Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham

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Abstract

High cholesterolemia is a key risk factor for the development of cardiovascular diseases, which are the main cause of mortality in developed countries. Most therapies are focused on the modulation of its biosynthesis through 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) inhibitors. In this sense, food-derived bioactive peptides might act as promising health alternatives through their ability to interact with crucial enzymes involved in metabolic pathways, avoiding the adverse effects of synthetic drugs. Dry-cured ham has been widely described as an important source of naturally-generated bioactive peptides exerting ACEI-inhibitory activity, antioxidant activity, and anti-inflammatory activity between others. Based on these findings, the aim of this work was to assess, for the first time, the in vitro inhibitory activity of HMG-CoAR exerted by dipeptides generated during the manufacturing of dry-cured ham, previously described with relevant roles on other bioactivities.

The in vitro inhibitory activity of the dipeptides was assessed by measuring the substrate consumption rate of the 3-hydroxy-3-methylglutaryl CoA reductase in their presence, with the following pertinent calculations. Further research was carried out to estimate the possible interactions of the most bioactive dipeptides with the enzyme by performing in silico analysis consisting of molecular docking approaches. Main findings showed DA, DD, EE, ES, and LL dipeptides as main HMG-CoAR inhibitors. Additionally, computational analysis indicated statin-like interactions of the dipeptides with HMG-CoAR.

This study reveals, for the first time, the hypocholesterolemic potential of dry-cured ham-derived dipeptides and, at the same time, converges in the same vein as many reports that experimentally argue the cardiovascular benefits of dry-cured ham consumption due to its bioactive peptide content.

Keywords: Dipeptides, Dry-cured ham, Bioactivity, HMG-CoA reductase

Introduction

Hypercholesterolemia leads to a pathogenic accumulation of low-density-lipoproteins (LDL) in blood vessels and the formation of atherosclerotic plaques, highly associated with the development of cardiovascular diseases (CVDs), which are one of the main global causes of death (Gallego et al. 2019a; Nagaoka 2019; Zalesin et al. 2011). Cholesterol synthesis consists of various steps and it is regulated at several points. The most relevant step is the reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate, by the enzyme HMG-CoA reductase (HMG-CoAR), using two NADPH as cofactors. This is the rate-limiting step of the overall synthesis of cholesterol and it constitutes a target on which many hypolipemic therapies are based on (Gesto et al. 2020). Statins are competitive inhibitors of HMG-CoAR but unfortunately, can cause neuromuscular disorders and rhabdomyolysis as secondary effects and are contraindicated for patients with previous liver diseases.
By controlling enzymatic pathways, the aim of prevention and treatment of human diseases has led to discover food compounds which may serve as agents against several disorders. In this sense, food-derived peptides can present low toxicity and accumulation in tissue (La Manna et al. 2018). Therefore, dietary bioactive peptides could mean a simple way of therapy avoiding treatments with side effects (Yao et al. 2018).

Dry-cured hams are protein-rich foods, and due to the proteolytic process associated with the dry-curing stage, they may constitute a highly potential source of bioactive peptides (Kęska & Stadnik 2016, 2017). As a result of endogenous proteolytic enzyme activities, the length of the polypeptides is progressively reduced, leading to the generation of short peptides (Toldrá et al. 2020) which are responsible of the final characteristics of the product, but also of its functional properties (Gallego et al., 2015; Gallego et al. 2019b, Mora et al. 2019). Regarding the biological activity, in vitro angiotensin I-converting enzyme (ACEI), dipeptidyl peptidase-IV (DPP-IV), α-amylase and α-glucosidase inhibitory activities, anti-inflammatory, antioxidant, and antilisterial activities have been reported (Castellano et al. 2016; Gallego et al. 2014; Gallego et al. 2016; Gallego et al. 2019a; Mora et al. 2020). Also several in vivo and clinical studies have been conducted, demonstrating antihypertensive and anti-inflammatory effects of dry-cured ham derived peptides (Escudero et al. 2012; Escudero et al. 2013; Martínez-Sánchez et al. 2017; Montoro-García et al. 2017).

The main limiting factor for peptides to exert beneficial effects is their susceptibility to a partial or total loss of activity as a result of food matrix interactions and further hydrolysis by digestive enzymes and intestinal microbiota. Finally, peptides must reach their target sites in an active form in significant quantity (Gallego et al. 2016; Toldrá et al. 2020). In this sense, dipeptides are of great therapeutic interest because they can be absorbed more efficiently in the intestinal tract (Bouglé & Bouhallab 2017; Guha & Majumder 2019) and arrive intact through the blood stream to the target sites of the organs where they could exert their functionalities.

According to this, the aim of this work was to test the in vitro HMG-CoAR inhibitory activity of different dipeptides previously reported to be generated during proteolysis in Spanish dry-cured ham. These dipeptides were selected for their potential to act as multifunctional peptides due to they have already been confirmed in previous experiments to exert other biological activities. Molecular docking computational analysis was carried out to predict statin-like interactions of the dipeptides with HMG-CoAR.

Results and discussion
The assayed dipeptides were chosen for being previously reported to be present in dry-cured ham by tandem mass spectrometry and in silico approaches (Kęska & Stadnik 2017; Mora et al. 2019; Zhou et al. 2020). The dipeptides were selected for their potential to act as multifunctional peptides due to they have already been confirmed in previous experiments to be able to exert other biological activities. Table 1 summarizes the protein of origin and main physicochemical characteristics attributed to the dipeptides of this study, whereas Table 2 includes other biological activities previously defined for these peptide sequences. In this sense, the dipeptide EE from β-conglycinin has been shown to reduce concentrations of endotelin-1 in human aortic endothelial cells, displaying a regulatory vasoactive substance release activity (Ringseis et al. 2005); while the dipeptide ES has also been previously described as an in vitro dipeptidyl peptidase IV inhibitor (Lan et al. 2015). Otherwise, the dipeptide DA has been proved as inhibitor of both ACEI and DPP-III enzymes (Cushman et al. 1981; Dhanda et al. 2007). On the other hand, AA has been documented as in vitro ACEI and DPP-IV inhibitor (Gallego et al. 2014; Sentandre & Toldrá 2007); AL and VH have been registered as in vitro DPP-IV inhibitors and to exert anti-inflammatory effects on lipopolysaccharide-induced RAW 264.7 macrophages (Lan et al. 2015; Non- gonierma et al. 2014; Zhao et al. 2016). AQ supplementation patients with severe burns can reduce the infection rate, wound healing time, intestinal permeability and serum endotoxin concentration at the same time that plasma Q levels are increased (Zhou et al. 2004). Moreover, this dipeptide, and also QQ, decreases the release of pro-inflammatory cytokines by polymorphonuclear leukocytes, while expression of the anti-inflammatory IL-10 is enhanced (Fürst et al. 2004). On other hand, LL was demonstrated to act as an in vitro DPP-IV inhibitor and as glucose uptake stimulating compound in L6 myotubes cell cultures (Bella et al. 1982; Morifuji et al. 2009), but also was predicted to have a role as anti-inflammatory via in silico analysis (Gupta et al. 2017). Finally, QQ was identified as in vitro DPP-IV inhibitor (Lan et al. 2015).

Regarding the variety of activities, it is important to consider that due to the link between metabolic pathways, bioactive peptides might act on different targets to regulate biological functions.

A further point is that the majority of the dipeptides of this work have also been correlated with different tastes that could influence on the typical organoleptic properties of dry-cured hams (Arai et al. 1972; Asao et al. 1987; Kim et al. 2015; Kuramitsu et al. 1996; Maheshi et al. 1999; Noguchi et al. 1975; Ohyama et al. 1988; Shim et al. 2015; Tamura et al. 1989; van den
Oord & van Wassenaar 1997). Hence, their relevance can take on a new sense in terms of their multifunctionality as bioactive and taste-active compounds.

**HMG-CoA inhibitory bioactivity of the selected dipeptides**

The anti-hypercholesterolemic activity of the dipeptides was assayed measuring the inhibition percentages of HMG-CoAR.

As it can be seen in Table 3, the dipeptides DA, EE, ES, and LL, tested at 1 Mm, showed the highest inhibition percentages (more than 40%), followed by the dipeptides DD and VH. The dipeptides AW, DG, EV, GA, LE, PA and VG showed null inhibition. Finally, the rest of the dipeptides showed approximately above 10% of inhibition, except for QQ. Pravastatin, used as positive control, reached inhibition values slightly higher than
70% with a concentration of 2.5 μM (Fig. 1). Those peptides with the highest inhibitory activity were tested at different concentrations, as it is shown in Fig. 2. Additionally, to evaluate the possibility of an in vitro synergistic effect, two sets of dipeptides at 1 mM were assayed. Set 1 was formed by the most active dipeptides DA, DD, EE, ES, LL, and VH, while Set 2 consisted of AA, AL, AQ, and QQ. However, no significative increment in the inhibition activity was observed (Table 3), thus, ruling out synergistic effects in the in vitro test. According to the inhibition activity of the Set 1, it might mean that dipeptides could act by a non-synergistic inhibition mechanism which relies on the global concentration of peptides with specific structural properties. Main results suggest that the acidic character of D and E of dipeptides DA, DD, EE, and ES could help them to bind the enzyme and reduce its activity. This fact may permit short peptides to access the catalytic site of the enzyme and simulate the HMG-like moiety of statins, which is bound in the narrow pocket of the active site (Pak et al. 2006). AA, AQ, AL, QQ and VH revealed the forming residues would have a less relevant role in inhibition of this enzyme. In general, the most active dipeptides meet the condition of having a pI under, or similar, to 1.0. Besides, they also present lower hydrophobicity values except DA. In addition, the steric hindrance seems to play a moderate influence, due to the fact that DA, DD, EE and ES are of those with the

### Table 2 Other bioactivities attributed to the dipeptides of this study

| Dipeptide | Bioactivity | Biological system | Reference |
|-----------|-------------|-------------------|-----------|
| AA        | in vitro ACEI inhibitor | cardiovascular | Gallego et al., 2014 |
| AL        | in vitro DPP-IV inhibitor | endocrine | Sentandreu & Toldrá, 2007 |
| AQ        | inhibition of NO production in RAW 264.7 | immune | Zhao et al., 2016 |
| AW        | in vitro antioxidant | cardiovascular | Liu et al., 2015 |
| DA        | in vitro ACEI inhibitor | cardiovascular | Cushman et al., 1981 |
| DD (absent) | | | |
| DG        | in vitro ACEI inhibitor | cardiovascular | Meisel et al., 2006 |
| EE        | stimulating vasoactive substance release | cardiovascular | Ringsseis et al., 2005 |
| ES        | in vitro DPP-IV inhibitor | endocrine | Lan et al., 2015 |
| EV        | in vitro ACEI inhibitor | cardiovascular | van Platerink et al., 2008 |
| GA        | ACEI inhibitor | cardiovascular | Cheung et al., 1980 |
| LE (absent) | | | |
| LL        | in vitro DPP-IV inhibitor | endocrine | Bella et al., 1982 |
| PA        | in vitro DPP-IV inhibitor | endocrine | Bella et al., 1982 |
| QQ        | in vitro DPP-IV inhibitor | endocrine | Lan et al., 2015 |
| VG        | in vitro ACEI inhibitor | cardiovascular | Cheung et al., 1980 |
| VH        | in vitro DPP-IV inhibitor | endocrine | Lan et al., 2015 |
|           | inhibition of NO production in RAW 264.7 | immune | Zhao et al., 2016 |

*Peptide sequences are given as amino acids one-letter code*
highest values. Globally, it can be said that aliphatic residues do not attribute any remarkable inhibition capacity (Table 1).

In this sense, T- and E-residues have been proposed to act as mimics of an HMG moiety for the HMG-CoA interacting site and facilitate the formation of additional H bonds that intensify the attachment with the binding site and the stabilization of the conformation (Pak et al. 2005a; Pak et al. 2007). In fact, the E-residue located at the C-terminus has been pointed out as an essential character for the recognition of the HMG binding site (Pak et al. 2012). In addition, it has been suggested that a positively charged residue at C-terminus is unfit within the positively charged residues of the HMG-CoA subpocket, whereas a D-residue in C-terminal position, makes the interaction between hydrophobicity and the inhibitory activity (Pak et al. 2006). Here, polar acidic E- and D-residues exhibited a more relevant role over the rest of the residues, as all peptides containing E and D amino acids exerted inhibition. Also, polar weak basic Q-residue seemed to attribute a fairly capacity of inhibition.

Additionally, certain structure–functional analyses focused on tetrapeptides showed that an E-residue in the C-terminus and L-, I- or Y-residues at N-terminal position, benefit the inhibitory bioactivity (Pak et al., 2005c), which could partially explain the inhibition observed on the dipeptide LL, although LE dipeptide did not showed a significant inhibitory activity. A- and V-residues could have a steric effect due to their aliphatic side chains at positions 2 and 3 in tetrapeptides, and that might be the reason of the inhibitory effect observed in AA, AL, AQ and VH dipeptides. A P-residue is also considered to mimic the nicotinamide moiety of NADPH, conferring a “turn” structure which promotes docking by imitating statins (Coelho et al. 2018; Pak et al. 2010). The presence of cationic amino acids such as H-residues has been correlated with a hypolipidemic effect, but mainly due to their ability to interact with the carboxylic groups of bile acids (Yao et al. 2018). Moreover, the analysis of the superposition of statins and peptide molecules showed a similar location of the iso-butyl (compactin and simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) with the side-chains of I- and Y-residues, respectively, and an aryl radical in the N-terminal increases the inhibitory activity of tetrapeptides (Pak et al. 2006). Basing on this, perhaps the imidazole ring system of H-residue, which has an aromatic character due to the presence of a planar ring containing 6 π-electrons, could contribute to an inhibitory effect although it is located at the C-terminus in the dipeptide VH. However, the residue position in a dipeptide may be not so essential as their small size might provide them with versatility in terms of spatial distribution.

Several reports have denoted the potential HMG-CoAR inhibitory effect of some peptides. Peptide extracts with molecular mass lower than 3 kDa from different sources, such as chia protein extracts and cowpea, reduced the HMG-CoAR enzymatic reaction velocity (Coelho et al. 2018; Marques et al. 2015). The presence of cationic amino acids such as H-residues has been correlated with a hypolipidemic effect, but mainly due to their ability to interact with the carboxylic groups of bile acids (Yao et al. 2018). Moreover, the analysis of the superposition of statins and peptide molecules showed a similar location of the iso-butyl (compactin and simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) with the side-chains of I- and Y-residues, respectively, and an aryl radical in the N-terminal increases the inhibitory activity of tetrapeptides (Pak et al. 2006). Basing on this, perhaps the imidazole ring system of H-residue, which has an aromatic character due to the presence of a planar ring containing 6 π-electrons, could contribute to an inhibitory effect although it is located at the C-terminus in the dipeptide VH. However, the residue position in a dipeptide may be not so essential as their small size might provide them with versatility in terms of spatial distribution.

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### Table 3: HMG-CoA reductase (HMG-CoAR) inhibitory activity of the assayed dipeptides at 1 mM ($n > 3$)

| Dipeptide | % Inhibition SD | % Inhibition SD |
|-----------|-----------------|-----------------|
| AA Ala-Ala | 13.80 $^{a,b}$ | 5.84 |
| AL Ala-Leu | 13.02 $^{a}$ | 1.82 |
| AQ Ala-Gln | 11.34 $^{c,d}$ | 3.55 |
| AW Ala-Trp | n. s. | n.s. |
| DA Asp-Ala | 49.57 $^{a}$ | 5.49 |
| DD Asp-Asp | 33.04 $^{a}$ | 6.23 |
| DG Asp-Gly | n. s. $^{e}$ | n.s. |
| EE Glu-Glu | 47.26 $^{a,b}$ | 7.40 |
| ES Glu-Ser | 45.49 $^{a,b}$ | 6.28 |
| EV Glu-Val | n. s. $^{f}$ | n.s. |
| GA Gly-Ala | n. s. $^{g}$ | n.s. |
| LE Leu-Glu | n. s. $^{h}$ | n.s. |
| LL Leu-Leu | 48.32 $^{a}$ | 5.87 |
| PA Pro-Ala | n. s. $^{i}$ | n.s. |
| QQ Gin-Gin | 4.71 $^{a,b}$ | 1.39 |
| VG Val-Gly | n. s. $^{j}$ | n.s. |
| VH Val-His | 28.63 $^{a,d}$ | 8.48 |

**SET 1** n. s. g n.s.

**SET 2** n. s. g n.s.

`Set 1 refers to the peptide group DA, DD, EE, ES, LL, and VH at 1 mM; Set 2 refers to the peptide group AA, AL, AQ, and QQ at 1 mM; Letters designate significant differences among the values at $P < 0.05$. n. s. indicates non-significant inhibition.`
**Fig. 1** Inhibition percentages of pravastatin at different concentrations (μM) (n > 3). Letters indicate significant differences among the values at $P < 0.05$.

**Fig. 2** Inhibition percentages of most bioactive dipeptides at different concentrations 0.1, 1, and 2 mM (n > 3). Letters designate significant differences among the values at $P < 0.05$. 
demonstrated that lupin peptides, soy β-conglycinin-derived peptides YVVNPDDEN and YVVNPDNENN; and LILPKHSAD and LTFPGSAED, derived from lupin β-conglutin, are able to regulate the cholesterol metabolism at HepG2 cell line (Lammi et al. 2014, 2015). Peptides GGV, IVG, and VGVL generated by in vitro hydrolysis of *Amaranthus cruentus* protein; and QDF, derived from cowpea β-vignin protein have been also reported as hypocholesterolemic peptides (Soares et al. 2015). More recently, a peptide from an Indonesian fermented fish and different peptide fractions from Japanese traditional fermented fishes have been described to inhibit HMG-CoAR enzyme (Rinto et al. 2017).

According to our knowledge, no evidence has been published to date about dry-cured ham hypocholesterolemic properties due to the presence of HMG-CoAR inhibitory dipeptides. It is known that dry-cured ham is a traditional Spanish food with numerous reports correlating the generation of bioactive peptides with counteracting effects related to hypertension, thrombotic issues, and inflammation processes, despite its salt content. A research on post-menopausal women, reviewed no prejudicial effects on the lipid profile after the consumption of acorn-fed Iberian ham rich in oleic acid. Total cholesterol, triglycerides (TG), LDL-cholesterol, and fibrinogen levels dropped significantly after the consumption of a diet rich in oleic acid, a part of which was from acorn-fed Iberian ham, while they were sustained after another diet whose oleic acid came from olive oil. Also, no changes were detected on high density lipoprotein (HDL)-cholesterol, Apo A and B and lipoprotein (Rebollo et al. 1998). In agreement, a prospective and dynamic epidemiologic cohort, recorded that after 6 years of consumption of dry-cured ham by university graduates, no association was found between higher levels of consumption of dry-cured ham and the incidence of cardiovascular disease, hypertension and weight gain (Ruiz-Canela López et al. 2009). More recently, a two-arm, cross-over, randomised controlled trial in healthy patients with hypertension, showed that consumption of dry-cured ham attenuates platelet and monocyte activation and it diminishes plasmatic P-selectin, monocyte chemoattractant protein-1 and interleukin 6 levels (Martínez-Sánchez et al. 2017). In accordance with these outcomes, another two-arm, cross-over, randomised controlled trial revealed regular dry-cured ham consumption had a lipid-lowering effect, with decreases in total cholesterol, LDL and TG levels, and in glycaemia. What is more, no significant changes were detected in HDL, TG/HDL-cholesterol, and LDL/HDL-cholesterol ratios after any treatment nor in blood pressure. Additionally, the study also identified several bioactive peptides in the interventional product with previously demonstrated antihypertensive bioactivity. Those observations suggested an intake-dependent improvement in the thrombogenic and inflammatory status and it might be due to the bioactivity of the peptides generated in dry-cured ham (Montoro-García et al. 2017).

Nevertheless, this is the first time that an inhibitory activity on HMG-CoAR is related to dipeptides generated in dry-cured ham.

**Docking results**

Those dipeptides showing the highest in vitro inhibitory activity were studied through molecular docking analyses to understand their possible mechanism of inhibition. As it is showed in Table 4 and Fig. 3, the computational process between HMG-CoAR and the studied dipeptides permits to estimate interacting residues, binding type, and binding energy of interactions.

Data suggested that pravastatin makes interactions with key enzyme residues as Ser684, Asp690, Lys691, Lys692 from one chain and Glu559, Lys735, His752, Asn755 and Leu853 from the other chain. These predications indicated that pravastatin establishes typical statin HMG-moieties interactions, some of them identical to those observed between protein and substrate HMG-CoA and presumably also with the reaction product mevalonate (Istvan 2001).

Pravastatin showed an estimated inhibition constant of 37.85 nM, which is in line with the fact that statins bind to the enzyme at nanomolar concentrations, whereas HMG-CoA binds at 4 μM (Carbonell & Freire 2005; Istvan 2001). Interestingly, EE was found to present an inhibition constant of the same order of units as pravastatin (569.34 nM), while the rest of the dipeptides showed micromolar units. Similar binding constant units and/or modulating mechanisms have been previously described with peptides such as QDF derived from cowpea β-vignin protein, and the synthetic peptide GFPTGG, caprine milk-derived sequences NMAIHPR, TNAIPYVR and TNAIPYVRL, and soy β-conglycinin-derived peptides YVVNPDDEN and YVVNPDNENN (Fatchiyah & Natasia 2018; Lammi et al. 2015; Lammi et al. 2016a, b; Pak et al. 2007; Silva et al. 2018; Zanoni et al. 2017).

According to these results, it was predicted that the studied dipeptides fit in the interface between the two subunits, quite similarly to the site occupied by pravastatin. As shown in Fig. 3, the dipeptides occupy the same binding site as the usual substrate HMG-CoA in the catalytic domain probably by the establishment of hydrogen bonds, hydrophobic interactions, and/or salt bridges. Docking studies have identified similar interacting residues with larger peptides (Fatchiyah & Natasia,
| Pravastatin Binding energy (kcal/mol) | Inhibition constant | Protein residues involved in H-bond interactions (chain:residue) | No. of H bonds | Protein residues involved in hydrophobic interactions (chain:residue) | Protein residues involved in salt bridges (chain:residue) |
|-------------------------------------|---------------------|---------------------------------------------------------------|----------------|---------------------------------------------------------------|---------------------------------------------------------------|
| AA −10.13 37.85 (nM)              | C:Ser684 (Donor, sd) | C:Asp690 (Acceptor), C:Asp690 (Acceptor), C:Asp690 (Donor) | 8              | C:Asn658, C:Lys662, D:His752, D:Leu853, D:Leu862, D:Val863 | C:Arg590 (Carboxilate), C:Lys692 (Carboxilate), D:Lys735 (Carboxilate) |
| AL −6.47 18.03 (μM)              | C:Ser684 (Donor, sd) | C:Lys691 (Donor, sd), D:Glu559 (Acceptor, sd), D:Ala751 (Acceptor) | 5              | D:His752, D:Leu853, D:Leu862 | C:Arg590 (Carboxilate), C:Lys692 (Carboxilate), D:Lys735 (Carboxilate) |
| AQ −6.41 20.08 (μM)              | C:Asp690 (Acceptor, sd) | C:Lys691 (Donor, sd), D:Glu559 (Acceptor, sd), D:His752 (Donor, sd), D:Asn755 (Donor, sd) | 6              | D:Glu559, D:Val863 | C:Arg590 (Carboxilate) |
| DA −6.58 15.08 (μM)              | C:Asn658 (Acceptor, sd) | C:Asn658 (Acceptor, sd), C:Ser661 (Donor, sd) | 6              | C:Asn658, C:Lys662, D:His752 | C:Arg590 (Carboxilate) |
| DD −6.92 8.51 (μM)              | C:Ser684 (Donor, sd) | C:Asp690 (Acceptor, sd), D:Glu559 (Acceptor, sd), D:Glu559 (Acceptor, sd), D:Ala751 (Acceptor) | 6              | C:Asn658, C:Lys662, D:His752 | C:Arg590 (Carboxilate) |
| EE −7.03 7.03 (μM)              | C:Arg590 (Donor, sd) | C:Arg590 (Donor, sd), C:Ser661 (Donor, sd) | 8              | D:Leu853 | C:Arg590 (Carboxilate) |
| ES −8.52 569.34 (nM)            | C:Asn658 (Donor, sd) | C:Ser684 (Donor, sd), C:Ser684 (Donor, sd), D:Glu559 (Acceptor, sd), D:Glu559 (Acceptor, sd), D:Asn755 (Donor, sd) | 6              | D:Leu853 | C:Arg590 (Carboxilate) |
| LL −6.83 9.85 (μM)              | C:Arg590 (Donor, sd) | D:Glu559 (Acceptor, sd), D:Glu559 (Acceptor, sd), D:His752 (Donor, sd), D:Asn755 (Donor, sd) | 9              | D:Leu853 | C:Arg590 (Carboxilate) |
| QQ −6.69 12.46 (μM)            | C:Asp690 (Acceptor, sd) | C:Asp690 (Acceptor, sd), D:Glu559 (Acceptor, sd), D:Glu559 (Acceptor, sd), D:His752 (Donor, sd), D:Asn755 (Donor, sd) | 5              | C:Asn658, C:Lys662, D:His752 | C:Arg590 (Carboxilate) |
| VH −5.91 46.66 (μM)            | C:Arg590 (Donor, sd) | C:Arg590 (Donor, sd), C:Ser684 (Donor, sd) | 11             | D:Leu853 | C:Arg590 (Carboxilate) |
supporting the idea that these compounds can potentially act as statin analogues. The lower binding energies obtained in this last case may reflect the influence of the peptide size on the number of possible interactions with the enzyme, both by the increase of side chains and by the ability to adopt optimal conformations (Lin et al. 2015; Pak et al. 2010). Notwithstanding, conformations have been calculated considering a single ligand, but there is also the possibility to avoid the entry of the substrate if various dipeptides cooperate to block the binding sites of the enzyme, or that side chains of the residues interact among them forming complexes which block the binding site in a different way than predicted in this study.

**Conclusion**

Dipeptides AA, AL, AQ, DA, DD, EE, ES, LL, QQ, and VH, generated in dry-cured ham have been reported for the first time to exert in vitro inhibitory activity of HMG-CoAR enzyme. Of all of them, DA, DD, EE ES and LL presented greater inhibition percentages. Therefore, D- and E-residues might play a key role on the interaction between dipeptides and the enzyme. In the other hand, according to the molecular docking analysis, these peptides may act as structural analogues of HMG-CoA docking within the active site in a similar statin-interacting behaviour or they might prevent the substrate recognition by covering up the active site. Nonetheless, further studies are needed to confirm the bioavailability and efficacy of these peptides in vivo. These results support that dipeptides generated during the proteolytic process of dry-cured ham, could act as in vitro modulators of HMG-CoA activity.

**Chemicals and reagents**

The dipeptides Ala-Ala (AA), Ala-Leu (AL), Ala-Gln (AQ), Ala-Trp (AW), Asp-Ala (DA), Asp-Asp (DD), Asp-Gly (DG), Glu-Glu (EE), Glu-Ser (ES), Glu-Val (EV), Gly-Ala (GA), Leu-Glu (LE), Leu-Leu (LL), Pro-Ala (PA), Gln-Gln (QQ), Val-Gly (VG) and Val-His (VH) were used in this study. Of all of them, DA, DD, EE ES and LL presented greater inhibition percentages. Therefore, D- and E-residues might play a key role on the interaction between dipeptides and the enzyme. In the other hand, according to the molecular docking analysis, these peptides may act as structural analogues of HMG-CoA docking within the active site in a similar statin-interacting behaviour or they might prevent the substrate recognition by covering up the active site. Nonetheless, further studies are needed to confirm the bioavailability and efficacy of these peptides in vivo. These results support that dipeptides generated during the proteolytic process of dry-cured ham, could act as in vitro modulators of HMG-CoA activity.

**Table 4 HMG-CoAR binding site residues involved in docking interactions with pravastatin and dipeptides, with docking scores (Continued)**

| Ligand | Binding energy (kcal/mol) | Inhibition constant | Protein residues involved in H-bond interactions (chain:residue) | No. of H bonds | Protein residues involved in hydrophobic interactions (chain:residue) | Protein residues involved in salt bridges (chain:residue) |
|--------|--------------------------|---------------------|----------------------------------------------------------------|--------------|----------------------------------------------------------------|------------------------------------------------------|
| C:Asp690 (Acceptor, sd) | | | | | | |
| C:Lys692 (Donor, sd) | | | | | | |
| D:Gly560 (Acceptor) | | | | | | |
| D:Ser565 (Acceptor, sd) | | | | | | |
| D:Ser565 (Donor, sd) | | | | | | |
| D:Lys735 (Donor, sd) | | | | | | |
| D:His752 (Donor, sd) | | | | | | |
| D:Asn755 (Donor, sd) | | | | | | |
| C:Arg590 (Donor, sd) | | | | | | |
| C:Asn658 (Acceptor, sd) | | | | | | |
| C:Asn658 (Acceptor, sd) | | | | | | |
| C:Asn658 (Donor, sd) | | | | | | |
| C:Ser661 (Donor, sd) | | | | | | |
| C:Lys691 (Donor, sd) | | | | | | |
| C:Arg590 (Carboxylate) | | | | | | |

Key residues of the binding site are highlighted in bold. Common residues whereby pravastatin and dipeptides interact with are coloured in purple.

2018), supporting the idea that these compounds can potentially act as statin analogues. The lower binding energies obtained in this last case may reflect the influence of the peptide size on the number of possible interactions with the enzyme, both by the increase of side chains and by the ability to adopt optimal conformations (Lin et al. 2015; Pak et al. 2010). Notwithstanding, conformations have been calculated considering a single ligand, but there is also the possibility to avoid the entry of the substrate if various dipeptides cooperate to block the binding sites of the enzyme, or that side chains of the residues interact among them forming complexes which block the binding site in a different way than predicted in this study.

**Conclusion**

Dipeptides AA, AL, AQ, DA, DD, EE, ES, LL, QQ, and VH, generated in dry-cured ham have been reported for the first time to exert in vitro inhibitory activity of HMG-CoAR enzyme. Of all of them, DA, DD, EE ES and LL presented greater inhibition percentages. Therefore, D- and E-residues might play a key role on the interaction between dipeptides and the enzyme. In the other hand, according to the molecular docking analysis, these peptides may act as structural analogues of HMG-CoA docking within the active site in a similar statin-interacting behaviour or they might prevent the substrate recognition by covering up the active site. Nonetheless, further studies are needed to confirm the bioavailability and efficacy of these peptides in vivo. These results support that dipeptides generated during the proteolytic process of dry-cured ham, could act as in vitro modulators of HMG-CoA activity.

**Chemicals and reagents**

The dipeptides Ala-Ala (AA), Ala-Leu (AL), Ala-Gln (AQ), Ala-Trp (AW), Asp-Ala (DA), Asp-Asp (DD), Asp-Gly (DG), Glu-Glu (EE), Glu-Ser (ES), Glu-Val (EV), Gly-Ala (GA), Leu-Glu (LE), Leu-Leu (LL), Pro-Ala (PA), Gln-Gln (QQ), Val-Gly (VG) and Val-His (VH) were used in this study. Of all of them, DA, DD, EE ES and LL presented greater inhibition percentages. Therefore, D- and E-residues might play a key role on the interaction between dipeptides and the enzyme. In the other hand, according to the molecular docking analysis, these peptides may act as structural analogues of HMG-CoA docking within the active site in a similar statin-interacting behaviour or they might prevent the substrate recognition by covering up the active site. Nonetheless, further studies are needed to confirm the bioavailability and efficacy of these peptides in vivo. These results support that dipeptides generated during the proteolytic process of dry-cured ham, could act as in vitro modulators of HMG-CoA activity.

**Table 4 HMG-CoAR binding site residues involved in docking interactions with pravastatin and dipeptides, with docking scores (Continued)**

| Pravastatin Binding energy (kcal/mol) | Inhibition constant | Protein residues involved in H-bond interactions (chain:residue) | No. of H bonds | Protein residues involved in hydrophobic interactions (chain:residue) | Protein residues involved in salt bridges (chain:residue) |
|--------------------------------------|---------------------|----------------------------------------------------------------|--------------|----------------------------------------------------------------|------------------------------------------------------|
| C:Asp690 (Acceptor, sd) | | | | | | |
| C:Lys692 (Donor, sd) | | | | | | |
| D:Gly560 (Acceptor) | | | | | | |
| D:Ser565 (Acceptor, sd) | | | | | | |
| D:Ser565 (Donor, sd) | | | | | | |
| D:Lys735 (Donor, sd) | | | | | | |
| D:His752 (Donor, sd) | | | | | | |
| D:Asn755 (Donor, sd) | | | | | | |
| C:Arg590 (Donor, sd) | | | | | | |
| C:Asn658 (Acceptor, sd) | | | | | | |
| C:Asn658 (Acceptor, sd) | | | | | | |
| C:Asn658 (Donor, sd) | | | | | | |
| C:Ser661 (Donor, sd) | | | | | | |
| C:Lys691 (Donor, sd) | | | | | | |
| C:Arg590 (Carboxylate) | | | | | | |

Key residues of the binding site are highlighted in bold. Common residues whereby pravastatin and dipeptides interact with are coloured in purple.
Fig. 3 (See legend on next page.)
HMG-CoAR activity assay

The assay is based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMG-CoAR in the presence of the substrate HMG-CoA.

The protocol was carried out according to the manufacturer’s instructions. Each reaction was prepared by adding the reagents in the following order: 1X assay buffer; dipeptide sample (1 μL) or positive control pravastatin (1 μL); NADPH (4 μL); substrate solution (12 μL); and finally, HMGCo-AR (2 μL). Subsequently, the samples were mixed, and the absorbance at 340 nm was read at 37 °C by a CLARIOstar microplate reader (BMG LABTECH, Ortenberg, Germany), from 0 to 10 min. The HMG-CoA dependent oxidation of NADPH in the absence (Control) and presence of inhibitors, was measured by the absorbance decline, which is directly proportional to enzyme activity. Then, enzyme inhibition was calculated as follows:

\[
\% \text{Inhibition} = \left( \frac{\Delta \text{Abs 100\%activity} - \Delta \text{Abs Sample}}{\Delta \text{Abs 100\%activity}} \right) \times 100
\]

Molecular docking

Based on the inhibitory results, the dipeptides with higher inhibitory activity were selected for the in silico analysis in order to predict their potential interacting mechanism. Dipeptide sequences in “sdf” format were obtained from PubChem (Kim et al. 2019) and pdb files were extracted using Discovery Studio Visualizer v20.1.0. 19,295 (Dassault Systèmes BIOVIA Corp, 2020).

The structure of human HMG-CoAR (protein data bank ID: 1DQ8), in complex with HMG and CoA (Istvan et al. 2000), was downloaded from Protein Data Bank (PDB) (Berman, 2000).

The catalytic portion of the human HMG-CoAR is a tetramer which comprises the range of residues from position 459 to 863 (Jawaid et al. 2010). The monomers are arranged in two dimers, each of which has two active sites, and each active site is formed by residues from both monomers. Monomers of the catalytic portion consist of three domains: an N-terminal “N-domain” (residues 460–527), a large and folded “L-domain” (residues 528–590 and 694–872) and a small “S-domain” (residues 592–682). The latter forms the binding site for NADP(H) and it is inserted into the L-domain (Istvan et al. 2000). The interface between both L- and S-domains originates the binding pocket for HMG (residues 684–692), which is the most important element in the binding of the substrate. If these residues interact with another molecule, it can no longer associate with HMG-CoA (Fatchiyah & Natasia, 2018). More specifically, the binding site is surrounded by key residues Arg590, Ser661, Val683, Ser684, Asp690, Lys691 from one subunit and Glu559, Cys561, Leu562, Ala564, Ser565, His752, Lys735, Asn755, Leu853, Ala856 from another subunit (Shiuan et al. 2015). Residues Tyr479, Asp767, and His866, also contribute to the catalytic process (Lateef et al. 2020).

Ligand-protein docking simulations were carried out using AutoDock tools v1.5.6 and AutoDock v4.2.5.1 (The Scripps Research Institute) programs (Morris et al. 2009; Sanner, 1999).

Gasteiger charges and hydrogens were added to all molecules, water molecules were also removed from the enzyme, and ligand torsions were detected by AutoDock. Structure data files were converted into the Protein Data Bank partial charge and atom type format.

Grid Boxes (60x60x60) were centred on one of the HMG, CoA and NADPH binding sites located at the interaction between chains C and D, with coordinates X = 17.146, Y = 16.96, and Z = -36.37, and spacing of 0.375 Å (Istvan et al. 2000). Fifty docking runs were performed, using a Lamarckian genetic algorithm between flexible ligand and rigid receptor, a population size of 150, a maximum of 2,500,000 generations and 2,500,000 evaluations for 50 GA runs. The root mean square deviation tolerance was set to 2.0 Å for the clustering of docking results. Analysis of the results was done by sorting the different complexes with respect to the predicted binding energy. The pose with lowest binding energy in each case was individually examined, and interactions were processed with online software Protein-Ligand Interaction Profiler (PLIP), to validate the interactions; and with ProteinsPlus, to obtain the two-dimensional representations by using PoseView algorithm (Fährrolfes et al. 2017; Salentin et al. 2015).
Results are presented as means of 3 replicates ± standard deviation. Statistical analysis was performed by one-way ANOVA and Fisher’s multiple range tests for inhibition data using the software XLSTAT 2011 v5.01 (Addinsoft, Barcelona, Spain). Statistically significant differences were considered at P < 0.05.

Abbreviations
A: Ala, L-alanine; ACEI: Angiotensin I-converting enzyme; CVDs: Cardiovascular diseases; D, Asp, L-aspartate; DPPs: Dipeptidyl peptidases; DPP-III: Dipeptidyl peptidase-III; DPP-IV: Dipeptidyl peptidase-IV; E, Glu: L-glutamate; F, Phe: L-phenylalanine; G, Gly: L-glycine; H, His: L-histidine; HDL: High-density-lipoproteins; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; HMG-CoAR: 3-hydroxy-3-methylglutaryl-CoA reductase; I, Ile: L-isoleucine; K, Lys: L-lysine; L, Leu: L-leucine; LDL: Low-density-lipoproteins; M, Met: L-methionine; N, Asn: L-asparagine; NADPH: Nicotinamide adenine dinucleotide phosphate; P, Pro: L-proline; Q, Gln: L-glutamine; R, Arg: L-arginine; S, Ser: L-serine; T, Thr: L-threonine; TG: Triglyceride; V, Val: L-valine; W, Trp: L-tryptophan; Y, Tyr: L-tyrosine.

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Authors’ contributions
AH developed the laboratory work and performed the experiments. AH and FT revised the manuscript. LM analyzed the data. AH drafted the manuscript. LM and FT revised the manuscript. All authors read and approved the final manuscript.

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