential health benefits. Some short and medium-sized peptides released from food proteins after their enzymatic hydrolysis may exhibit hypocholesterolemic activity. Hypocholesterolemic peptides act either by targeting exogenous cholesterol in the gastrointestinal (GI) tract or by modulating endogenous cholesterol levels via cholesterol metabolism pathways in the liver after being absorbed.

Scope and Approach

This paper provides a comprehensive review of current pieces of evidence regarding the production, transepithelial transport, and cellular mechanisms underlying the hypocholesterolemic activities of food-derived peptides.

Key Findings and Conclusions

The molecular mechanisms of hypocholesterolemic peptides involve bile acid binding, inhibition of cholesterol micellar solubility, statin-like effects through the modulation of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCoAR), as well as the targeting of interactions between proprotein convertase subtilisin/kexin type 9 (PCSK9) and low-density lipoprotein receptor (LDLR), sterol regulatory element-binding protein 2 (SREBP-2), and hepatocyte nuclear factor 1α (HNF-1α) pathways. Furthermore, some peptides exhibit multiple biological activities, such as anti-inflammatory and antioxidant activities, besides cholesterol-lowering properties, thereby safeguarding cellular components against high levels of cholesterol-induced damage. However, since only a few studies have evaluated the *in vivo* effects of hypocholesterolemic peptides, further studies carried out in animal models or human are necessary to exploit these ingredients in the prevention and management of hypercholesterolemia.

Keywords: hypocholesterolemic peptides, transepithelial transport, molecular mechanisms, bioavailability, cholesterol

1. Introduction

Food proteins, in addition to comprehensive energetic and nutritional functions, are well known for their biological properties. To exert their biological effects, dietary proteins must undergo enzymatic digestion to yield bioactive peptides, i.e. specific protein fragments typically ranging from 2 to 20 amino acid residues, which are encrypted within the protein primary sequences, ultimately contributing to a beneficial impact on health conditions (Xu, Hong, Wu, & Yan, 2019). For instance, bioactive peptides released from proteins by enzymatic hydrolysis (exogenous or endogenous proteolytic enzymes) provide several biological effects, including the lowering of high cholesterol levels, as demonstrated in *in vitro*, *in vivo*, and human studies (Gu, et al., 2017; Lammi, Zanoni, Arnoldi, & Vistoli, 2015; Udenigwe, Abioye, Okagu, & Obeme-Nmom, 2021). Hypercholesterolemia is a metabolic condition characterized by elevated blood cholesterol levels, one of the most critical factors of cardiovascular disease. High levels of plasma cholesterol, particularly low-density lipoprotein (LDL) cholesterol (LDL-C), may cause arteriosclerosis by developing plaques in the arteries, with implications for cardiovascular disease outcomes. Available evidence shows that hypocholesterolemic peptides act either by targeting exogenous cholesterol, or by modulating endogenous cholesterol levels via cholesterol metabolism pathways (Boachie, Yao, & Udenigwe, 2018).

An increase in plasma cholesterol concentrations can be attributed to the disturbed balance between endogenous cholesterol, dietary cholesterol, and the excretion of bile acids and cholesterol in feces. Numerous studies have established that peptides exert hypocholesterolemic effects by affecting exogenous cholesterol or modulating endogenous cholesterol levels (Singh, Aluko, Hati, & Solanki, 2022). Regarding the regulation of exogenous cholesterol, the inhibition of the intestinal absorption of

dietary cholesterol is the main mechanism by which the peptides act, involving the hindering of the enterohepatic circulation of bile acids and reduction of cholesterol micellar solubility in small intestinal epithelial cells (Nagaoka, Nakamura, Shibata, & Kanamaru, 2010; J. Wang, Shimada, Kato, Kusada, & Nagaoka, 2015). Apart from obtaining cholesterol through the diet, the majority of cholesterol is synthesized endogenously in the body. The endogenous cholesterol may be modulated if the peptides are absorbed into the blood circulating system and are bioavailable in the targeted organ or tissues (e.g. liver and adipose tissues). Several studies have reported specific hypocholesterolemic mechanisms of food-derived peptides, mainly focusing on the inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) activity, whereas proprotein convertase subtilisin/kexin type 9 (PCSK9) has also received some attention due to its association with LDL receptor (LDLR) degradation (Lammi, Aiello, Boschin, & Arnoldi, 2019). Meanwhile, the effect of food-derived peptides on the expression of proteins involved in cholesterol metabolism has been evaluated, including transcription factors sterol regulatory element binding protein 2 (SREBP-2) and the hepatocyte nuclear factor 1α (HNF-1α).

In light with these observations, this review takes into consideration all studies reporting the hypocholesterolemic activity of food-derived peptides with special references to their production strategies, digestion, transportation, absorption, and mechanisms of action, highlighting the roles played in the prevention and management of hypercholesterolemia (**Figure 1**).

2. Production of hypocholesterolemic peptides

The generation of hypocholesterolemic peptides from food can be accomplished by a number of strategies, generally based on enzymatic hydrolysis, microbial

fermentation, or chemical hydrolysis. Enzymatic hydrolysis is the most common method to obtain hypocholesterolemic peptides having the advantages of the use of mild temperature and pH conditions, the selectivity of commercial enzymes compared with chemical hydrolysis, the absence of secondary products that may be often produced during microbial fermentations, and the absence of chemicals that makes this type of hydrolysis more sustainable for the environment (Xue, Yin, Howell, & Zhang, 2021). The process of enzymatic hydrolysis is simple and easy to inactivate and, once optimized, it may provide high yields of good quality bioactive peptides.

Different enzymes used in enzymatic hydrolysis possess specific substrate preferences that determine their cleavage sites on proteins. This specificity leads to the creation of distinct peptide profiles, influencing the properties of the resulting peptides. For instance, trypsin is known for its preference to cleave peptide bonds following positively charged amino acids, such as arginine and lysine, resulting in the production of peptides with basic properties. In contrast, chymotrypsin targets hydrophobic amino acids, yielding peptides with varying characteristics. Many natural peptides are produced from food proteins during the normal human digestion process, hydrolyzed by gastrointestinal enzymes, such as pepsin, pancreatin, trypsin, α-chymotrypsin, and peptidases. Enzymes from plants, food, bacteria and fungi, and commercial enzymes are also commonly used to produce peptides from various sources. For instance, food-grade enzyme (i.e., Alcalase) can release hypocholesterolemic peptides from different plant proteins, such as lupin, soy, hempseed, and olive kernel (Cerrato, et al., 2023; Prados, Marina, & García, 2018; Santos-Sánchez, et al., 2022). The type of hypocholesterolemic peptides produced after hydrolysis depends on the type of protease selected, as several enzymes have different cleavage sites and could produce different peptides even from the same

substrate. For instance, when white lupin (*Lupinus albus*) protein is hydrolyzed by pepsin or trypsin, peptides with different amino acid sequences and HMGCoAR-inhibitory activity are produced. In fact, the hydrolysate produced by pepsin showed lower HMGCoAR-inhibitory activity *in vitro* (−17%) at the maximum tested dose (2.5 mg/mL), whereas the trypsin hydrolysate significantly inhibited the HMGCoAR activity *in vitro* by 57% at the same concentration (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014). Moreover, the Alcalase hydrolysate from narrow-leaf lupin (*Lupinus angustifolius*) caused a reduction of HMGCoAR activity *in vitro* by 51.5% at the concentration of 2.5 mg/mL (Santos-Sánchez, et al., 2022). Interestingly, hempseed was digested with Alcalase and pepsin, and both hydrolysates showed HMGCoAR-inhibitory activity. However, the hydrolysate from Alcalase decreased the PCSK9 protein level in HepG2 cells (Cerrato, et al., 2023), whereas the pepsin hydrolysate showed an opposite activity raising the expression of PCSK9 (Zanoni, Aiello, Arnoldi, & Lammi, 2017a). Furthermore, the combination of different enzymes could further influence peptide activity. The utilization of multiple enzymes for hydrolysis is a widely adopted strategy due to the distinctive cleavage preferences of each enzyme. This diversity in enzymatic action leads to the generation of a broad range of peptides with diverse bioactive properties. This multifaceted approach not only enhances the overall efficiency and yield of peptide production but also improves the bioavailability of bioactive peptides, thereby increasing their potential health benefits. Notably, some known hypocholesterolemic peptides from plant proteins have been generated by a multi-enzyme system that simulates gastrointestinal digestion, as reported for rice protein hydrolysates (Tong, et al., 2017). Thus, the selection of the enzyme exerting suitable endo- and exopeptidase activities is a crucial step in the production of hypocholesterolemic peptides.

In addition, numerous processing methods, including microwave, pulsed electric field, high hydrostatic pressure, and ultrasound, can be combined with enzymatic hydrolysis to raise protein digestibility and peptide release (Marciniak, Suwal, Naderi, Pouliot, & Doyen, 2018; Ulug, Jahandideh, & Wu, 2021). It is believed that the processing techniques may cause the protein to unfold thus increasing the accessibility of the enzyme to break the peptide bonds. A study reported that the rate of β-lactoglobulin hydrolysis was raised 5–10 times under treatment at high hydrostatic pressure (300 or 450 MPa) together with specific enzymes (trypsin, chymotrypsin and a protease from *Bacillus licheniformis*) (Knudsen, Otte, Olsen, & Skibsted, 2002). High pressure can affect the conformation of β-lactoglobulin, causing it to unfold and to expose some hydrophobic areas, thereby increasing the enzyme-substrate collision rate. This in turn strengthens enzymatic activity, increasing the rate of protein hydrolysis and promoting the release of active peptides. In another study, high-pressure-assisted hydrolysis with commercial enzymes was employed, increasing levels of active peptides in the *Spirulina platensis* hydrolysates, and two HMGCoAR-inhibiting peptides (RCD and SNV) were identified (Chen & Yang, 2021). Likewise, cholesterol micelle formation inhibitory peptides were released from a fermented seabass byproduct through high hydrostatic pressure-assisted protease hydrolysis (Chen, Lin, Huang, Lin, & Lin, 2021).

Ovalbumin (Quirós, Chichón, Recio, & López-Fandiño, 2007), chickpea protein (Zhang, Jiang, Miao, Mu, & Li, 2012) and pinto bean protein (Garcia-Mora, et al., 2016), have been explored in this context. Besides high hydrostatic pressure, ultrasound-assisted technology has also been used in the production of hypocholesterolemic peptides because of its ability to unfold protein structure and strengthen the affinity between enzymes and proteins (Umego, He, Ren, Xu, & Ma,

2021). For example, mung bean hydrolysate exhibited higher inhibition of cholesterol solubilization after pre-treatment with thermosonication (Ashraf, et al., 2020). Ultrasound-assisted sodium bisulfite pre-treatment improved the cholesterol-lowering activity of soybean protein hydrolysates after simulated gastrointestinal digestion by loosening soybean protein structure and exposing more hydrophobic groups (Huang, et al., 2021).

Moreover, peptides with different hypocholesterolemic activities may also result from these various procedures. For example, the peptides from the protein isolate of raw cowpeas inhibit HMGCoAR activity, whereas peptides from cooked cowpeas are more effective in inhibiting micellar cholesterol solubility (Marques, Soares Freitas, et al., 2015). This may be due to the treatment temperature causing greater protein denaturation and release of various bioactive peptides. Overall, processing technologies are being applied in the production of hypocholesterolemic peptides and have been found to reduce time and costs of processing and to improve the yield of bioactive peptides.

On the other hand, modern in silico strategies based on simulation using bioinformatic tools are also supplying large amounts of data compared to the traditional empiric approaches. Modern in silico strategies use computational methods, including database mining, sequence analysis, structure-activity relationships, virtual screening, de novo design, bioavailability prediction, and safety assessment, to predict and design hypocholesterolemic peptides, streamlining the discovery process and enhancing efficiency and precision. For higher hydrolysis rates and larger production, continuous reactors are being developed by using membranes or immobilized enzymes (Sitanggang, Sumitra, & Budijanto, 2021). Although much research has been

189 performed at laboratory scale, further research is needed to overcome the challenges
190 related to large-scale production of hypocholesterolemic peptides.

191 **3. Digestion, transportation, and absorption of hypocholesterolemic peptides**

192 The hypocholesterolemic effect of peptides *in vitro* does not determine their
193 cholesterol-lowering effect *in vivo*, because several physical and biological barriers
194 have to be overcome. The hypocholesterolemic peptides can modulate *in vivo* the
195 endogenous cholesterol only if they are transported across the intestinal barrier into
196 the circulation in an intact or active form with adequate concentrations reaching their
197 target organs and tissues.

198 **3.1 Gastrointestinal digestion**

199 The digestive enzymes in the GI tract may act upon the hypocholesterolemic peptides,
200 and resistance of a peptide to the digestive enzymes depends on whether there are
201 cleavage sites for digestive enzymes in its amino acid sequence and whether these
202 cleavage sites are exposed. Most peptides generated from proteins enter the intestine,
203 which plays a key role in absorption. The intestinal brush-border membrane, highly
204 folded, provides a large surface area for metabolic activities, such as enzyme secretion
205 and transporter presentation. Some hypocholesterolemic peptides can be produced in
206 the GI tract during protein digestion by multiple microbial or digestive enzymes in the
207 brush-border membrane. Generally, *in vitro* digestion systems can be used to produce
208 hypocholesterolemic peptides and study their resistance to GI degradation. Enzymes,
209 including pepsin, trypsin, pancreatic protease, elastase, α-chymotrypsin, and
210 carboxypeptidases A and B are commonly used to mimic the process of human GI
211 digestion. For instance, two hypocholesterolemic peptides, VKP and VKK, identified
212 from freshwater clam hydrolysate with *in vitro* GI digestion, display bile-acid-binding

capacity and inhibitory activity on cholesterol micelle formation (Lin, Tsai, & Chen, 2017). Additionally, in this context, hypocholesterolemic peptides have been generated from various sources, including GCTLN, IAF, QGF, and QDF from cowpea bean; RCD and SNV from *S. platensis* protein; SAQ, PW, and VGGT from sea bass hydrolysates; and GEQQQQPGM from rice protein (Chen, et al., 2021; Chen & Yang, 2021; Marques, Fontanari, Pimenta, Soares-Freitas, & Arêas, 2015; Tong, et al., 2017).

The stability of peptides in GI digestion depends on the length and molecular size and structural characteristics. Firstly, the length and molecular size play a significant role. Numerous studies have shown that peptides with a molecular weight above 3 kDa are more likely to be hydrolyzed by GI enzymes than those below 3 kDa. Another study suggested that small peptides (2 ~ 6 amino acids) are less susceptible to hydrolysis by digestive enzymes, probably due to a reduced number of enzyme-susceptible peptide bonds and less structural flexibility (Xu, et al., 2019). Secondly, structural properties, including hydrophobicity, net charge, acid-base properties, C- and N-terminal amino acid residues, amino acid sequence, and amino acid composition, all have an impact on the digestive stability of peptides (Pei, et al., 2022).

Generally, peptides containing high content of proline residues, especially at the C-terminal, are more resistant to degradation by digestive enzymes (Dupont & Mackie, 2015). This resistance is due to proline unique structural characteristics, notably its cyclic structure, which restricts the flexibility of the peptide backbone. Consequently, this structural constraint hinders the proteolytic enzyme's ability to cleave effectively adjacent peptide bonds. This observation aligns with experimental evidence that many tripeptides with proline residues were detected in human blood plasma after oral ingestion of corn and wheat hydrolysates, demonstrating marked stability to *in vivo*

digestive conditions (Akika, Megumi, Yasushi, & Kenji, 2018). Peptides containing acidic amino acids were reported to display higher resistance to GI enzymes in comparison with peptides containing neutral and basic amino acids, such as Arg, His and Lys (C. Wang, Wang, & Li, 2016). Moreover, net negatively charged peptide fractions with higher acidic amino acid contents were reported to easily escape from *in vitro* GI digestion than positively charged fractions containing a higher amount of basic and aromatic residues (Ao & Li, 2013). This enhanced resistance to GI enzymes is attributed to the negatively charged nature of acidic amino acids, enabling them to form ionic bonds with positively charged enzyme groups. These interactions impair the enzymatic degradation. Regarding hydrophobicity, high numbers of hydrophobic amino acids, such as Val and Leu, within the peptide structure can have a profound impact on their stability and render them more susceptible to enzymatic digestion, particularly within the gastrointestinal environment. Peptides containing an elevated concentration of hydrophobic amino acids exhibit decreased solubility and are more likely to aggregate or form secondary structures due to the hydrophobic interactions between their constituent amino acids. These structural changes can expose vulnerable sites in the peptide, making them more accessible to digestive enzymes, such as trypsin and chymotrypsin, which preferentially cleave peptide bonds adjacent to hydrophobic residues. As a result, peptides rich in hydrophobic amino acids may experience more extensive enzymatic hydrolysis in the intestinal milieu. A recent review reported that Leu was completely absent at the C-terminal of stable peptides but accounted for a large proportion of C-terminal cleavages in unstable peptides (Ahmed, Sun, & Udenigwe, 2022). This finding is consistent with the specificity of carboxypeptidase A1, preferentially cleaving at C-terminal hydrophobic residues such as Leu. In addition, the cyclization induced by disulfide bond linkage would

potentially prevent susceptible peptide bonds from enzymatic cleavage during GI digestion (Góngora-Benítez, Tulla-Puche, & Albericio, 2014).

3.2 Intestinal transport and potential bioavailability of hypocholesterolemic peptides

It is important to note that there is not any specific mechanism of transport for hypocholesterolemic peptides, which are transported using the same mechanisms as other peptides (Xu, et al., 2019). Peptides can be transported across the intestinal epithelial cells through one or more routes, including peptide transport 1 (PepT1) - mediated routes, the paracellular route via tight junctions, transcytosis via vesicles and passive transcellular diffusion. Trans-epithelial transport and routes of transport of peptides vary based on the physicochemical properties, including net charge, hydrophobicity, chain length, and sequence of the peptide (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011).

Commonly, di- and tri-peptides can be actively transported intact across the brush border membrane of the epithelial cells into enterocytes via PepT1, which is responsible for the transportation of small peptides (< 500 Da). PepT1 is mainly distributed in the intestinal brush border membrane and is a high-capacity and low-affinity transporter that takes advantage of the proton gradient between the intestinal lumen (pH 5.5–6.0) and epithelial cells (pH 7.0). However, thousands of transported di- and tri-peptides are reported as having anti-hypertensive, antioxidant, antidiabetic, and anti-inflammatory properties (Xu, et al., 2019), whereas few literature data have reported the mechanism of transport of di- or tri-peptides with a cholesterol-lowering activity. For instance, dry-cured ham derived di-peptides DA, DD, EE, ES, and LL (Heres, Mora, & Toldrá, 2021), *Amaranthus cruentus* derived tri-peptides GGV and

IVG (Tovar-Pérez, Lugo-Radillo, & Aguilera-Aguirre, 2019), and cowpea bean β-vignin protein derived tri-peptides IAF, QGF, and QDF (M. Silva, et al., 2021), are identified as HMGCoAR inhibitors *in vitro* without information about the mechanism of transport. As mentioned in a recent review, 400 di-peptides and 8,000 tri-peptides can be recognized and transported by PepT1 (Xue, et al., 2021), without selecting for a specific amino acid sequence. Therefore, the mechanism of transport of these HMGCoAR inhibitory di- and tri-peptides may involve a PepT1-mediated route, but this needs to be verified.

Paracellular transport is a passive, energy-independent mechanism for the absorption of water-soluble peptides. It involves the transportation of peptides through water-filled channels between enterocytes. The hydrophilicity of these peptides plays a crucial role in their paracellular transport. Additionally, the paracellular route is the main pathway for the transportation of low molecular weight peptides. For example, peptides such as VPP derived from cheese and HLPLP derived from casein can be transported intact across the Caco-2 monolayer via paracellular transport (Xue, et al., 2021). The presence of tight junctions, which mediate the paracellular route, explains why smaller peptides exhibit a higher transport rate compared to larger ones. This is due to the diameter of the pores formed by tight junctions, which measures approximately 5-6 nm in the crypts and 0.4-0.9 nm in the villi of the intestinal membrane. The tight junctions of Caco-2 cell monolayers contain a substantial number of pores with a diameter of 1.2-2.1 nm, suggesting their ability to transport peptides smaller than 27 amino acids (the estimated diameter being approximately 2.1 nm) (Xu, et al., 2019). However, peptides with high hydrophobicity are more easily transported by simple passive transcellular diffusion or by transcytosis (Xu, et al., 2019; Xue, et al., 2021). A recent study (Lammi, et al., 2021), examined the intestinal

transport ability in the differentiated Caco-2 cell model of white lupin peptide LILPKHSDAD with a dual HMGCoAR/PCSK9 inhibitory activity. Since LILPKHSDAD is a decapeptide with a net charge (−1) and hydrophobicity (+17.79 kcal/mol), it might be preferentially transported by passive transcellular diffusion or by transcytosis.

It is difficult to assess the transport through the passive diffusion route due to the lack of regulators of this route, whereas wortmannin can be used as a transcytosis inhibitor to investigate the transcytosis route (Vij, Reddi, Kapila, & Kapila, 2016). In the presence of wortmannin, the transport of LILPKHSDAD was significantly impaired, which suggested that LILPKHSDAD is mainly transported by the transcytotic route. Another study investigated the intestinal trans-epithelial transport of the hempseed peptide IGFLIIWV with hypocholesterolemic activity, and results suggested that this peptide may be preferentially transported by the paracellular route and/or by transcytosis due to its hydrophobic property (Bollati, et al., 2022). Generally, intestinal transport and route of transport of hypocholesterolemic peptides, especially via transcytosis, have been shown to depend on molecular weight, net charge and hydrophobicity, with small-sized, positively charged and hydrophobic peptides being generally more permeable than others (Shimizu & Ok Son, 2007). The current understanding of the structural requirements for peptides in transepithelial transport is limited. Nevertheless, this knowledge would be very important for providing functional foods with enhanced prevention potential. Moreover, it can facilitate the precise delivery of bioactive peptides, enabling the development of targeted delivery systems. Addressing the gaps in knowledge regarding peptide transport is crucial for evidence-based healthcare and nutrition practices. Furthermore, studying the relationship between structure and transport ensures the safety and efficacy of these

processes for regulatory purposes. In summary, further research in this field is essential to unlock the full potential of bioactive peptides, offering opportunities for better healthcare, personalized nutrition, and more effective therapies, ultimately advancing human health. This underscores the importance of conducting comprehensive investigations into the structure-transport relationship by using well-established bioavailable peptides and physiologically relevant intestinal models.

Furthermore, *in vivo* bioactivities of some peptides may be also directly associated with their fragments generated by the action of peptidases during intestinal transport (Daroit & Brandelli, 2021; Karaś, 2019). For example, the peptide LPKHSDAD was produced by hydrolysis of LILPKHSDAD by Caco-2 cell peptidases and transported across the cell monolayer via a passive diffusion mechanism or the paracellular route, not by intracellular transcytosis being unaffected by wortmannin. LPKHSDAD was also proved to exert a hypocholesterolemic behavior and shared the same mechanism of action with its native peptide (Lammi, et al., 2021). However, in some cases, metabolism under the action of peptidases may generate a fragment whose activity is enhanced and/or shifted to different targets. The HMGCoAR inhibitory peptide LTFPGSAED from white lupin protein hydrolysates was reported to be hydrolyzed to LTFPG by Caco-2 cell peptidases, and both the native peptide and its fragment were transported across the cell monolayer (Lammi, et al., 2020). In particular, LTFPGSAED was transported across the cell monolayer by the transcellular route, whereas the mechanism of transport of LTFPG may involve the paracellular route. Although LTFPG showed a poor ability to reduce the *in vitro* HMGCoAR activity, it is an effective hypotensive peptide whose activity has been demonstrated both *in vitro* and *in vivo*. Based on *in vitro* bioactivity, the transported peptides are strong

candidates for further evaluation of hypocholesterolemic properties and/or other health-promoting activities *in vivo*.

In addition, despite their permeability across the intestinal epithelium, many bioactive peptides are not bioavailable in substantial amounts *in vivo*. An 8.5% decrease in plasma PCSK9 level followed by a cholesterol-lowering effect was observed in mildly hypercholesterolemic humans who consumed 30 g of lupin protein/day for 4 weeks (Lammi, Zanoni, Calabresi, & Arnoldi, 2016). Although this may suggest that the hypocholesterolemic peptides were absorbed, detection and quantification of the parent peptides in serum and tissues will be crucial in validating their biostability and bioavailability. Based on the limited literature in this area, short chain hydrophobic peptides are hypothesized to be more resistant to hydrolysis by intestinal brush border proteases and to across the intestinal epithelium in their intact form (Daniel, 2004). Some hypocholesterolemic peptides that are not absorbed through the intestine can also offer health benefits by binding bile acids and inhibiting cholesterol micellar solubility modulating dietary cholesterol metabolism in the gut (Nagaoka, et al., 2010). Unabsorbed bioactive peptides may also influence gut microbiota population and metabolism in a special way thus exerting a positive cholesterol-lowering condition (Ashaolu, 2020). Given that gut microbiota has been reported to mediate other health-promoting effects of bioactive peptides, it is imperative to explore how the gut microbiota may influence the bioavailability and activity of these peptides, particularly in the context of their cholesterol-lowering properties. The gut microbiota can metabolize unabsorbed bioactive peptides, potentially leading to structural modifications or the generation of biologically active metabolites (Guo, et al., 2021; Wu, et al., 2021; Yu, Amorim, Marques, Calhau, & Pintado, 2016). This microbial metabolism plays a pivotal role in shaping the absorption and efficacy of these

386 peptides, ultimately impacting their hypocholesterolemic potential and broader health
387 benefits. Therefore, understanding the interplay between gut microbiota and bioactive
388 peptides is crucial in comprehending their physiological effects.

389 **4. Molecular mechanisms of hypocholesterolemic peptides**

390 **4.1 Effect of food-derived peptides on bile acids and micelles**

391 Hypocholesterolemic peptides interact with exogenous cholesterol through interaction
392 with bile acids, salts, and lipids, as summarized in **Table 1**.

393 During GI absorption, dietary cholesterol forms micelles with bile acids, which
394 promote its solubility and facilitate absorption in the intestine. Therefore, by
395 suppressing the solubility of dietary cholesterol in micelles, the process of
396 emulsifying bile acid and cholesterol can be effectively prevented. This, in turn,
397 inhibits the formation of cholesterol micelles and ultimately contributes to the
398 reduction of cholesterolemia.

399 In recent years, several studies have shown that food-derived proteins possess the
400 ability to prevent bile acid reabsorption, stimulate the conversion of liver cholesterol
401 to supplementary bile acids, and diminish the solubility of cholesterol micelles in the
402 cells that line the small intestine. This process effectively lowers excessive serum
403 elevated cholesterolemia. For instance, both major royal jelly protein 1 (MRJP1) and
404 rice bran protein have shown a remarkable ability to bind to taurocholate, effectively
405 inhibiting the micellar solubility of cholesterol *in vitro*, compared to casein (Kashima,
406 et al., 2014; J. Wang, et al., 2015). This hypocholesterolemic effect of both proteins
407 was further demonstrated in rat studies, leading to increased excretion of fecal
408 steroids, including cholesterol and bile acids, and enhanced hepatic cholesterol
409 catabolism (Kashima, et al., 2014; J. Wang, et al., 2015). In addition, the tryptic

hydrolysate of MRJP1 has exhibited a capacity to increase the cholesterol 7α-hydroxylase (CYP7A1) mRNA and protein levels in hepatocytes compared with that of casein tryptic hydrolysate (Kashima, et al., 2014). Similarly, protein hydrolysates derived from a range of food sources, including bovine milk β-lactoglobulin, soybean, chickpea (*Cicer arietinum L.*), cowpea bean and olive seed, have demonstrated a definite capacity to bind bile acids/salts or lipids and effectively inhibit the solubility of cholesterol in micelles. In these studies, the β-lactoglobulin tryptic hydrolysate exhibits hypocholesterolemic effects by inhibiting the micellar solubility of cholesterol, thereby reducing both serum and liver cholesterol levels in rats (Nagaoka, et al., 2001). Meanwhile, the identified peptide IIAEK from β-lactoglobulin has also confirmed a hypocholesterolemic activity (Nagaoka, et al., 2001). In addition, a hydrolysate of soy protein digested with Alcalase showed the highest inhibition of micellar solubility inhibition, i.e. 48.6% when the protein hydrolysis rate reached 18% (Zhong, Liu, Ma, & Shoemaker, 2007). In a mouse-feeding study, levels of LDL-C + Very-low-density lipoprotein (VLDL)-C went down by 34% and 45%, respectively, when mice consumed high-cholesterol diets with the soy protein hydrolysate (0.5 and 2.5 g/kg body weight), compared to animal fed only the high fat diet (Zhong, Liu, et al., 2007). After further purification, the hydrolysate yielded an active peptide sequence recognized as WGAPSL, with the highest inhibition rate of 94.3% against micellar solubility (Zhong, Zhang, Ma, & Shoemaker, 2007). Moreover, the other two peptides IAVPGEVA and VAWWMY (soystatin), derived from soybean glycinin, exhibited a significant bile acid-binding capacity with IAVPGEVA specifically enhancing cholesterol metabolism in plasma, while soystatin displayed clearly inhibited micellar solubility and cholesterol absorption in rats (Nagaoka, et al., 2010; Pak, Koo, Kasymova, & Kwon, 2005). In a study with chickpea protein, a hydrolysate

was obtained with Alcalase and Flavourzyme, exhibiting a significant inhibition rate of up to 50% for cholesterol micellar solubility (Yust, Millán-Linares, Alcaide-Hidalgo, Millán, & Pedroche, 2012). Such a direct interaction with lipids was also observed for protein hydrolysates digested from olive seed, cooked cowpea and flaxseed, respectively, all inhibiting micellar cholesterol solubility *in vitro* (Bao, Yuan, Li, & Liu, 2022; Marques, Fontanari, et al., 2015; Prados, et al., 2018). In particular, the flaxseed-derived peptide IPPF exhibited the highest cholesterol micelle solubility inhibition rate of 93.47% and effectively modulated the protein expression levels of cholesterol transporters Niemann-Pick C1-Like 1 (NPC1L1) and ATP-binding cassette transporter G5/G8 (ABCG5/8) in Caco-2 cells, thereby inhibiting cholesterol intestinal absorption (Bao, et al., 2022).

This mechanism was also demonstrated in hydrolysates derived from both freshwater clam (*Corbicula fluminea*) residual meat byproducts and sea bass byproducts. The hydrolysate from freshwater clam residual meat byproducts exhibited a cholic acid-binding ability of 35.9% and an inhibitory activity of 18.5% against cholesterol micelle formation (Lin, Tsai, Hung, & Pan, 2010, 2011). Moreover, the total cholesterol levels in plasma and liver were decreased by 26.1% and 50.0%, respectively, in Sprague–Dawley rats with hyperlipidemia after consuming the clam meat hydrolysate (16.6% in the diet) (Lin, et al., 2010, 2011). The purified peptides VKP and VKK from clam meat hydrolysate had inhibitory efficiency ratios of 64.8% and 10.2% mg/mL, respectively, by binding bile acids and inhibiting cholesterol micelle formation (Lin, et al., 2017).

In another study, after lactic fermentation, the byproducts of sea bass were hydrolyzed by high hydrostatic pressure (HHP)-assisted Protease N, followed by GI enzymatic hydrolysis. After fractionation by gel filtration chromatography, the most active

fraction included three di-peptide inhibitors of cholesterol micelle formation, SAQ, PW, and VGGT, with inhibitory efficiency ratios of 361.7, 3230.0, and 302.9%/mg/mL, respectively (Chen, et al., 2021). In addition, a recent study revealed hypocholesterolemic effects of silk sericin-derived oligopeptides, inhibiting cholesterol uptake in monolayer Caco-2 cells and decreasing serum total and non-high-density lipoprotein cholesterol (HDL-C) levels in rats, likely attributed to a direct interaction between silk sericin-derived oligopeptides and cholesterol/bile acids (Lapphanichayakool, Sutheerawattananonda, & Limpeanchob, 2017).

However, there is still limited *in vivo* and clinical evidence to support the hypocholesterolemic effects resulting from the interactions between bile acid/lipid and peptides derived from food proteins. Meanwhile, although numerous studies have demonstrated the importance of the presence of hydrophobic cores in peptides for cholesterol and bile acid binding (Boachie, et al., 2018), information on the structure–function relationship of bile acid/lipid interactions with food protein-derived peptides is still scarce. Therefore, further studies on the hypocholesterolemic activity of protein hydrolysates, novel peptides, and identification of their peptide sequences are necessary to understand their possible mechanism of action and structure–function relationship.

Table 1 summarizes *in vitro* and *in vivo* food-derived peptides related to exogenous cholesterol.

480 *Table 1. Effects of food-derived peptides on cholesterol reduction via interaction with bile acids, salts, and lipids in the gastrointestinal tract.*

Peptide sequence	**Protein source**	**Hydrolytic enzyme**	***In vitro* or *in vivo***	**Mechanism of action**	**Hypocholesterolemic effect/micellar solubility**	**References**
Peptide mixtures	Bovine milk β-lactoglobulin	Trypsin	*In vivo* (rats)	Inhibition of micellar solubility of cholesterol	↓Serum and liver cholesterol levels	(Nagaoka, et al., 2001)
IIAEK	Bovine milk β-lactoglobulin hydrolysate	Trypsin	*In vivo* (rat)	Inhibition of micellar solubility of cholesterol	↓Serum and liver cholesterol levels	(Nagaoka, et al., 2001)
Peptide mixtures	Chickpea (*Cicer arietinum L.*)	Alcalae and Flavourzyme	*In vitro*	Inhibition of micellar solubility of cholesterol	Identification of highest cholesterol micellar solubility inhibition rate --50%	(Yust, et al., 2012)
Peptide mixtures	Soy	Alcalase	*In vivo* (mice)	Inhibition of micellar solubility of cholesterol	The highest cholesterol micellar solubility inhibition rate was 48.6% (when the protein hydrolysis rate reached 18%) ↓Serum level of LDL-C + VLDL-C (decreased by 34% and 45%, respectively, at 0.5 and 2.5 g/kg b.w.)	(Zhong, Liu, et al., 2007)
WGAPSL	Soy protein hydrolysate	Alcalase	*In vitro*	Inhibition of micellar solubility of cholesterol	The highest cholesterol micellar solubility inhibition rate was 94.3%	(Zhong, Zhang, et al., 2007)

IAVPGEVA	Soy glycinin (11S-globulin) hydrolysate	Pepsin	*In vitro*	Binding to bile acids	↓Reabsorption of bile acids ↑—Cholesterol metabolism in plasma	(Pak, et al., 2005)
Soystatin (VAWWMY)	Soy glycinin		*In vitro* and *in vivo* (rats)	Binding to bile acids, and inhibition of micellar cholesterol solubility and cholesterol absorption	↓Micellar solubility and cholesterol absorption in rats	(Nagaoka, et al., 2010)
Oligopeptides	Sericin (silk cocoon)	Protease (from *Bacillus* species)	*In vitro* and *in vivo* (rats)	Binding to bile acids/salts or lipids, and inhibition of micellar cholesterol solubility	↓Cholesterol solubility in lipid micelles ↓Cholesterol uptake in monolayer Caco-2 cells ↓Serum total and non-HDL cholesterol levels in rats (three doses: 10 mg kg^{-1} day^{-1}, 50 mg kg^{-1} day^{-1}, and 200 mg kg^{-1} day^{-1}, respectively)	(Lapphanichayakool, et al., 2017)
Protein (MRJP1) and MRJP1 tryptic hydrolysate	Royal jelly	Trypsin	*In vitro* and *in vivo* (rats)	Binding to bile acids/salts, and inhibition of micellar cholesterol solubility	↓Micellar cholesterol solubility *in vitro* ↑Liver bile acids levels, and CYP7A1 mRNA and protein ↑Fecal bile–acid and cholesterol excretion in rats	(Kashima, et al., 2014; Mureșan, et al., 2022)

Protein	Rice bran		*In vitro* and *in vivo* (rats)	Binding to bile acids and inhibition of micellar cholesterol solubility	↓Micellar cholesterol solubility *in vitro* ↓Serum total cholesterol levels in rats ↑Excretion of fecal cholesterol and bile acids in rats	(J. Wang, et al., 2015)
Peptide mixtures	Olive seed	Alcalase	*In vitro*	Inhibition of micellar cholesterol solubility	↓Micellar cholesterol solubility Inhibition of cholesterol esterase and lipase enzymes	(Prados, et al., 2018)
IPPF	Flaxseed (*Linum usitatissimum*)	Protease M	*In vitro*	Inhibition of intestinal cholesterol absorption in Caco-2 cells and hepatic cholesterol synthesis in HepG2 cells	↓Micellar cholesterol solubility (The highest cholesterol micelle solubility inhibition rate was 93.47%) Caco-2 cells: (cholesterol transporters) ↓NCP1L1 protein levels ↑Protein levels of ABCG5 and ABCG8 in HepG2 cells (cell transporters) ↓mRNA levels of SREBP-2 and HMGCoAR	(Bao, et al., 2022)

Peptide mixtures	Freshwater clam meat	Protamex	*In vitro* and *in vivo* (rats)	Binding to bile acids and inhibited formation of cholesterol micelles	A binding capacity of 35.9% with cholic acid and an inhibitory capacity of 18.5% against cholesterol micelle formation, *in vitro* ↓Cholesterol (content in plasma and liver reduced by 26.1% and 50.5%, respectively)	(Lin, et al., 2010, 2011)
VKP and VKK	Muscles of freshwater clams hydrolysate	Pepsin	*In vitro*	Bind to bile acids and inhibit formation of cholesterol micelles	The inhibitory efficiency ratios are 64.8% and 10.2% mg/mL, respectively	**(Lin, et al., 2017)**
Peptide mixtures	Sea bass byproducts	High hydrostatic pressure (HHP)-assisted Protease N hydrolysis after lactic fermentation	*In vitro*	Inhibit formation of cholesterol micelles	The inhibitory activity on formation of cholesterol micelles is 88.4%	(Chen, et al., 2021)
SAQ, PW and VGGT	Sea bass hydrolysates	Gastrointestinal digestion	*In vitro*	Inhibit the formation of cholesterol micelles	The inhibitory efficiency ratios are 361.7, 3230.0, and 302.9%/mg/mL, respectively	(Chen, et al., 2021)

481 Micellar solubility observed in food-derived peptides in the different *in vitro* and *in vivo* models. ↑, increased; ↓, decreased.
482

483

4.2 Modulation of endogenous cholesterol levels via cholesterol synthesis pathways

Table 2 summarizes the *in vitro* and *in vivo* effects of food-derived peptides related to the cholesterol synthesis pathway, as also illustrated in **Figure 2**.

489 *Table 2. Effects of food-derived peptides on cholesterol reduction via modulate of cholesterol synthesis pathway.*

Peptide sequence	**Protein source**	**Hydrolytic enzyme**	***In vitro* or *in vivo***	**Mechanism of action**	**Hypocholesterolemic effect**	**References**
Peptide mixtures	Chia protein	Alcalase, Flavourzyme and sequential Alcalase-Flavourzyme	*In vitro*	Inhibition of HMGCoAR activity	↓*In vitro* HMGCoAR activity (More effective at 3 mg/mL)	(Coelho, Soares-Freitas, Arêas, Gandra, & Salas-Mellado, 2018)
HPP and SGQR	Silkworm pupae protein	Neutral proteinase	*In vitro*	Inhibition of HMGCoAR activity	↓mRNA and protein level of HMGCoAR (1.2- to 1.7-fold decrease at 0.5 mg/mL)	(Sun, et al., 2021)
DA, DD, EE, ES, and LL	Dry-cured ham	Generated during manufacturing	*In vitro*	Inhibition of HMGCoAR activity (statin-like interactions of the dipeptides with HMGCoAR)	↓*In vitro* HMGCoAR activity (More than 40% at 1 mM)	(Heres, et al., 2021)
RCD and SNV	*Spirulina platensis*	Gastrointestinal digestion	*In vitro*	Inhibition of HMGCoAR activity	↓*In vitro* HMGCoAR activity (IC_{50}: 6.9 μM and 20.1 μM, respectively)	(Chen & Yang, 2021)

Lunasin	Soybean		*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity	↓HMGCoAR activity ↑LDLR expression	(Galvez, 2012)
Peptide mixtures	Olive seed	Alcalase	*In vitro* and *in vivo* (mice)	Inhibition of HMGCoAR activity	↓HMGCoAR activity ↑LDLR expression (At dose of 200 or 400 mg/kg/day)	(Prados, Orellana, Marina, & García, 2020)
Peptide mixtures	Cowpea (raw and cooked beans)	Gastrointestinal digestion	*In vitro*	Inhibition of HMGCoAR activity and micellar cholesterol solubility	↓*In vitro* HMGCoAR activity ↓The micellar solubility of cholesterol (Peptides from the protein isolate of raw cowpeas inhibit HMGCoAR activity, whereas ~~the~~ peptides from cooked cowpeas are more effective in inhibiting the micellar cholesterol solubility)	(Marques, Soares Freitas, et al., 2015)
GCTLN	Cowpea bean	Gastrointestinal digestion	*In vitro*	Inhibition of HMGCoAR activity and micellar cholesterol solubility	↓*In vitro* HMGCoAR activity ↓Micellar cholesterol solubility	(Marques, Fontanari, et al., 2015)
IAF, QGF, and QDF	Cowpea bean β-vignin protein	Gastrointestinal digestion	*In vitro*	Inhibition of HMGCoAR activity (Lower cholesterol synthesis through a statin-like mechanism)	↓*In vitro* HMGCoAR activity (At 500 µM concentration, IAF, QGF, and QDF reduced HMGCoAR activity by 69%, 77% and 78%)	(M. Silva, et al., 2021; M. B. d. C. e. Silva, et al., 2018)

GGV, IVG, and VGVL	*Amaranthus cruentus*	Multi-enzyme system	*In vitro*	Inhibition of HMGCoAR activity	↓*In vitro* HMGCoAR activity (IC_{50} of VGVL: 50 μM)	(Soares, Mendonça, De Castro, Menezes, & Arêas, 2015)
GEQQQQPGM	Rice protein α-globulin	Pepsin and Trypsin sequential *in vitro* digestion	*In vivo* (hamsters)	Lower plasma LDL cholesterol	↓LDL cholesterol (100 mg/kg b.w.)	(Tong, et al., 2017)
Peptide mixtures	Pigeon pea milling by-product	Pepsin and pancreatic enzymes	*In vivo* (rats)	Inhibition of HMGCoAR activity	↓mRNA expression levels (SREBP2, HMGCoAR, PPARγ, and CYP7A1) ↑mRNA expression levels (LDLR, PPARα, LPL, Insig1, and Insig2)	(Kumar, Muthu Kumar, & Tiku, 2021)
PFVKSEPIPETNNE	Pigeon pea milling by-product	Pepsin and pancreatic enzymes	*In vitro* and *in vivo* (rats)	Inhibition of HMGCoAR activity Increase SREBP-2 and LDLR protein levels	*In vitro* (HepG2 cells): ↓*In vitro* HMGCoAR activity ↑mRNA and protein expression of HMGCoAR, LDLR and SERBP-2 ↑LDL uptake *In vivo* (Wistar rats): less effective in reducing liver cholesterol	(Kumar, et al., 2021; Kumar, et al., 2019)
Peptide mixtures	Chickpea	Alcalase	*In vivo* (high-fat diet-induced obese rats)	Inhibition of HMGCoAR activity and micellar cholesterol solubility	↓HMGCoAR ↑LDLR	(Shi, Hou, Guo, & He, 2019)

VFVRN	Chickpea	Identified from chickpea peptides using a pharmacophore model	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity	↓*In vitro* HMGCoAR activity (0.4 mM inhibited HMGCoAR by 64.38%) in HepG2 cells	(Shi, et al., 2019)
Peptide mixtures Peptide mixtures	White lupin (*Lupinus*	Pepsin Trypsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity and PCSK9-LDLR binding, *in vitro* Increasing SREBP-2 and LDLR protein levels and decreasing PCSK9 production via HNF-1α protein	↓*In vitro* HMGCoAR activity (17% reduction by peptic peptides and 57% by tryptic peptides at 2.5 mg/mL) ↓PCSK9-LDLR binding ↓PCSK9 ↓HNF-1α ↑SREBP-2 ↑LDLR expression ↑LDL uptake ↑Activation of PI3K/Akt/GSK3β kinases	(Lammi, Aiello, et al., 2016; Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016; Lammi, Zanoni, Calabresi, et al., 2016; Lammi, et al., 2014)
YDFYPSSTKDQQS	White lupin (*L. albus*) β-conglutin	Pepsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity (Modulates cholesterol metabolism in HepG2 cells via SREBP-1 activation)	↓*In vitro* HMGCoAR activity ↑LDLR expression ↑LDL uptake ↑SREBP-1	(Lammi, Zanoni, Arnoldi, & Aiello, 2018)

GQEQSHQDEGVIVR	Lupin (*albus*) β-conglutin	Trypsin	*In vitro* (HepG2 cells)	Modulates the mutant PCSK9^{D374Y} pathway, a dual mechanism of action involving either the modulation of the PCSK9^{D374Y} or LDLR pathways	↓PCSK9^{D374Y}-LDLR binding (IC_{50}: 285.6 ± 2.46 μM) ↓PCSK9^{D374Y}-FLAG protein ↓HNF-1α ↓*In vitro* HMGCoAR activity (IC_{50}: 99.5 ± 0.56 μM) ↓HMGCoAR ↑LDLR ↑LDL uptake ↑SREBP-2	(Grazioso, Bollati, Sgrignani, Arnoldi, & Lammi, 2018; Lammi, Bollati, Lecca, Abbracchio, & Arnoldi, 2019)
GQRQWKQAEGVMVR	Analogs of GQEQSHQDEGVIVR (Computational design)	Computational design	*In vitro*	Inhibits the mutant PCSK9^{D374Y} activity	↓PCSK9^{D374Y}-LDLR binding (IC_{50} : 147.8 ± 3.23 μM)	(Lammi, Sgrignani, Roda, Arnoldi, & Grazioso, 2019)

LILPKHSDAD	Lupin (*Lupinus albus*) β-conglutin	Pepsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity and PCSK9-LDLR binding; raises SREBP-2 and LDLR protein levels and decreases PCSK9 production via the HNF-1α protein	↓*In vitro* HMGCoAR activity (IC_{50}: 147 µM) ↓PCSK9 ↓HNF-1α ↓PCSK9-LDLR binding ↑pHMGCoAR (Ser 872) ↑pAMPK (Thr 172) ↑LDLR ↑LDL uptake ↑SREBP-2	(Zanoni, Aiello, Arnoldi, & Lammi, 2017b)
LPKHSDAD	Metabolite of LILPKHSDAD, during epithelial transport experiments	Pepsin and intestinal peptidases	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity and PCSK9-LDLR binding, raises SREBP-2 and LDLR protein levels and decreases PCSK9 production via HNF-1α protein	↓*In vitro* HMGCoAR activity (IC_{50}: 175.3 µM) ↓PCSK9 ↓HNF-1α ↓PCSK9-LDLR binding (IC_{50}: 1.7 µM) ↑pHMGCoAR (Ser 872) ↑pAMPK (Thr 172) ↑LDLR ↑LDL uptake ↑SREBP-2	(Lammi, et al., 2021)

LYLPKHSDRD, LILPKASDAD, and LILPKHADAD	Analogs of LILPKHSDAD	Computational design	*In vitro*	Inhibition of HMGCoAR activity and PCSK9-LDLR binding, raises SREBP-2 and LDLR protein levels and lowers PCSK9 production via effect on HNF-1α protein	Showed the same/similar effects of LILPKHSDAD ↓*In vitro* HMGCoAR activity (IC_{50}: 88.9 μM, 74.4 μM, and 73.8 μM) ↓PCSK9-LDLR binding (IC_{50}: 0.7 μM, 9.0 μM, and 1.45 μM)	(Lammi, et al., 2022)
LTFPGSAED	Lupin (*Lupinus albus*) β-conglutin	Pepsin	*In vitro* (HepG2 cells)	Inhibits HMGCoAR activity Increasing SREBP2 and LDLR protein levels	↓*In vitro* HMGCoAR activity (IC_{50}: 68 μM) ↑pHMGCoAR (Ser 872) ↑pAMPK (Thr 172) ↑LDLR ↑LDL uptake ↑SREBP-2	(Zanoni, et al., 2017b)
LTFPG	Metabolite of LTFPGSAED, during epithelial transport experiments	Pepsin and intestinal peptidases	*In vitro*	Inhibition of HMGCoAR activity	↓ HMGCoAR activity (Inhibits the enzyme by 4.7 ± 0.3 and 10.3 ± 0.8% at 100 and 250 μM)	(Lammi, et al., 2020)

Peptide mixtures	Lupin (*Lupinus angustifolius*)	Alcalase	*In vivo* (western diet-fed ApoE$^{-/-}$ mice)	Hypocholesterolemic effects in Western diet-fed ApoE$^{-/-}$ mice by modulation of LDLR and PCSK9 pathways	↓ HMGCoAR activity (Decrased by 51.5 ± 0.6% at 2.5 mg/mL) ↓PCSK9 ↓HNF-1α ↓HMGCoAR ↑pHMGCoAR (Ser 872) ↑pAMPK (Thr 172) ↓LDLR ↑LDL uptake ↓SREBP-2	(Santos-Sánchez, et al., 2022)
IAVPGEVA, IAVPTGVA, and LPYP	Soy glycinin	Pepsin or Trypsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity Increased SREBP2 and LDLR protein levels via the activation of AMPK and ERK 1/2	↓*In vitro* HMGCoAR activity (IC_{50}: 222 ± 90, 274 ± 95, and 300 ± 150 μM) ↑LDLR ↑LDL uptake ↑SREBP-2 ↑pAMPK (Thr 172) ↑pHMGCoAR (Ser 872) ↑pERK 1/2 (Thr 202/Tyr 204)	(Lammi, Zanoni, & Arnoldi, 2015)
YVVNPDNDEN and YVVNPDNNEN	Soy β-conglycinin	Pepsin or Trypsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity Increased SREBP2 and LDLR protein levels	↓-HMGCoAR activity (IC_{50}: 150 and 200 μM) ↑LDLR ↑LDL uptake ↑SREBP-2	(Lammi, Zanoni, Arnoldi, et al., 2015)

Peptide mixtures (Including 90 identified peptides belonging to 33 proteins)	Hempseed (*Canabis sativa*)	Pepsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity (Hypocholesterolemic effects with a statin-like mechanism)	↓ HMGCoAR activity ↑pHMGCoAR (Ser 872) ↑pAMPK (Thr 172) ↑LDLR ↑LDL uptake ↑SREBP-2 ↑PCSK9	(Zanoni, et al., 2017a)
Short-chain peptide mixture, medium-chain peptide mixture, and total hydrolysate	Hempseed	Alcalase	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity. Increased SREBP-2 and LDLR protein levels and reduced PCSK9 production via HNF-1α protein	↓HMGCoAR activity ↓PCSK9 ↓HNF-1α ↑LDLR ↑SREBP-2	(Cerrato, et al., 2023)
IGFLIIWV	Hempseed (*Cannabis sativa*)	Pepsin	*In vitro* (HepG2 cells)	Inhibition of HMGCoAR activity Increased SREBP-2 and LDLR protein levels and lower PCSK9 production via HNF-1α protein	↓ HMGCoAR activity ↓PCSK9 ↓HNF-1α ↑pHMGCoAR (Ser 872) ↑pAMPK (Thr 172) ↑LDLR ↑LDL uptake ↑SREBP-2	(Li, et al., 2022)

Lunasin (a 43-amino acid polypeptide)	Soybean	*In vitro* (HepG2 cells) and *in vivo* ($ApoE^{-/-}$ mice)	Inhibits PCSK9 expression by down-regulating HNF-1α and enhances LDLR expression via PI3K/Akt-mediated activation of SREBP-2 pathway	↓PCSK9 at mRNA and protein levels ↓HNF-1α ↑LDLR ↑LDL uptake	(Fernández-Tomé & Hernández-Ledesma, 2019; Gu, et al., 2017)

Hypocholesterolemic effects observed in food-derived peptides in the different *in vitro* and *in vivo* models. ↑, increased; ↓, decreased.

493 **4.2.1 HMGCoAR-inhibiting effect of food-derived peptides**

494 The most common pharmacological strategy for hypercholesterolemia control is
495 based on the inhibition of HMGCoAR, the rate-controlling enzyme in the mevalonate
496 pathway an a key factor in endogenous cholesterol biosynthesis, thereby elevating the
497 LDLR expression, increasing the LDL particle uptake from the circulation. The most
498 representative oral agents targeted to HMGCoAR for the prevention and treatment of
499 cardiovascular diseases associated to hypercholesterolemia are the statins, such as
500 lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, pitavastatin and
501 rosuvastatin, all reversible competitive inhibitors of HMGCoAR. Although statins are
502 effective medications for primary and secondary prevention of CVD and are taken by
503 approximately 25% of the world older population (> 65 years), patients treated with
504 statins may have undesirable side effects, such as muscle pain, including muscle
505 weakness, myalgia, stiffness, cramps, and arthralgia (Reiner, 2014). Moreover, other
506 limitations of statins are the considerable variability of individual LDL-C reduction
507 after statin therapy (varying from 5 to 70%) and the inability to reduce LDL-C to
508 desirable and safe levels for ~ 50% of the patients (Taylor & Thompson, 2016).
509 Limitations of statins have stimulated research towards discovering new drugs for
510 cholesterol management, and food-derived peptides, due to their very low toxic or
511 adverse effects.

512 Over the years, some food-derived peptides were found to inhibit HMGCoAR activity
513 *in vitro*, and/or to lower endogenous cholesterol levels *in vivo* statin-like effects with
514 other mechanisms. For instance, white lupin protein hydrolysates digested by pepsin
515 or trypsin were found to reduce HMGCoAR activity *in vitro* (-17% for peptic peptides
516 and -57% for tryptic peptides at 2.5 mg/mL) and improved the capacity of HepG2

cells to take up LDL-C from the extracellular environment by way of the LDLR (Lammi, et al., 2014). Furthermore, white lupin peptides LILPKHSDAD and LTFPGSAED inhibited HMGCoA activity *in vitro* with an IC_{50} values of 147 μM and 68 μM, respectively; an *in-silico* investigation further predicted the potential binding mode to the catalytic site of this enzyme (Zanoni, et al., 2017b). Peptides LPKHSDAD and LTFPG are the metabolites of LILPKHSDAD and LTFPGSAED, respectively, during epithelial transport experiments, which also shown the inhibitor activity of HMGCoAR *in vitro* (Lammi, et al., 2021; Lammi, et al., 2020). In addition, this feature was shown *in vitro* with computational design analogs of LILPKHSDAD, including LYLPKHSDRD (IC_{50} ~ 88.9 μM), LILPKASDAD (IC_{50} ~ 74.4 μM), and LILPKHADAD (IC_{50} ~ 73.8 μM) (Lammi, et al., 2022). Similarly, HMGCoAR-inhibitory peptides were also found in hempseed protein. A study on a culture of HepG2 cells demonstrated that the cholesterol-lowering effect of hempseed protein hydrolysate digested by pepsin, is due to the inhibition of HMGCoAR activity with a statin-like mechanism (Zanoni, et al., 2017a). Moreover, the HMGCoAR inhibitory activities were observed also with hempseed protein derived short-chain peptide mixture (IC_{50} ~ 0.18 mg/mL), medium-chain peptide mixture (IC_{50} ~ 0.25 mg/mL), and total hydrolysate (IC_{50} ~ 0.38 mg/mL), generated by Alcalase. Especially, the short-chain peptide mixture is more active on cholesterol metabolism pathway through the modulation of LDLR activity (Cerrato, et al., 2023). Another study identified IGFLIIWV from hempseed protein, a multifunctional octapeptide, with antioxidant and anti-inflammatory activities, inhibiting HMGCoAR activity *in vitro* dose-dependently (IC_{50} ~ 59 μM) (Cerrato, et al., 2023).

One of the major sources of peptides in the search of the cholesterol-lowering effects targeted to HMGCoAR is soybean protein. Three peptides (IAVPGEVA,

IAVPTGVA, and LPYP) produced from soy glycinin can inhibit HMGCoAR activity with IC_{50} of 222, 274, and 300 μM *in vitro*, respectively (Lammi, Zanoni, & Arnoldi, 2015). The two soy β-conglycinin-derived peptides YVVNPDNDEN and YVVNPDNNEN exhibited higher HMGCoAR inhibitory activity with IC_{50} of 150 and 175 μM, respectively (Lammi, Zanoni, Arnoldi, et al., 2015). Moreover, Lunasin, a 43-amino acid polypeptide initially isolated from soybean, has been shown to significantly reduce HMGCoAR expression in HepG2 cells grown in cholesterol-free media (Galvez, 2012).

In addition, peptides released from raw or cooked cowpea bean, chickpea, and olive seed, are capable of decreasing the HMGCoAR activity in addition to their ability to reduce micellar cholesterol solubility (Marques, Soares Freitas, et al., 2015; Prados, et al., 2018; Prados, et al., 2020; Shi, et al., 2019). Moreover, smaller peptides, such as GCTLN (Marques, Fontanari, et al., 2015), IAF, QGF, and QDF (M. Silva, et al., 2021; M. B. d. C. e. Silva, et al., 2018) derived from cowpea bean protein, GGV, IVG and VGVL isolated from *Amaranthus cruentus* protein (Soares, et al., 2015), VFVRN derived from chickpea protein (Shi, et al., 2019), and DA, DD, EE, ES, and LL derived from dry-cured ham (Heres, et al., 2021), were also found to inhibit HMGCoAR activity *in vitro* and some showed statin-like interactions with HMGCoAR. Small peptides, especially di- and tri-peptides, are generally considered to be carried across the intestinal epithelium by the pepT1 transporter or by other transport routes in an intact form, and be bioavailable where activity is needed (Daniel, 2004), exerting a hypocholesterolemic effect. Based on these findings, although food-derived peptides hold promise for the treatment of hypercholesterolemia by targeting the cholesterol biosynthetic pathway, there is

limited knowledge on their structure–activity relationship, bioavailability, and *in vivo* related research, thus making it necessary to conduct further investigations.

4.2.2 PCSK9-mediated effects of food-derived peptides

As a promising therapeutic target for endogenous cholesterol regulation, PCSK9 has gained increasing attention, and its biological mechanism for cholesterol modulation is also now well-established. PCSK9 is a major regulator of hepatocyte LDLR concentrations by inhibiting the receptor recycling pathway, thus causing elevation of plasma LDL-C levels, subsequently accelerating atherosclerosis. Specifically, the LDLR is responsible for the cellular uptake and subsequent degradation of LDL, playing a crucial role in cholesterol homeostasis. Extracellular LDL can bind to the N-terminal domain of LDLR to form an LDL: LDLR complex internalized by a receptor-mediated endocytosis and then migrated to the endosome, where the low pH condition drives the LDLR to release LDL and recycle it back to the cell surface. Subsequently, separated LDL is shifted to the lysosome where it is degraded to provide cholesterol or amino acids to the cell. PCSK9 can facilitate the catabolism of the LDLR within the lysosomes and block its normal recycling to the hepatocyte surface (Lambert, Charlton, Rye, & Piper, 2009). Therefore, inhibition of PCSK9 reduces the LDLR degradation, thereby lowering LDL-C plasma concentrations, offering an additional therapeutic option for patients with primary and secondary cardiovascular prevention.

Over the years, considerable research has been devoted to discovering peptides for PCSK9 regulation. In the case of white lupin, in addition to the HMGCoAR inhibitory property, protein hydrolysates have the ability to impair the protein-protein interaction between PCSK9 and LDLR *in vitro* and to reduce PCSK9 protein levels in HepG2

cells (Lammi, Zanoni, Aiello, et al., 2016). Meanwhile, another protein hydrolysate generated from narrow-leaf lupin by Alcalase showed hypocholesterolemic effects in western diet-fed ApoE$^{-/-}$ mice through the modulation of PCSK9 and LDLR pathways (Santos-Sánchez, et al., 2022). Two peptides LILPKHSDAD and GQEQSHQDEGVIVR, isolated from the white lupin protein hydrolysate, competitively bound to PCSK9 at micromolar concentrations and could normalize the LDL uptake (Lammi, Zanoni, Aiello, et al., 2016). LILPKHSDAD showed a higher inhibitory activity on the protein-protein interaction between PCSK9 and LDLR with an IC_{50} of 1.6 μM and, further, lowered PCSK9 protein levels and secretion in HepG2 cell. Moreover, the inhibitory activity on the interaction between LDLR and PCSK9 for the metabolite LPKHSDAD (IC_{50} ~ 1.7 μM) of LILPKHSDAD, as well as of its analogs LYLPKHSDRD (IC_{50} ~ 0.7 μM), LILPKASDAD (IC_{50} ~ 9.0 μM), and LILPKHADAD (IC_{50} ~ 1.45 μM), were also observed (Lammi, et al., 2021; Lammi, et al., 2022). In addition, the peptide GQEQSHQDEGVIVR not only intervened on the PPI between PCSK9 and LDLR with an IC_{50} of 320 μM, but also inhibited the PCSK9^{D374Y}:LDLR interaction with an IC_{50} of 285.6 μM (Lammi, Sgrignani, et al., 2019). In contrast, the most active compound against wild-type PCSK9, LILPKHSDAD, was inactive against PCSK9^{D374Y}, identified as the familial hypercholesterolemia (FH) associated gain-of-function PCSK9 mutant (Grazioso, et al., 2018; Lammi, Bollati, et al., 2019). Optimization of GQEQSHQDEGVIVR by computational design (GQRQWKQAEGVMVR) raised two-fold the PCSK9^{D374Y}:LDLR antagonist (IC_{50} ~ 147.8 μM)) activity and restored cellular LDLR function more efficiently (Lammi, Sgrignani, et al., 2019). This inhibitory behavior of white lupin protein hydrolysate and its derived peptides led to an

improved ability of treated HepG2 cells to take up extracellular LDL with a final hypocholesterolemic effect.

Although hempseed (*C. sativa*) pepsin hydrolysate exerts hypocholesterolemic effects with a statin-like mechanism leading to increased PCSK9 levels, the identified peptide IGFLIIWV from this hydrolysate can reduce PCSK9 protein levels and subsequent secretion of mature PCSK9 in HepG2 cells (Li, et al., 2022). Moreover, short-chain and medium-chain peptide mixtures and total hydrolysate digested by Alcalase from hempseed protein were tested in HepG2 cells, resulting in a decreased expression of PCSK9 protein (Cerrato, et al., 2023). Soybean-derived peptide Lunasin has been previously reported to inhibit HMGCoAR, and to down-regulate PCSK9 expression as a new mechanism to increase cell-surface LDLR level and enhance LDL uptake (Fernández-Tomé & Hernández-Ledesma, 2019; Gu, et al., 2017). Lunasin was found to inhibit PCSK9 expression at the mRNA and protein levels in HepG2 cells in a dose-and-time dependent manner, thereby contributing to increasing LDLR level and functionally enhancing LDL uptake. ApoE$^{-/-}$ mice receiving Lunasin by intraperitoneal injection at doses of 0.125~0.5 μmol/kg/day for 4 weeks had significantly lower PCSK9 and higher LDLR levels in the liver, as well as remarkably reduced plasma LDL-cholesterol versus controls (Gu, et al., 2017). Interestingly, HMGCoAR-inhibiting peptides also inhibited or modulated the expression of PCSK9, showing a unique synergistic and dual HMGCoAR/PCSK9 inhibitory ability (Lammi, et al., 2021; Zanoni, et al., 2017b)**.** The activity of these peptides indicates them as promising starting points for a further optimization in the development of new hypocholesterolemic compounds. Although these studies suggest the potential hypocholesterolemic effects of food-derived peptides inhibiting PCSK9 expression,

only a few have been investigated and more efforts are necessary to exploit these dual inhibitory peptides as effective cholesterol-regulating agents.

4.2.3 Regulatory effect of food-derived peptides on transcription factors

In the cholesterol biosynthetic pathway, SREBP-2 is a crucial player, being a master transcriptional regulator of cholesterol biosynthesis. SREBP-2 is synthesized as an endoplasmic reticulum (ER) anchored precursor, consisting of an N-terminal transcription factor domain, two transmembrane segments, and a C-terminal regulatory domain that interacts with the domain of the SREBP-cleavage activating protein (SCAP). To become active, the complex of SREBP-2 and SCAP membrane needs to undergo a successive two-step cleavage process in the Golgi to liberate the N- terminal fragment from the membrane. Subsequently, the processed SREBP-2 enters the nucleus as a homodimer, binds to the sterol regulatory element (SRE) sequence in the promoters of target genes, including HMGCoAR, LDLR, and PCSK9, and upregulates their transcription (Sato, 2010). Thus, SREBP-2 activation is important for cholesterol homeostasis and food-derived peptides have been found to influence SREBP-2-mediated processes with a resulting hypocholesterolemic activity. For instance, peptides isolated from white lupin can increase the expression of LDLR at the protein level by the activation of SREBP-2 pathway, resulting in an improved capacity of HepG2 cells to take up LDL from the extracellular environment (Lammi, et al., 2014). The up-regulation of SREBP-2 is associated with the activation of the PI3K/Akt/GSK3b pathway in cultured hepatocytes. This is also a major feature of the identified peptides GQEQSHQDEGVIVR (Lammi, Bollati, et al., 2019) and LILPKHSDAD (Zanoni, et al., 2017b), effectively raising SREBP-2 and LDLR proteins followed by improvement of LDL-uptake by HepG2 cells. Soy glycinin-

662 derived peptides, IAVPGEVA, IAVPTGVA and LPYP, also modulated cholesterol
663 metabolism in HepG2 cells by activation of the LDR/SREBP-2 pathway (Lammi,
664 Zanoni, & Arnoldi, 2015). Moreover, two peptides YVVNPDNDEN and
665 YVVNPDNNEN from β-conglycinin also raise SREBP-2 protein levels, leading to
666 elevated LDLR and LDL uptake (Lammi, Zanoni, Arnoldi, et al., 2015). A similar up-
667 regulation is exerted by Lunasin via PI3K/Akt-mediated activation of SREBP-2 (Gu,
668 et al., 2017). Total protein hydrolysates, medium-chain peptide mixture and short-
669 chain peptide mixtures from hempseed similarly raise SREBP-2 with concomitantly
670 LDLR and HMGCoAR (Cerrato, et al., 2023), a mechanism clearly described for a
671 specific peptide, IGFLIIWV, identified in hempseed protein (Li, et al., 2022). The
672 pigeon milling by-product peptide PFVKSEPIPETNNE increases protein and mRNA
673 expression of HMGCoAR, LDLR, and SREBP-2, enhancing LDL uptake in HepG2
674 cells by modulating the LDLR/SREBP2 pathway (Kumar, et al., 2019). However, it
675 exhibits lower effectiveness in reducing liver cholesterol in high cholesterol-fed rats
676 compared to pigeon pea by-product hydrolysate, potentially attributed to the peptide
677 tissue-specific diversity and stability (Kumar, et al., 2021).
678 Unlike SREBP-2, the transcription factor HNF-1α (hepatic nuclear factor-1α)
679 transcriptionally upregulates PCSK9 by binding to the HNF1 site on the PCSK9
680 promoter without direct effect on LDLR and HMGCoAR expression, regulated by
681 only SREBP-2 transcription factor. HNF-1α knockdown reduces circulating PCSK9
682 protein levels and accumulation of intracellular cholesterol in HepG2 and primary
683 hepatocytes of normolipidemic mice (Shende, et al., 2015). The absence of HNF-1α
684 causes accumulation of lipid droplets and increases intracellular cholesterol-in HepG2
685 cells transfected with HNF-1α siRNA (Hu, Huang, Han, & Ji, 2020). Furthermore,
686 HNF-1α can directly upregulate the transcription of microRNA (miR)-122 to enhance

miR-122-inhibited SCAP expression interfering with the maturation of SREBP-2, leading to ~~a~~ decreased lipid biosynthesis and lipid uptake by HepG2 cells (Liu, Zhu, Jiang, Li, & Lv, 2022). HNF-1α thus plays an important role in the regulation of intracellular cholesterol metabolism. The hempseed peptide IGFLIIWV has been found to decrease the protein levels of PCSK9 by down-regulating expression of HNF-1α, independent of SREBP2, thus showing a distinct hypocholesterolemic mechanism in HepG2 cells (Li, et al., 2022), occurring after exposure to short-chain, medium-chain peptide mixtures, and total hydrolysate (Cerrato, et al., 2023), respectively. White lupin peptide LILPKHSDAD, its metabolite LPKHSDAD and its analogs (LYLPKHSDRD, LILPKASDAD, and LILPKHADAD) reduce PCSK9 levels by decreasing HNF-1α, thereby improving the functional ability of HepG2 to take up extracellular LDL (Lammi, et al., 2021; Lammi, et al., 2022). HepG2 cells treated with Lunasin inhibited PCSK9 expression at the mRNA and protein levels in a dose-and-time dependent manner by down-regulating HNF-1α, thereby raising increasing LDLR level and LDL uptake (Gu, et al., 2017).

A strategic combination of food-sourced peptides that focus on various metabolic and signaling pathways will result in significant hypocholesterolemic effects, reducing the side effects associated with a single approach, particularly HMGCoA reductase inhibition.

5. Hypocholesterolemic peptides exhibiting multifunctional behavior

Several peptides, identified in different food protein hydrolysates, are also endowed with significant biological activities. Lunasin (Lammi, Aiello, et al., 2019) among others, they have potential anti-cancer activities, possibly linked to antioxidant, hypocholesterolemic, and anti-inflammatory properties. Furthermore, a

hypocholesterolemic peptide with antioxidant and anti-inflammatory properties was identified in hempseed. The octapeptide IGFLIIWV from the pepsin hydrolysate of hempseed is transported intact by differentiated Caco-2 cells and exerts cholesterol-lowering effects in HepG2 cells. Briefly, it inhibits the HMGCoAR activity *in vitro* in a dose-dependent manner with an IC_{50} of 59 μM. Furthermore, the activation of the SREBP-2 transcription factor, followed by increased LDLR protein levels, was observed in HepG2 cells treated with IGFLIIWV at 25 μM. Similar to statins, IGFLIIWV raised the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) at the Thr172 residue, in turn inhibiting the intracellular HMGCoAR activity through phosphorylation on the Ser872 residue (the inactive form of HMGCoAR). Consequently, the increased LDLR on cell membranes improved the ability of HepG2 cells to take up extracellular LDL with a positive effect on cholesterolemia. The additional reduction of PCSK9 protein levels via decreased transcription factor HNF-1α provides an additional cholesterol lowering mechanism (Li, et al., 2022).

The antioxidant properties of peptide IGFLIIWV have been confirmed *in vitro* and at cellular level (Bollati, et al., 2022). *In vitro,* at the concentration of 25 μM, IGFLIIWV (1) scavenged the 2,2-azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic) acid (ABTS) radical by 146.1%**,** (2) had 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity of 29.8%, (3) scavenged the peroxyl radicals generated by 2,2′-azobis (2-methylpropionamidine) dihydrochloride up to 181.8% in oxygen radical absorbance capacity (ORAC) test, and (4) increased the ferric reducing antioxidant power (FRAP) by 299.3%. When evaluating cellular assays, IGFLIIWV lowered the hydrogen peroxide (H_2O_2)-induced reactive oxygen species (ROS) and lipid peroxidation by 23.2% and 44% at 25 μM versus HepG2 cells treated with H_2O_2

alone, respectively. The reduction of H_2O_2-induced nitric oxide (NO) production was observed after treatment of HepG2 cells with IGFLIIWV, associated with the regulation of inducible NO synthase (iNOS). Moreover, IGFLIIWV suppressed the H_2O_2-induced oxidant stress by modulating the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway, playing a crucial role in the protection against oxidative stress and responsible for the maintenance of homeostasis and redox balance in cells and tissues.

Inflammation can be triggered by a wide variety of stimuli, including pathogens, damaged cells, toxins, and allergens. The release of these inflammatory mediators, such as cytokines, tumor necrosis factor α (TNF-α), prostaglandins (PGs), nitric oxide (NO), and leukotrienes (LTs), is a key aspect of the inflammatory process. These mediators play a central role in coordinating the immune response and orchestrating the various cellular and physiological processes involved in the healing and repair of damaged tissue (Chakrabarti, Jahandideh, & Wu, 2014). It is also worth mentioning that the balance between pro- and anti-inflammatory mediators is critical for the proper inflammation resolution. As far as the anti-inflammatory activity is concerned, the regulation by IGFLIIWV is consequent to its capacity to modulate production and release of cytokines the nuclear factor-κB (NF-κB) and iNOS pathways. Since the NF-κB pathway plays a major role in the pro-inflammatory response, IGFLIIWV (25 μM) has the ability to reduce both NF-κB and its more active phosphorylated form (p(Ser276)NF-κB) in lipopolysaccharide (LPS)-stimulated HepG2 cells, thus antagonizing the inflammatory effect. In fact, peptide IGFLIIWV has been shown to effectively suppress the production of pro-inflammatory cytokines (IFN-γ: −13.1 ± 2.0%, TNF: −20.3 ± 1.7%, and IL-6: −15.1 ± 6.5%), while promoting also the expression of the anti-inflammatory cytokine IL-10 (+26.0 ± 2.3%). A reduction of

761 the iNOS protein level and NO production was observed as well (Cruz-Chamorro, et
762 al., 2022).

763 **6. Challenges and perspective**

764 There are several challenges before hypocholesterolemic peptides can be
765 commercialized and used as dietary supplements or functional foods. The primary
766 concern is their efficacy, which can be compromised by extensive hydrolysis during
767 processing conditions as well as by proteolytic enzymes of the digestive tract.
768 Processing conditions, such as temperature and duration of hydrolysis or fermentation,
769 may result in non-reproducible peptide profiles, especially when the substrate
770 contains mixtures of differently expressed proteins. The interaction of
771 hypocholesterolemic peptides with other components such as polyphenols,
772 carbohydrates, and lipids may result in undesirable substances that possess toxic,
773 allergenic, or carcinogenic properties in food products (Daliri, Lee, & Oh, 2018).
774 Moreover, some microbial proteases used in hypocholesterolemic peptide production
775 lack established safety evidence. Thus, critical hydrolysis parameters must be
776 optimized for each protein/substrate couple and each selected enzyme or enzymes
777 combination should be maintained constant during the reaction to ensure an efficient
778 peptide release.

779 In recent decades, a number of papers focuses on the functional attributes of
780 hypocholesterolemic peptides, primarily based on *in vitro* data. However, the *in vitro*
781 efficacy of hypocholesterolemic peptides may not consistently align with their *in vivo*
782 effect, as they can be impaired within the GI tract, vascular system, and liver.
783 Hypocholesterolemic peptides must remain active during GI digestion, and some
784 hypocholesterolemic peptides must be transported across the intestinal epithelial cells

into the bloodstream to exert their hypocholesterolemic effect, as discussed in Section 3. For this reason, further studies, specifically more *in vivo* research, including animal and clinical studies, with a focus on hypocholesterolemic activity and toxicity, are required. More studies are needed to understand fully the biological activity of the hypocholesterolemic peptides and their specific molecular mechanisms, as discussed in Section 4. This knowledge is crucial in refining the application of hypocholesterolemic peptides and optimizing their use for human health and well-being.

In addition, knowledge of hypocholesterolemic peptide stability in the GI tract and site-specific delivery at target locations in the body should be improved in future research studies. This can be achieved through the development of nanoparticles or nanoconjugates to encapsulate, stabilize and deliver these peptides. Various colloidal systems like chitosan nanoparticles, nanoliposomes, and biopolymer-based microgels have been recommended for this purpose (McClements, 2018). Therefore, extensive studies are needed to demonstrate significant evidence of improved bioavailability of cholesterol-lowering peptides upon encapsulation.

Another important aspect is the regulatory approval of hypocholesterolemic peptides. While, as of now, several hypocholesterolemic peptides have been identified from various sources of food protein, only a few are marketed as functional foods, such as LunaRich® X, a concentrated form of lunasin, is currently marketed by Reliv (USA) as a dietary supplement for lowering cholesterol (López-Barrios, Gutiérrez-Uribe, & Serna-Saldívar, 2014). To validate functional effects, more animal experiments and randomized human intervention trials are needed to allow the use of hypocholesterolemic peptides as preventive or management treatments.

7. Conclusion

As discussed in this review, emerging reports have demonstrated that some food-derived peptides have demonstrated their cholesterol-lowering properties via one or more following mechanisms: (1) binding to bile acids/salts or lipids, and inhibition of micellar cholesterol solubility, (2) blocking the mevalonate pathway and cholesterol biosynthesis by inhibition of HMGCoAR activity, and (3) modulation of LDLR and PCSK9 pathways. While some peptides lower cholesterol by binding to bile acids, salts, or lipids and inhibiting micellar cholesterol solubility in the GI tract, others must be absorbed and reach specific target tissues, such as the liver, to modulate cholesterol synthesis pathway (**Figure 2**). Thus, stability in the GI tract, ADMET profiles (including absorption, distribution, metabolism, excretion, and toxicity), which are directly related to the effect of peptides on endogenous cholesterol metabolism, and bioavailability should be established well, to help understand how these food-derived hypocholesterolemic peptides exert their cholesterol-lowering effect. Moreover, to date, most of the demonstrated hypocholesterolemic effects of peptides have been reported *in vitro* and free-cell systems. For this reason, further studies in animals and/or in humans are needed to confirm these *in vitro* hypocholesterolemic activities. In addition, the specific molecular targets of hypocholesterolemic peptides, such as the CYP7A1/SREBP-2/HNF-1α/PCSK9-mediated effects, need to be identified for better understanding of their structure-function relationships. Undoubtedly, this review opens the field for exploring the beneficial effects of hypocholesterolemic peptides and building the evidence base for future human studies, facilitating the application of hypocholesterolemic peptides as nutraceuticals to enhance human health and well-being.

Funding

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3 - Call for tender No. 341 of 15 March 2022 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU; Project code PE00000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001, Project title "**ON Foods - Research and innovation network on food and nutrition Sustainability, Safety and Security – Working ON Foods**".

Declaration of Competing Interest

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

Acknowledgement

The authors gratefully acknowledge the financial support from the China Scholarship Council for a fellowship to J.L. I.C.-C. was supported by a postdoctoral fellowship from the Andalusian Government Ministry of Economy, Knowledge, Business, and University (DOC_00587/2020).

References

Ahmed, T., Sun, X., & Udenigwe, C. C. (2022). Role of structural properties of bioactive peptides in their stability during simulated gastrointestinal digestion: A systematic review. *Trends in Food Science & Technology, 120*, 265-273. https://doi.org/10.1016/j.tifs.2022.01.008.

Akika, E., Megumi, N., Yasushi, A. S., & Kenji, S. (2018). Identification of food-derived peptides in human blood after ingestion of corn and wheat gluten hydrolysates. *Journal of Food Bioactives, 2*, 104–111. https://doi.org/10.31665/JFB.2018.2145.

Ao, J., & Li, B. (2013). Stability and antioxidative activities of casein peptide fractions during simulated gastrointestinal digestion in vitro: Charge properties of peptides affect digestive stability. *Food Research International, 52*, 334-341. https://doi.org/10.1016/j.foodres.2013.03.036.

Ashaolu, T. J. (2020). Soy bioactive peptides and the gut microbiota modulation. *Applied Microbiology and Biotechnology, 104*, 9009-9017. https://doi.org/10.1007/s00253-020-10799-2.

Ashraf, J., Liu, L., Awais, M., Xiao, T., Wang, L., Zhou, X., Tong, L.-T., & Zhou, S. (2020). Effect of thermosonication pre-treatment on mung bean (Vigna radiata) and white kidney bean (Phaseolus vulgaris) proteins: Enzymatic hydrolysis, cholesterol lowering activity and structural characterization. *Ultrasonics Sonochemistry, 66*, Article 105121. https://doi.org/10.1016/j.ultsonch.2020.105121.

Bao, X., Yuan, X., Li, X., & Liu, X. (2022). Flaxseed-derived peptide, IPPF, inhibits intestinal cholesterol absorption in Caco-2 cells and hepatic cholesterol synthesis in HepG2 cells. *Journal of Food Biochemistry, 46*, e14031. https://doi.org/10.1111/jfbc.14031.

Boachie, R., Yao, S., & Udenigwe, C. C. (2018). Molecular mechanisms of cholesterol-lowering peptides derived from food proteins. *Current Opinion in Food Science, 20*, 58-63. https://doi.org/10.1016/j.cofs.2018.03.006.

Bollati, C., Cruz-Chamorro, I., Aiello, G., Li, J., Bartolomei, M., Santos-Sánchez, G., Ranaldi, G., Ferruzza, S., Sambuy, Y., Arnoldi, A., & Lammi, C. (2022). Investigation of the intestinal trans-epithelial transport and antioxidant activity of two hempseed peptides WVSPLAGRT (H2) and IGFLIIWV (H3). *Food Research International, 152*, Article 110720. https://doi.org/10.1016/j.foodres.2021.110720.

Cerrato, A., Lammi, C., Laura Capriotti, A., Bollati, C., Cavaliere, C., Maria Montone, C., Bartolomei, M., Boschin, G., Li, J., Piovesana, S., Arnoldi, A., & Laganà, A. (2023). Isolation and functional characterization of hemp seed protein-derived short- and medium-chain peptide mixtures with multifunctional properties for metabolic syndrome prevention. *Food Research International, 163*, Article 112219. https://doi.org/10.1016/j.foodres.2022.112219.

Chakrabarti, S., Jahandideh, F., & Wu, J. (2014). Food-Derived Bioactive Peptides on Inflammation and Oxidative Stress. *BioMed Research International, 2014*, Article 608979. https://doi.org/10.1155/2014/608979.

Chen, G.-W., Lin, H.-T. V., Huang, L.-W., Lin, C.-H., & Lin, Y.-H. (2021). Purification and Identification of Cholesterol Micelle Formation Inhibitory Peptides of Hydrolysate from High Hydrostatic Pressure-Assisted Protease Hydrolysis of Fermented Seabass Byproduct. *International Journal of Molecular Sciences, 22*, 5295. https://doi.org/10.3390/ijms22105295.

Chen, G.-W., & Yang, M.-H. (2021). Production and Purification of Novel Hypocholesterolemic Peptides from Lactic Fermented Spirulina platensis through High Hydrostatic Pressure-Assisted

Protease Hydrolysis. *Catalysts, 11*, 873. https://doi.org/10.3390/catal11080873.

Coelho, M. S., Soares-Freitas, R. A. M., Arêas, J. A. G., Gandra, E. A., & Salas-Mellado, M. d. l. M. (2018). Peptides from Chia Present Antibacterial Activity and Inhibit Cholesterol Synthesis. *Plant Foods for Human Nutrition, 73*, 101-107. https://doi.org/10.1007/s11130-018-0668-z.

Cruz-Chamorro, I., Santos-Sánchez, G., Bollati, C., Bartolomei, M., Li, J., Arnoldi, A., & Lammi, C. (2022). Hempseed (Cannabis sativa) Peptides WVSPLAGRT and IGFLIIWV Exert Anti-inflammatory Activity in the LPS-Stimulated Human Hepatic Cell Line. *Journal of Agricultural and Food Chemistry, 70*, 577-583. https://doi.org/10.1021/acs.jafc.1c07520.

Daliri, E. B.-M., Lee, B. H., & Oh, D. H. (2018). Current trends and perspectives of bioactive peptides. *Critical Reviews in Food Science and Nutrition, 58*, 2273-2284. https://doi.org/10.1080/10408398.2017.1319795.

Daniel, H. (2004). Molecular and Integrative Physiology of Intestinal Peptide Transport. *Annual Review of Physiology, 66*, 361-384. https://doi.org/10.1146/annurev.physiol.66.032102.144149.

Daroit, D. J., & Brandelli, A. (2021). In vivo bioactivities of food protein-derived peptides – a current review. *Current Opinion in Food Science, 39*, 120-129. https://doi.org/10.1016/j.cofs.2021.01.002.

Dupont, D., & Mackie, A. R. (2015). Static and dynamic in vitro digestion models to study protein stability in the gastrointestinal tract. *Drug Discovery Today: Disease Models, 17-18*, 23-27. https://doi.org/10.1016/j.ddmod.2016.06.002.

Fernández-Tomé, S., & Hernández-Ledesma, B. (2019). Current state of art after twenty years of the discovery of bioactive peptide lunasin. *Food Research International, 116*, 71-78. https://doi.org/10.1016/j.foodres.2018.12.029.

Galvez, A. F. (2012). Abstract 10693: Identification of Lunasin as the Active Component in Soy Protein Responsible for Reducing LDL Cholesterol and Risk of Cardiovascular Disease. *Circulation, 126*, A10693-A10693. https://doi.org/10.1161/circ.126.suppl_21.A10693.

Garcia-Mora, P., Peñas, E., Frias, J., Zieliński, H., Wiczkowski, W., Zielińska, D., & Martínez-Villaluenga, C. (2016). High-Pressure-Assisted Enzymatic Release of Peptides and Phenolics Increases Angiotensin Converting Enzyme I Inhibitory and Antioxidant Activities of Pinto Bean Hydrolysates. *Journal of Agricultural and Food Chemistry, 64*, 1730-1740. https://doi.org/10.1021/acs.jafc.5b06080.

Góngora-Benítez, M., Tulla-Puche, J., & Albericio, F. (2014). Multifaceted Roles of Disulfide Bonds. Peptides as Therapeutics. *Chemical Reviews, 114*, 901-926. https://doi.org/10.1021/cr400031z.

Grazioso, G., Bollati, C., Sgrignani, J., Arnoldi, A., & Lammi, C. (2018). First Food-Derived Peptide Inhibitor of the Protein–Protein Interaction between Gain-of-Function PCSK9D374Y and the Low-Density Lipoprotein Receptor. *Journal of Agricultural and Food Chemistry, 66*, 10552-10557. https://doi.org/10.1021/acs.jafc.8b03233.

Gu, L., Wang, Y., Xu, Y., Tian, Q., Lei, G., Zhao, C., Gao, Z., Pan, Q., Zhao, W., Nong, L., & Tan, S. (2017). Lunasin functionally enhances LDL uptake via inhibiting PCSK9 and enhancing LDLR expression in vitro and in vivo. *Oncotarget, 8*, 80826-80840. https://doi.org/10.18632/oncotarget.20590.

Guo, Z., Yi, D., Hu, B., Shi, Y., Xin, Y., Gu, Z., Liu, H., & Zhang, L. (2021). The alteration of gut microbiota by bioactive peptides: a review. *Systems Microbiology and Biomanufacturing, 1*, 363-377. https://doi.org/10.1007/s43393-021-00035-x.

Heres, A., Mora, L., & Toldrá, F. (2021). Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham. *Food Production, Processing and Nutrition, 3*, Article 18. https://doi.org/10.1186/s43014-021-00058-w.

Hu, M., Huang, X., Han, X., & Ji, L. (2020). Loss of HNF1α Function Contributes to Hepatocyte Proliferation and Abnormal Cholesterol Metabolism via Downregulating miR-122: A Novel Mechanism of MODY3. *Diabetes Metab Syndr Obes, 13*, 627-639. https://doi.org/10.2147/dmso.S236915.

Huang, S., Li, Y., Li, C., Ruan, S., Roknul Azam, S. M., Ou Yang, N., Ye, X., Wang, Y., & Ma, H. (2021). Effects of ultrasound-assisted sodium bisulfite pretreatment on the preparation of cholesterol-lowering peptide precursors from soybean protein. *International Journal of Biological Macromolecules, 183*, 295-304. https://doi.org/10.1016/j.ijbiomac.2021.04.125.

Karaś, M. (2019). Influence of physiological and chemical factors on the absorption of bioactive peptides. *International Journal of Food Science & Technology, 54*, 1486-1496. https://doi.org/10.1111/ijfs.14054.

Kashima, Y., Kanematsu, S., Asai, S., Kusada, M., Watanabe, S., Kawashima, T., Nakamura, T., Shimada, M., Goto, T., & Nagaoka, S. (2014). Identification of a Novel Hypocholesterolemic Protein, Major Royal Jelly Protein 1, Derived from Royal Jelly. *PLOS ONE, 9*, Article e105073. https://doi.org/10.1371/journal.pone.0105073.

Knudsen, J. C., Otte, J., Olsen, K., & Skibsted, L. H. (2002). Effect of high hydrostatic pressure on the conformation of β-lactoglobulin A as assessed by proteolytic peptide profiling. *International Dairy Journal, 12*, 791-803. https://doi.org/10.1016/S0958-6946(02)00078-X.

Kumar, V., Muthu Kumar, S. P., & Tiku, P. K. (2021). Hypocholesterolemic Effect of Potent Peptide and Bioactive Fraction from Pigeon Pea By-Products in Wistar Rats. *International Journal of Peptide Research and Therapeutics, 27*, 2403-2415. https://doi.org/10.1007/s10989-021-10261-5.

Kumar, V., Sharma, P., Bairagya, H. R., Sharma, S., Singh, T. P., & Tiku, P. K. (2019). Inhibition of human 3-hydroxy-3-methylglutaryl CoA reductase by peptides leading to cholesterol homeostasis through SREBP2 pathway in HepG2 cells. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1867*, 604-615. https://doi.org/10.1016/j.bbapap.2019.04.002.

Lambert, G., Charlton, F., Rye, K.-A., & Piper, D. E. (2009). Molecular basis of PCSK9 function. *Atherosclerosis, 203*, 1-7. https://doi.org/10.1016/j.atherosclerosis.2008.06.010.

Lammi, C., Aiello, G., Bollati, C., Li, J., Bartolomei, M., Ranaldi, G., Ferruzza, S., Fassi, E. M., Grazioso, G., Sambuy, Y., & Arnoldi, A. (2021). Trans-Epithelial Transport, Metabolism, and Biological Activity Assessment of the Multi-Target Lupin Peptide LILPKHSDAD (P5) and Its Metabolite LPKHSDAD (P5-Met). *Nutrients, 13*, 863. https://doi.org/10.3390/nu13030863.

Lammi, C., Aiello, G., Boschin, G., & Arnoldi, A. (2019). Multifunctional peptides for the prevention of cardiovascular disease: A new concept in the area of bioactive food-derived peptides. *Journal of Functional Foods, 55*, 135-145. https://doi.org/10.1016/j.jff.2019.02.016.

Lammi, C., Aiello, G., Dellafiora, L., Bollati, C., Boschin, G., Ranaldi, G., Ferruzza, S., Sambuy, Y., Galaverna, G., & Arnoldi, A. (2020). Assessment of the Multifunctional Behavior of Lupin Peptide P7 and Its Metabolite Using an Integrated Strategy. *Journal of Agricultural and Food Chemistry, 68*, 13179-13188. https://doi.org/10.1021/acs.jafc.0c00130.

Lammi, C., Aiello, G., Vistoli, G., Zanoni, C., Arnoldi, A., Sambuy, Y., Ferruzza, S., & Ranaldi, G. (2016). A multidisciplinary investigation on the bioavailability and activity of peptides from lupin

protein. *Journal of Functional Foods, 24*, 297-306. https://doi.org/10.1016/j.jff.2016.04.017.

Lammi, C., Bollati, C., Lecca, D., Abbracchio, M. P., & Arnoldi, A. (2019). Lupin Peptide T9 (GQEQSHQDEGVIVR) Modulates the Mutant PCSK9D374Y Pathway: in vitro Characterization of its Dual Hypocholesterolemic Behavior. *Nutrients, 11*, 1665. https://doi.org/10.3390/nu11071665.

Lammi, C., Fassi, E. M. A., Li, J., Bartolomei, M., Benigno, G., Roda, G., Arnoldi, A., & Grazioso, G. (2022). Computational Design and Biological Evaluation of Analogs of Lupin Peptide P5 Endowed with Dual PCSK9/HMG-CoAR Inhibiting Activity. *Pharmaceutics, 14*, 665. https://doi.org/10.3390/pharmaceutics14030665.

Lammi, C., Sgrignani, J., Roda, G., Arnoldi, A., & Grazioso, G. (2019). Inhibition of PCSK9D374Y/LDLR Protein–Protein Interaction by Computationally Designed T9 Lupin Peptide. *ACS Medicinal Chemistry Letters, 10*, 425-430. https://doi.org/10.1021/acsmedchemlett.8b00464.

Lammi, C., Zanoni, C., Aiello, G., Arnoldi, A., & Grazioso, G. (2016). Lupin Peptides Modulate the Protein-Protein Interaction of PCSK9 with the Low Density Lipoprotein Receptor in HepG2 Cells. *Scientific Reports, 6*, Article 29931. https://doi.org/10.1038/srep29931.

Lammi, C., Zanoni, C., & Arnoldi, A. (2015). IAVPGEVA, IAVPTGVA, and LPYP, three peptides from soy glycinin, modulate cholesterol metabolism in HepG2 cells through the activation of the LDLR-SREBP2 pathway. *Journal of Functional Foods, 14*, 469-478. https://doi.org/10.1016/j.jff.2015.02.021.

Lammi, C., Zanoni, C., Arnoldi, A., & Aiello, G. (2018). YDFYPSSTKDQQS (P3), a peptide from lupin protein, absorbed by Caco-2 cells, modulates cholesterol metabolism in HepG2 cells via SREBP-1 activation. *Journal of Food Biochemistry, 42*, e12524. https://doi.org/10.1111/jfbc.12524.

Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. (2015). Two Peptides from Soy β-Conglycinin Induce a Hypocholesterolemic Effect in HepG2 Cells by a Statin-Like Mechanism: Comparative in Vitro and in Silico Modeling Studies. *Journal of Agricultural and Food Chemistry, 63*, 7945-7951. https://doi.org/10.1021/acs.jafc.5b03497.

Lammi, C., Zanoni, C., Calabresi, L., & Arnoldi, A. (2016). Lupin protein exerts cholesterol-lowering effects targeting PCSK9: From clinical evidences to elucidation of the in vitro molecular mechanism using HepG2 cells. *Journal of Functional Foods, 23*, 230-240. https://doi.org/10.1016/j.jff.2016.02.042.

Lammi, C., Zanoni, C., Scigliuolo, G. M., D'Amato, A., & Arnoldi, A. (2014). Lupin Peptides Lower Low-Density Lipoprotein (LDL) Cholesterol through an Up-regulation of the LDL Receptor/Sterol Regulatory Element Binding Protein 2 (SREBP2) Pathway at HepG2 Cell Line. *Journal of Agricultural and Food Chemistry, 62*, 7151-7159. https://doi.org/10.1021/jf500795b.

Lapphanichayakool, P., Sutheerawattananonda, M., & Limpeanchob, N. (2017). Hypocholesterolemic effect of sericin-derived oligopeptides in high-cholesterol fed rats. *Journal of Natural Medicines, 71*, 208-215. https://doi.org/10.1007/s11418-016-1050-9.

Li, J., Bollati, C., Bartolomei, M., Mazzolari, A., Arnoldi, A., Vistoli, G., & Lammi, C. (2022). Hempseed (Cannabis sativa) Peptide H3 (IGFLIIWV) Exerts Cholesterol-Lowering Effects in Human Hepatic Cell Line. *Nutrients, 14*, 1804. https://doi.org/10.3390/nu14091804.

Lin, Y.-H., Tsai, J.-S., & Chen, G.-W. (2017). Purification and identification of hypocholesterolemic peptides from freshwater clam hydrolysate with in vitro gastrointestinal digestion. *Journal of Food Biochemistry, 41*, Article e12385. https://doi.org/10.1111/jfbc.12385.

Lin, Y.-H., Tsai, J.-S., Hung, L.-B., & Pan, B. S. (2010). Hypocholesterolemic effect of compounded freshwater clam protein hydrolysate and Gracilaria. *Food Chemistry, 123*, 395-399. https://doi.org/10.1016/j.foodchem.2010.04.053.

Lin, Y.-H., Tsai, J.-S., Hung, L.-B., & Pan, B. S. (2011). Plasma lipid regulatory effect of compounded freshwater clam hydrolysate and Gracilaria insoluble dietary fibre. *Food Chemistry, 125*, 397-401. https://doi.org/10.1016/j.foodchem.2010.09.016.

Liu, F., Zhu, X., Jiang, X., Li, S., & Lv, Y. (2022). Transcriptional control by HNF-1: Emerging evidence showing its role in lipid metabolism and lipid metabolism disorders. *Genes & Diseases, 9*, 1248-1257. https://doi.org/10.1016/j.gendis.2021.06.010.

López-Barrios, L., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bioactive Peptides and Hydrolysates from Pulses and Their Potential Use as Functional Ingredients. *Journal of Food Science, 79*, R273-R283. https://doi.org/10.1111/1750-3841.12365.

Marciniak, A., Suwal, S., Naderi, N., Pouliot, Y., & Doyen, A. (2018). Enhancing enzymatic hydrolysis of food proteins and production of bioactive peptides using high hydrostatic pressure technology. *Trends in Food Science & Technology, 80*, 187-198. https://doi.org/10.1016/j.tifs.2018.08.013.

Marques, M. R., Fontanari, G. G., Pimenta, D. C., Soares-Freitas, R. M., & Arêas, J. A. G. (2015). Proteolytic hydrolysis of cowpea proteins is able to release peptides with hypocholesterolemic activity. *Food Research International, 77*, 43-48. https://doi.org/10.1016/j.foodres.2015.04.020.

Marques, M. R., Soares Freitas, R. A. M., Corrêa Carlos, A. C., Siguemoto, É. S., Fontanari, G. G., & Arêas, J. A. G. (2015). Peptides from cowpea present antioxidant activity, inhibit cholesterol synthesis and its solubilisation into micelles. *Food Chemistry, 168*, 288-293. https://doi.org/10.1016/j.foodchem.2014.07.049.

McClements, D. J. (2018). Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and microparticle systems: A review. *Advances in Colloid and Interface Science, 253*, 1-22. https://doi.org/10.1016/j.cis.2018.02.002.

Mureşan, C. I., Dezmirean, D. S., Marc, B. D., Suharoschi, R., Pop, O. L., & Buttstedt, A. (2022). Biological properties and activities of major royal jelly proteins and their derived peptides. *Journal of Functional Foods, 98*, 105286. https://doi.org/10.1016/j.jff.2022.105286.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., Tadashi, K., & Kuwata, T. (2001). Identification of Novel Hypocholesterolemic Peptides Derived from Bovine Milk β-Lactoglobulin. *Biochemical and Biophysical Research Communications, 281*, 11-17. https://doi.org/10.1006/bbrc.2001.4298.

Nagaoka, S., Nakamura, A., Shibata, H., & Kanamaru, Y. (2010). Soystatin (VAWWMY), a Novel Bile Acid-Binding Peptide, Decreased Micellar Solubility and Inhibited Cholesterol Absorption in Rats. *Bioscience, Biotechnology, and Biochemistry, 74*, 1738-1741. https://doi.org/10.1271/bbb.100338.

Pak, V. V., Koo, M. S., Kasymova, T. D., & Kwon, D. Y. (2005). Isolation and Identification of Peptides from Soy 11S-Globulin with Hypocholesterolemic Activity. *Chemistry of Natural Compounds, 41*, 710-714. https://doi.org/10.1007/s10600-006-0017-6.

Pei, J., Gao, X., Pan, D., Hua, Y., He, J., Liu, Z., & Dang, Y. (2022). Advances in the stability challenges of bioactive peptides and improvement strategies. *Current Research in Food Science, 5*, 2162-2170. https://doi.org/10.1016/j.crfs.2022.10.031.

Prados, I. M., Marina, M. L., & García, M. C. (2018). Isolation and identification by high resolution liquid chromatography tandem mass spectrometry of novel peptides with multifunctional lipid-lowering capacity. *Food Research International, 111*, 77-86. https://doi.org/10.1016/j.foodres.2018.05.009.

Prados, I. M., Orellana, J. M., Marina, M. L., & García, M. C. (2020). Identification of Peptides Potentially Responsible for In Vivo Hypolipidemic Activity of a Hydrolysate from Olive Seeds. *Journal of Agricultural and Food Chemistry, 68*, 4237-4244. https://doi.org/10.1021/acs.jafc.0c01280.

Quirós, A., Chichón, R., Recio, I., & López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry, 104*, 1734-1739. https://doi.org/10.1016/j.foodchem.2006.10.050.

Reiner, Ž. (2014). Primary Prevention of Cardiovascular Disease with Statins in the Elderly. *Current Atherosclerosis Reports, 16*, Article e420. https://doi.org/10.1007/s11883-014-0420-6.

Santos-Sánchez, G., Cruz-Chamorro, I., Bollati, C., Bartolomei, M., Pedroche, J., Millán, F., Millán-Linares, M. d. C., Capriotti, A. L., Cerrato, A., Laganà, A., Arnoldi, A., Carrillo-Vico, A., & Lammi, C. (2022). A Lupinus angustifolius protein hydrolysate exerts hypocholesterolemic effects in Western diet-fed ApoE−/− mice through the modulation of LDLR and PCSK9 pathways. *Food & Function, 13*, 4158-4170. https://doi.org/10.1039/D1FO03847H.

Sato, R. (2010). Sterol metabolism and SREBP activation. *Archives of Biochemistry and Biophysics, 501*, 177-181. https://doi.org/10.1016/j.abb.2010.06.004.

Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D., & Hernandez-Escalante, V. M. (2011). Bioavailability of Bioactive Peptides. *Food Reviews International, 27*, 213-226. https://doi.org/10.1080/87559129.2011.563395.

Shende, V. R., Wu, M., Singh, A. B., Dong, B., Kan, C. F. K., & Liu, J. (2015). Reduction of circulating PCSK9 and LDL-C levels by liver-specific knockdown of HNF1α in normolipidemic mice[S]. *J Lipid Res, 56*, 801-809. https://doi.org/10.1194/jlr.M052969.

Shi, W., Hou, T., Guo, D., & He, H. (2019). Evaluation of hypolipidemic peptide (Val-Phe-Val-Arg-Asn) virtual screened from chickpea peptides by pharmacophore model in high-fat diet-induced obese rat. *Journal of Functional Foods, 54*, 136-145. https://doi.org/10.1016/j.jff.2019.01.001.

Shimizu, M., & Ok Son, D. (2007). Food-Derived Peptides and Intestinal Functions. *Current Pharmaceutical Design, 13*, 885-895. http://dx.doi.org/10.2174/138161207780414287.

Silva, M., Philadelpho, B., Santos, J., Souza, V., Souza, C., Santiago, V., Silva, J., Souza, C., Azeredo, F., Castilho, M., Cilli, E., & Ferreira, E. (2021). IAF, QGF, and QDF Peptides Exhibit Cholesterol-Lowering Activity through a Statin-like HMG-CoA Reductase Regulation Mechanism: In Silico and In Vitro Approach. *International Journal of Molecular Sciences, 22*, 11067. https://doi.org/10.3390/ijms222011067.

Silva, M. B. d. C. e., Souza, C. A. d. C., Philadelpho, B. O., Cunha, M. M. N. d., Batista, F. P. R., Silva, J. R. d., Druzian, J. I., Castilho, M. S., Cilli, E. M., & Ferreira, E. S. (2018). In vitro and in silico studies of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitory activity of the cowpea Gln-Asp-Phe peptide. *Food Chemistry, 259*, 270-277. https://doi.org/10.1016/j.foodchem.2018.03.132.

Singh, B. P., Aluko, R. E., Hati, S., & Solanki, D. (2022). Bioactive peptides in the management of lifestyle-related diseases: Current trends and future perspectives. *Critical Reviews in Food Science and Nutrition, 62*, 4593-4606. https://doi.org/10.1080/10408398.2021.1877109.

Sitanggang, A. B., Sumitra, J., & Budijanto, S. (2021). Continuous production of tempe-based bioactive peptides using an automated enzymatic membrane reactor. *Innovative Food Science & Emerging Technologies, 68*, 102639. https://doi.org/10.1016/j.ifset.2021.102639.

Soares, R. A., Mendonça, S., De Castro, L. Í. A., Menezes, A. C., & Arêas, J. A. (2015). Major Peptides from Amaranth (Amaranthus cruentus) Protein Inhibit HMG-CoA Reductase Activity. *International Journal of Molecular Sciences, 16*, 4150-4160. https://doi.org/10.3390/ijms16024150.

Sun, S., Wang, W., Wang, N., Zhang, Y., Zhu, Z., Li, X., Wang, J., Chen, Q., Ahmed Sadiq, F., Yang, H., Qi, Q., & Zhang, G. (2021). HPP and SGQR peptides from silkworm pupae protein hydrolysates regulated biosynthesis of cholesterol in HepG2 cell line. *Journal of Functional Foods, 77*, 104328. https://doi.org/10.1016/j.jff.2020.104328.

Taylor, B. A., & Thompson, P. D. (2016). Statins and Their Effect on PCSK9—Impact and Clinical Relevance. *Current Atherosclerosis Reports, 18*, Article e46. https://doi.org/10.1007/s11883-016-0604-3.

Tong, L.-T., Ju, Z., Qiu, J., Wang, L., Liu, L., Zhou, X., & Zhou, S. (2017). Peptide GEQQQQPGM derived from rice α-globulin reduces the risk of atherosclerosis in hamsters by improving vascular endothelial cells injury. *RSC Advances, 7*, 49194-49203. https://doi.org/10.1039/C7RA08304A.

Tovar-Pérez, E. G., Lugo-Radillo, A., & Aguilera-Aguirre, S. (2019). Amaranth grain as a potential source of biologically active peptides: a review of their identification, production, bioactivity, and characterization. *Food Reviews International, 35*, 221-245. https://doi.org/10.1080/87559129.2018.1514625.

Udenigwe, C. C., Abioye, R. O., Okagu, I. U., & Obeme-Nmom, J. I. (2021). Bioaccessibility of bioactive peptides: recent advances and perspectives. *Current Opinion in Food Science, 39*, 182-189. https://doi.org/10.1016/j.cofs.2021.03.005.

Ulug, S. K., Jahandideh, F., & Wu, J. (2021). Novel technologies for the production of bioactive peptides. *Trends in Food Science & Technology, 108*, 27-39. https://doi.org/10.1016/j.tifs.2020.12.002.

Umego, E. C., He, R., Ren, W., Xu, H., & Ma, H. (2021). Ultrasonic-assisted enzymolysis: Principle and applications. *Process Biochemistry, 100*, 59-68. https://doi.org/10.1016/j.procbio.2020.09.033.

Vij, R., Reddi, S., Kapila, S., & Kapila, R. (2016). Transepithelial transport of milk derived bioactive peptide VLPVPQK. *Food Chemistry, 190*, 681-688. https://doi.org/10.1016/j.foodchem.2015.05.121.

Wang, C., Wang, B., & Li, B. (2016). Bioavailability of peptides from casein hydrolysate in vitro: Amino acid compositions of peptides affect the antioxidant efficacy and resistance to intestinal peptidases. *Food Research International, 81*, 188-196. https://doi.org/10.1016/j.foodres.2015.12.013.

Wang, J., Shimada, M., Kato, Y., Kusada, M., & Nagaoka, S. (2015). Cholesterol-lowering effect of rice bran protein containing bile acid-binding proteins. *Bioscience, Biotechnology, and Biochemistry, 79*, 456-461. https://doi.org/10.1080/09168451.2014.978260.

Wu, S., Bekhit, A. E.-D. A., Wu, Q., Chen, M., Liao, X., Wang, J., & Ding, Y. (2021). Bioactive peptides and gut microbiota: Candidates for a novel strategy for reduction and control of neurodegenerative diseases. *Trends in Food Science & Technology, 108*, 164-176. https://doi.org/10.1016/j.tifs.2020.12.019.

Xu, Q., Hong, H., Wu, J., & Yan, X. (2019). Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review. *Trends in Food Science & Technology, 86*, 399-411. https://doi.org/10.1016/j.tifs.2019.02.050.

Xue, L., Yin, R., Howell, K., & Zhang, P. (2021). Activity and bioavailability of food protein-derived angiotensin-I-converting enzyme–inhibitory peptides. *Comprehensive Reviews in Food Science and Food Safety, 20*, 1150-1187. https://doi.org/10.1111/1541-4337.12711.

Yu, Y.-J., Amorim, M., Marques, C., Calhau, C., & Pintado, M. (2016). Effects of whey peptide extract on the growth of probiotics and gut microbiota. *Journal of Functional Foods, 21*, 507-516. https://doi.org/10.1016/j.jff.2015.10.035.

Yust, M. d. M., Millán-Linares, M. d. C., Alcaide-Hidalgo, J. M., Millán, F., & Pedroche, J. (2012). Hypocholesterolaemic and antioxidant activities of chickpea (Cicer arietinum L.) protein hydrolysates. *Journal of the Science of Food and Agriculture, 92*, 1994-2001. https://doi.org/10.1002/jsfa.5573.

Zanoni, C., Aiello, G., Arnoldi, A., & Lammi, C. (2017a). Hempseed Peptides Exert Hypocholesterolemic Effects with a Statin-Like Mechanism. *Journal of Agricultural and Food Chemistry, 65*, 8829-8838. https://doi.org/10.1021/acs.jafc.7b02742.

Zanoni, C., Aiello, G., Arnoldi, A., & Lammi, C. (2017b). Investigations on the hypocholesterolaemic activity of LILPKHSDAD and LTFPGSAED, two peptides from lupin β-conglutin: Focus on LDLR and PCSK9 pathways. *Journal of Functional Foods, 32*, 1-8. https://doi.org/10.1016/j.jff.2017.02.009.

Zhang, T., Jiang, B., Miao, M., Mu, W., & Li, Y. (2012). Combined effects of high-pressure and enzymatic treatments on the hydrolysis of chickpea protein isolates and antioxidant activity of the hydrolysates. *Food Chemistry, 135*, 904-912. https://doi.org/10.1016/j.foodchem.2012.05.097.

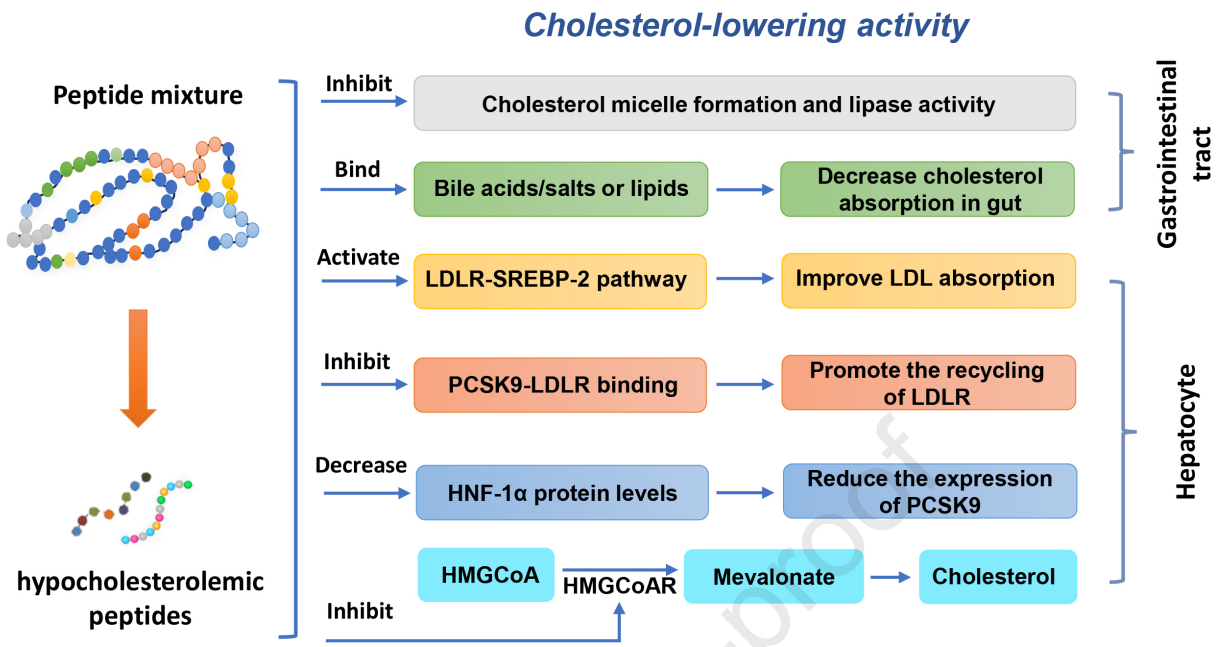
Zhong, F., Liu, J., Ma, J., & Shoemaker, C. F. (2007). Preparation of hypocholesterol peptides from soy protein and their hypocholesterolemic effect in mice. *Food Research International, 40*, 661-667. https://doi.org/10.1016/j.foodres.2006.11.011.

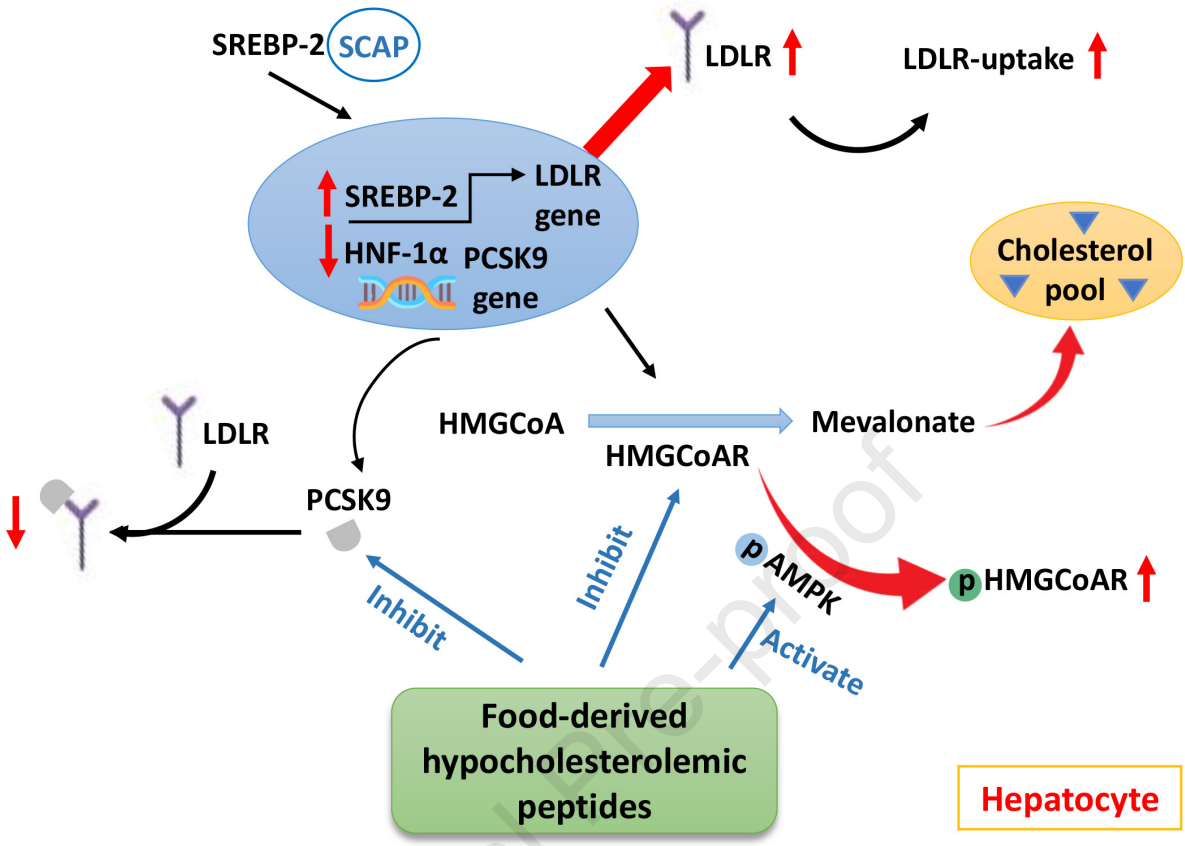
Zhong, F., Zhang, X., Ma, J., & Shoemaker, C. F. (2007). Fractionation and identification of a novel hypocholesterolemic peptide derived from soy protein Alcalase hydrolysates. *Food Research International, 40*, 756-762. https://doi.org/10.1016/j.foodres.2007.01.005.

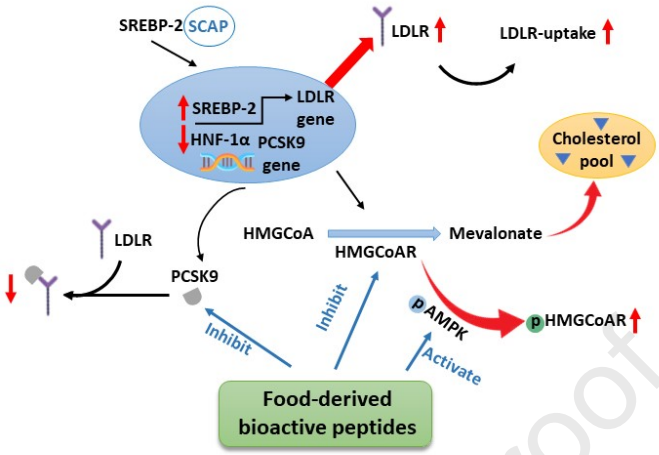
Figure Caption

Figure 1. Schematic representation of the food-derived hypocholesterolemic peptides for lowering cholesterol.

Figure2. Mechanistic pathways of food-derived hypocholesterolemic peptides in hepatocytes.







Highlight

- Some peptides exert their activity through the modulation of HMGCoAR.
- Some peptides inhibit the interactions between PCSK9 and LDL receptor.
- They may be used in the prevention of hypercholesterolemia.