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ARTICLE

Host defense peptides identified in human apolipoprotein B as natural food bio-preservatives: Evaluation of their biosafety and digestibility

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Abstract

The employment of chemical agents in the food industry is raising several concerns by consumers and is leading to an increasing interest in natural food preservatives. Among alternatives, host defense peptides (HDPs) have attracted great interest for their ability to preserve food samples from contamination without altering their quality, taste, and organoleptic properties. Recently, we evaluated the applicability of ApoB-derived peptides as novel food bio-preservatives and demonstrated their ability to prevent chicken meat sample contamination when immobilized on chitosan films. To perform a further step towards the applicability of these peptides in the food field, here we evaluated peptides biosafety and digestibility. To do this, we used a multidisciplinary approach including the evaluation of the peptides' toxicity and antimicrobial activity, the analysis of resistance phenotype development, an in silico prediction of the peptides' susceptibility to proteases and the evaluation of the peptides' stability in simulated gastric and intestinal fluids. ApoB-derived peptides were found to be nontoxic when tested on human gastric carcinoma cells SNU-1 and on human colon-rectal adenocarcinoma cells HT-29, and not to induce resistance phenotype in *Salmonella* strains. Bioinformatic analyses showed that the peptides are susceptible to several proteases, as also confirmed by experiments in simulated gastric and intestinal fluids. Altogether these findings open interesting perspectives to the future applicability of ApoB-derived peptides as novel food biopreservatives.

KEYWORDS

antimicrobial peptides, antimicrobial resistance, food biopreservatives, host defense peptides, peptide biocompatibility, peptide digestibility

Abbreviations: AMPs, antimicrobial peptides; ApoB, apolipoprotein B; HDPs, host defense peptides; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SGF, simulated gastric fluid; SIF, simulated intestine fluid.

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1 | INTRODUCTION

1.1 | Cryptic bioactive peptides

In recent decades, it has become evident that the limited number of genes is not enough to explain the complexity of the organism and the key role played by the proteins[1] present in a cell, tissue or organ in a given time and under specific conditions[2] has been highlighted. The situation is even more complicated if we consider that most of the proteins are processed to produce a pool of bioactive encrypted peptides endowed with functions often not directly related to those of the precursor proteins. The term "cryptome" has been used to define a subset of the proteome comprising cryptic peptides with distinct bioactivities, that have been named cryptides.[3,4] They have been found to be endowed with a wide range of bioactivities, such as host defense, antithrombotic, protease inhibition, blood pressure regulation, opioid, analgesic, signaling, angiogenesis, and wound healing.[5–10]

1.2 | Host defense peptides

Among cryptides, a special class is represented by antimicrobial peptides (AMPs), then named host defense peptides (HDPs) because of their involvement in the innate immune system of living organisms. HDPs generally share crucial features, such as the length ranging between 12 and 50 amino acids, the high content of hydrophobic aminoacidic residues (+50%) and the positive net charge at physiological pH values.[11] These features are the basis of HDPs ability to selectively interact with and target negatively charged bacterial membranes.[10] In the case of vertebrates, HDPs are not only able to directly affect and kill bacteria but they also play a role in augmenting the adaptive response.[6–8,10]

1.3 | Applicability of HDPs in food industry

Because of their wide range of activities and their peculiar mechanism of action, HDPs have attracted great interest as good candidates to be employed in biomedical, cosmeceutical and food fields. In the case of food industry, traditional procedures, such as the addition of chemical additives or food thermal processing, have been found to negatively affect food quality and nutritional properties,[12] with a consequent increase of the interest of consumers towards untreated foods and natural biopreservatives. In this scenario, HDPs have attracted the interest of researchers as novel biopreservatives able to protect food samples from microbial contaminations while not affecting food quality. The introduction in the market of a novel food ingredient requires detailed analyses on its biocompatibility, allergenicity and safety for human health.[13]

1.4 | ApoB-derived HDPs

In the present work, we analyzed HDPs previously identified in human apolipoprotein B (ApoB) and found to be endowed with antimicrobial, anti-biofilm, antifungal, wound healing, and immunomodulatory properties.[14–17] MIC values determined for ApoB-derived peptides were found to be comprised between 1.25 and 20 µM depending on the bacterial strains tested.[15] Moreover, peptides were also found to be able to synergistically act in combination with conventional antibiotics[18–22] and to exert antimicrobial and antibiofilm properties when tested on *Salmonella* cells.[23] ApoB-derived peptides were found to be effective on both *Salmonella enteriditis* 706 RIVM and *Salmonella typhimurium* ATCC® 14028 bacterial strains with MIC values ranging from 2.5 to 5 µM.[23] Furthermore, a cost-effective production procedure of peptides in bacterial cells has been set up and it has been estimated that, when peptides production scale is 1,000 mg/batch, the production cost is about 42 €/mg, a competitive value for a new compound that has to be introduced in the market.[16] The production procedure has been also scaled-up and found to be suitable to produce the amounts of peptides required for their applicability.[16] Even more importantly, ApoB-derived peptides were found to be able to preserve chicken meat samples from microbial contamination when employed to functionalize chitosan edible films.[24–26] Starting from these very promising results, here we investigated ApoB-derived peptides biosafety as novel food biopreservatives. We demonstrated that peptides are biocompatible, do not induce the development of resistance phenotype upon a prolonged incubation with *Salmonella* cells, and are characterized by a high propensity to be fragmented during simulated digestion. Altogether obtained results open interesting perspectives to the applicability of ApoB-derived peptides as novel biopreservatives to be employed in food industry.

2 | MATERIALS AND METHODS

According to material data sheets, all the necessary precautions for potential safety or environmental hazards were followed.

2.1 | Chemical reagents

All the reagents were purchase from Sigma-Merck (Milan, Italy), unless differently specified. ApoB-derived peptides r(P)ApoB$_S^{Pro}$ PHVALKPGKLKFIIPSPKRPVKLLSG, r(P)ApoB$_L^{Pro}$ PHVALKPGKLKFIIPSPKRPVKLLSGGNTLHLVSTTKT, and r(P)ApoB$_L^{Ala}$ PHVALKAGKLKFIIPSPKRPVKLLSGGNTLHLVSTTKT were recombinantly produced in bacterial cells as previously described.[17]

2.2 | Cell cultures and their authentication

Human gastric carcinoma cells SNU-1 (ATCC® CRL-5971™) and human colon-rectal adenocarcinoma cells HT-29 (ATCC® HTB-38™) were purchased from American Type Culture Collection (ATCC) that validated them via short tandem repeat (STR) profiling. Cells were cultured in the laboratory for less than 6 months and were routinely tested for mycoplasma by MycoSEQ™ Mycoplasma Detection Kit (Applied Biosystems™, Thermo Fisher Scientific, Milan, Italy). Adherent human HT-29 cells were

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cultured in Dulbecco's modified Eagle's medium, whereas human SN-1 cells were grown in suspension in RPMI-1640 medium. In both cases, media were supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Lifescience, Chicago, IL) and antibiotics, and cells were grown in a 5% CO_2 humidified atmosphere at 37°C.

2.3 | Cytocompatibility assays

Cytocompatibility of r(P)ApoB$_L^{Pro}$, r(P)ApoB$_S^{Pro}$ and r(P)ApoB$_L^{Ala}$ has been evaluated by performing experiments on human HT-29 and SNU-1 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[27] To this purpose, HT-29 and SNU-1 cells were plated into 96-well plates at a density of 5×10^3 cells/well in 100 μL of medium, and incubated overnight at 37°C. Afterwards, cells were treated with increasing concentrations (0–20 μM) of ApoB-derived peptides for 24 and 48 h at 37°C.

Following incubation, in the case of HT-29 cells, the peptide-containing medium was removed, and 100 μL of MTT reagent, dissolved in DMEM without phenol red, were added to the cells (100 μL/well) at a final concentration of 0.5 mg/mL. After 4 h at 37 °C, culture medium was removed, and the resulting formazan salts were dissolved by the addition of isopropanol containing 0.1 N HCl (100 μL/well). Absorbance values of blue formazan were determined at 570 nm by using an automatic plate reader (Microbeta Wallac 1420, Perkin Elmer). In the case of SNU-1 cell line, instead, upon treatment with peptides, plates were centrifuged at 1000 rpm for 10 min. Following incubation, 50 μL of MTT solubilized in DMEM without phenol red were added to the cells. Upon 4 h at 37°C, DMSO was added to each sample in a ratio of 1:5 (vol/vol) and samples were incubated for 24 h at room temperature. Absorbance values of blue formazan were determined at 570 nm by using an automatic plate reader (Microbeta Wallac 1420, Perkin Elmer). In all the cases, cell survival was expressed as the percentage of viable cells in the presence of the peptides with respect to control cells grown in the absence of the peptides. The experiments have been carried out in triplicate with triplicate determinations.

2.4 | Bacterial strains and growth conditions

Bacterial strains *Salmonella typhimurium* ATCC® 14028 and *Salmonella enteritidis* RIVM 706 were grown in Muller Hinton Broth (MHB, Becton Dickinson Difco, Franklin Lakes, NJ) and on Tryptic Soy Agar (TSA; Oxoid Ltd., Hampshire, UK). In all the experiments, bacteria were inoculated and grown overnight in MHB at 37°C. After about 24 h, bacteria were transferred into a fresh MHB tube and grown to mid-logarithmic phase.

2.5 | Evaluation of resistance development in *Salmonella* strains

To verify whether *Salmonella typhimurium* (ATCC® 14028) and *Salmonella enteritidis* RIVM 706 bacterial cells develop resistance to r(P)ApoB$_L^{Pro}$ or to conventional antibiotic ciprofloxacin, first of all MIC_{100} values of the antimicrobial agents were determined. To this purpose, bacterial cells were grown to mid-logarithmic phase in Nutrient Broth (NB, Difco, Becton Dickinson, Franklin Lakes, NJ) at 37°C. Cells were then diluted to a final concentration of 2×10^6 CFU/mL in 0.5× NB and were plated into 96-well plates in the presence of increasing amounts of the antimicrobials. For each agent under test, starting from a stock solution, two-fold serial dilutions were carried out, accordingly to broth microdilution method.[28] Following an over-night incubation, MIC_{100} values were determined as the lowest drug concentration responsible for no visible bacterial growth. Moreover, for each treatment, the MIC_{100} value was verified by plating the cells on LB agar. Once detected the MIC_{100} values for each antimicrobial compound against the bacterial strains tested, bacterial cells that survived to exposure at a subinhibitory (MIC/2) concentration were regrown and re-exposed to the peptide or the antibiotic. Strains that developed resistance to the compound under test were characterized by higher MIC_{100} values at subsequent passages.

2.6 | In silico analyses to verify ApoB-derived peptides susceptibility to proteases

The prediction of cutting sites for pepsin and trypsin enzymes has been performed by using "Peptide cutter tool" available on ExPASy server as previously described.[29] In addition, PROSPER software was used to perform an in silico prediction of ApoB-derived peptides susceptibility to 24 proteases.[30] Peptide cutter tool was used to generate the scoring function based on the occurrence of aminoacidic consensus sequences. PROSPER, instead, integrates substrate specificity profiles derived from the MEROPS, CutDB and PMAP database to construct trained models for 24 proteases that have at least 40 experimentally verified substrates and include the four major catalytic types (aspartic, cysteine, metallo and serine proteases).[30] The 24 proteases contained in PROSPER dataset are cathepsin K, calpain-1, caspases (−1, −3, −7, −6, −8), matrix metallopeptidase (−2, −9, −3, −7), chymotrypsin A, granzyme B (human), elastase-2, cathepsin-G, granzyme B (mouse), thrombin, plasmin, glutamyl peptidase I, furin, signal peptidase I, thylakoidalpeptidase I, signalase, and the HIV-1 retropepsin.

2.7 | Evaluation of ApoB-derived peptides stability in in vitro simulated gastric fluid (SGF) and simulated intestine fluid (SIF)

Evaluation of in vitro stability of ApoB-derived peptides has been performed as previously described[31] with some modifications. Briefly, a total amount of 20 μg of r(P)ApoB$_L^{Pro}$, r(P)ApoB$_S^{Pro}$ or r(P)ApoB$_L^{Ala}$ have been analyzed in 100 μL of simulated gastric fluid (SGF) containing 0.32% (w/v) pepsin and 34 mM NaCl at pH 1.2,[32] and in simulated intestinal fluid (SIF) containing 10 mg/mL trypsin in 50 mM KH_2PO_4 at pH 7.5.[31] Preliminarily to the assay, both SGF and SIF mixtures have been preincubated at 37°C for 3 min in the absence of ApoB-derived peptides.

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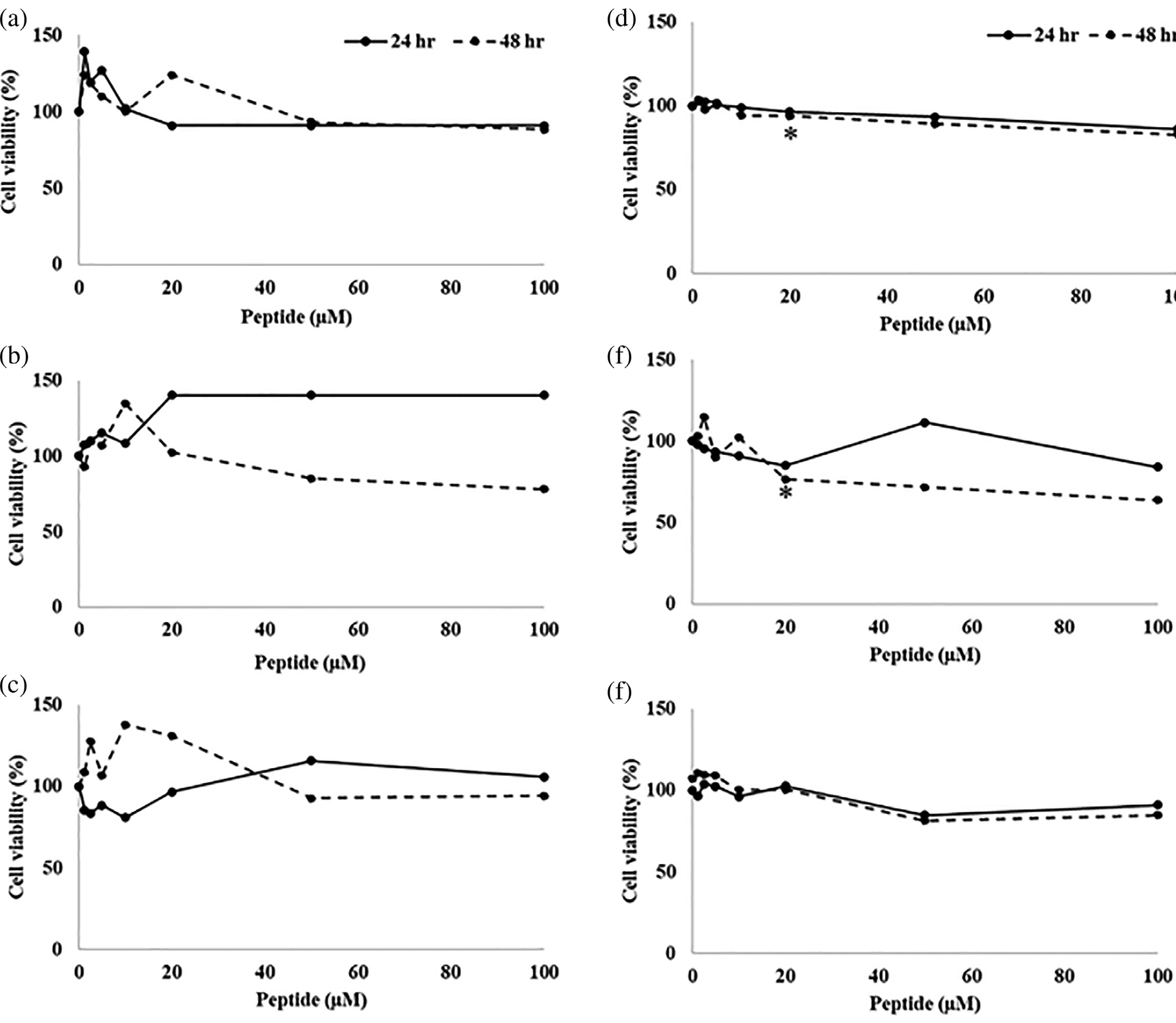

FIGURE 1 Effects of r(P)ApoB$_L$^{Pro} (A, D), r(P)ApoB$_L$^{Ala} (B, E) or r(P)ApoB$_S$^{Pro} (C, F) on the viability of HT-29 (A–C) and SNU-1 (D–F) cell lines. MTT reduction assays were performed by treating cells with increasing concentrations (0–100 μM) of each peptide for 24 and 48 h. Experiments were performed in triplicate with triplicate determinations. Significant differences were indicated as *(P < 0.05) for treated versus untreated samples.

Then, 20 μg of each peptide have been added to SGF or SIF at 1:1 and 1:20 ratio (v/v), respectively. The reaction mixtures have been subsequently incubated at 37°C for 0, 10, 30, 60, 120, and 180 min. Assays have been performed in duplicate for each experimental condition. Reactions have been stopped by adding 200 mM Na_2CO_3 (1:1 v/v ratio) and by heating at 100°C for 3 min. Degradation of full-length peptides r(P)ApoB$_L$^{Pro}, r(P)ApoB$_S$^{Pro} and r(P)ApoB$_L$^{Ala} upon incubation in SGF and SIF have been monitored by SDS-PAGE 18%[33] followed by densitometric analyses. Experiments have been carried out in duplicate.

2.8 | Statistical analyses

Statistical analyses were performed by using Student's t test. Significant differences were indicated as *(P < 0.05), **(P < 0.01) or ***(P < 0.001).

3 | RESULTS

3.1 | Analysis of ApoB-derived peptides cytocompatibility

The employment of ApoB-derived peptides as novel food additives and bio-preservatives is strictly dependent from their cytocompatibility. For this reason, we performed experiments to evaluate peptides' effects on the viability of human colon-rectal adenocarcinoma cells HT-29 and human gastric carcinoma cells SNU-1, selected as prototypes of stomach and intestine targets of peptide deposition during digestion. To this purpose, cells were plated and incubated with increasing concentrations of r(P)ApoB$_L$^{Pro}, r(P)ApoB$_L$^{Ala} or r(P)ApoB$_S$^{Pro} for 24 and 48 h. Interestingly, all the three peptides were found not to affect cell viability (Figure 1). Slight toxic effects were detected

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only upon incubation of SNU-1 cells with r(P)ApoB$_L$^{Ala} peptide at concentrations starting from 20 μM (Figure 1). Altogether obtained results suggest that the peptides are cytocompatible when tested at concentrations known to affect the viability of bacterial *Salmonella* cells (2.5–5 μM).

3.2 | Analysis of resistance evolution upon treatment of bacterial cells with ApoB-derived peptides

Since most of conventional antibiotics employed in food field are responsible for the development of resistance phenotype in foodborne pathogens, we decided to evaluate whether a prolonged exposure of *Salmonella* cells to ApoB-derived peptides is responsible for the insurance of resistance. To this purpose, we treated *S. typhimurium* ATCC® 14028 and the clinically isolated *S. enteritidis* RIVM 706 strains with either r(P)ApoB$_L$^{Pro} peptide or with conventional antibiotic ciprofloxacin for a prolonged time. As shown in Figure 2a,b, upon reiterated treatments with concentration gradients of the above-mentioned antimicrobials, both *Salmonella* strains were found to remain sensitive to r(P)ApoB$_L$^{Pro} peptide antimicrobial activity. Indeed, peptide MIC values were found to remain stable upon treatment of *S. enteritidis* RIVM 706 cells for a prolonged time interval (Figure 2b and Table 1). As shown in Table 1, MIC value detected for r(P)ApoB$_L$^{Pro} peptide in the case of *S. enteritidis* RIVM 706 strain was found to be 5 μM both at time 0 and after 7 rounds of treatment. In the case of *S. typhimurium* ATCC® 14028 strain, only a slight increase of peptide MIC value, which was found to be about 4-fold higher with respect to treatment 1, was observed from treatment 3 to treatment 7 (Figure 2a and Table 1). Indeed, as shown in Table 1, MIC values were found to be 2.5 and 10 μM at time 0 and after 3 rounds of treatment, respectively. However, it has to be highlighted that no further increase of MIC values was detected from treatment 3 to treatment 7 (Figure 2a and Table 1), thus indicating that bacterial cells remain sensitive to peptide toxic effects even if a slightly higher dose of peptide is required to completely inhibit bacterial growth. Resistance development was, instead, detected upon treatment of both *Salmonella* strains with the conventional antibiotic ciprofloxacin. Indeed, as shown in Figure 2, at treatment 3, which corresponds to the third day of treatment with ciprofloxacin, a MIC value 67-fold higher with respect to treatment 1 was detected for both *Salmonella* strains, what is indicative of resistance acquisition. Indeed, as shown in Table 1, in the case of both *Salmonella* strains, ciprofloxacin MIC value was found to increase from 0.3 μg/mL at time 0 to 20 μg/mL after 3 rounds of treatment. In the case of both strains, at treatment 4, it is observed a reversion of resistance development with a MIC value found to be only 4-fold higher with respect to treatment 1. Indeed, at treatment 4, ciprofloxacin MIC value was found to decrease from 20 to 1.25 μg/mL in the case of both *Salmonella* strains. However, it has to be highlighted that, in the case of *S. typhimurium* ATCC® 14028, MIC value was found to further increase at treatment 7 becoming again 20 μg/mL, that is 67-fold higher with respect to treatment 1 (Figure 2a and Table 1), a trend that is indicative of a two-step selection of resistant cells and that

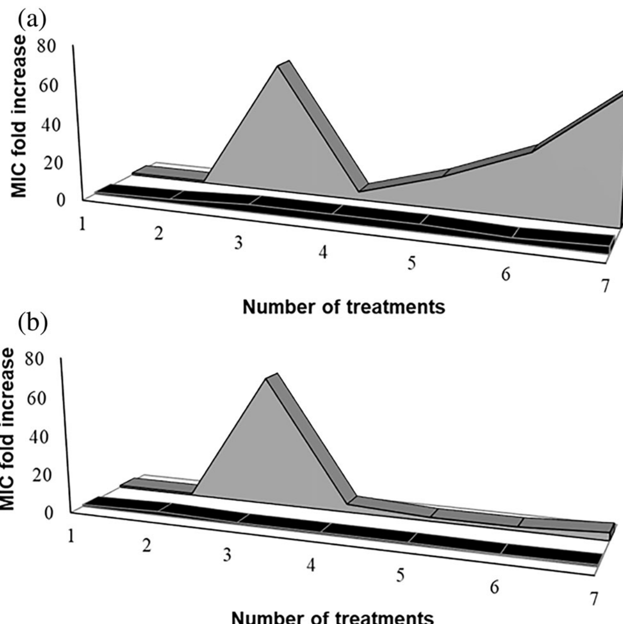

FIGURE 2 Analysis of MIC fold increase upon treatment of *S. typhimurium* ATCC® 14028 (a) or *S. enteritidis* RIVM 706 (b) with r(P)ApoB$_L$^{Pro} (black) or ciprofloxacin (gray) for a prolonged time interval.

TABLE 1 MIC values obtained at time 0 and after several rounds of treatment of bacterial cells with ciprofloxacin or r(P)ApoB$_L$^{Pro} peptide.

	CIPROFLOXACIN				r(P)ApoB$_L$^{Pro}			
	S. Typhimurium ATCC 14028		*S. Enteriditis* 706 RIVM		*S. Typhimurium* ATCC 14028		*S. Enteriditis* 706 RIVM	
Number of treatments	MIC (μg/mL)	Fold increase	MIC (μg/mL)	Fold increase	MIC (μM)	Fold increase	MIC (μM)	Fold increase
1	0.3	1	0.3	1	2.5	1	5	1
2	0.3	1	0.3	1	5	2	5	1
3	20	67	20	67	10	4	5	1
4	1.25	4	1.25	4	10	4	5	1
5	5	17	0.6	2	10	4	5	1
6	10	33	0.6	2	10	4	5	1
7	20	67	1.25	4	10	4	5	1

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indicates the occurrence of different mechanisms of resistance.[34,35] This generally happens when the fitness costs of antibiotic resistance development are not fully tolerated by bacterial cells that try to find alternative ways to overcome antibiotic effects. In the case of *S. enteriditis* RIVM 706, instead, upon reversion of resistance, MIC value was found to remain substantially stable at 1.25 μg/mL from treatment 4 to treatment 7, that is 4-fold higher with respect time 0, thus indicating a more robust reversion of resistance. However, it has to be highlighted that the development of resistance, even if temporary, provides an obstacle to the successful eradication of bacterial infections also considering that a greatly higher dose of effective antibiotic drug is consequently required.

3.3 | In silico analyses to identify cleavage sites in ApoB-derived peptides sequence

In order to evaluate ApoB-derived peptides digestibility in the gastro-intestinal tract, an in silico prediction of peptides proteolysis has been performed as first by using Peptide Cutter software.[29] As shown in Figure 3a,b, pepsin and trypsin cleavage sites have been identified in all the peptides even if a different number of cutting sites has been detected for the three peptides. In particular, r(P)ApoB$_L$^{Pro} peptide was found to contain 9 proteolytic cleavage sites recognized by pepsin enzyme (Figure 3a), whereas an additional site was found to be present in r(P)ApoB$_L$^{Ala} peptide in correspondence of leucine residue preceding the substituted alanine (Figure 3a). Peptide r(P)ApoB$_S$^{Pro}, lacking the last 12 amino acids, was found to contain only 5 cleavage sites recognized by pepsin enzyme. Similar findings have been obtained when trypsin cleavage sites have been analyzed. Indeed, r(P)ApoB$_L$^{Ala} has been found to contain the highest number (6) of trypsin cutting sites, with an additional cleavage site with respect to r(P)ApoB$_L$^{Pro} in correspondence of a lysine residue adjacent to the substituted alanine (Figure 3b). Due to its shorter sequence, also in this case, r(P)ApoB$_S$^{Pro} was found to contain the lowest number (4) of trypsin cleavage sites (Figure 3b). The differences in proteolytic susceptibility between r(P)ApoB$_L$^{Ala} and r(P)ApoB$_L$^{Pro} peptides appear related to the high influence exerted by amino acid residues flanking the cleavage site recognized by pepsin and trypsin enzymes.[36,37]

Starting from these findings, we decided to perform an in silico analysis of ApoB-derived peptides susceptibility to 24 proteases by using PROSPER integrated feature-based server.[30] As shown in Table 2, we found that all the three peptides are susceptible to some of the analyzed proteases comprising cysteine, metallo- and serine proteases. Peptides r(P)ApoB$_L$^{Pro} and r(P)ApoB$_L$^{Ala} have been found to contain overall 7 proteolytic sites recognized by the analyzed proteases. It is interesting to notice that the presence in position 7 of Pro or Ala residue is responsible for a different susceptibility of the peptide molecules to analyzed proteases. Indeed, r(P)ApoB$_L$^{Ala} was found to be susceptible to chymotrypsin A (cattle-type) cleavage (AGKL | KFII) in position 10, whereas r(P)ApoB$_L$^{Pro} was found to be susceptible to matrix metallopeptidase-9 cleavage in position 9 (Table 2). In agreement with its shorter length, r(P)ApoB$_S$^{Pro} peptide was found to contain only 5 sites recognized by the analyzed proteases (Table 2).

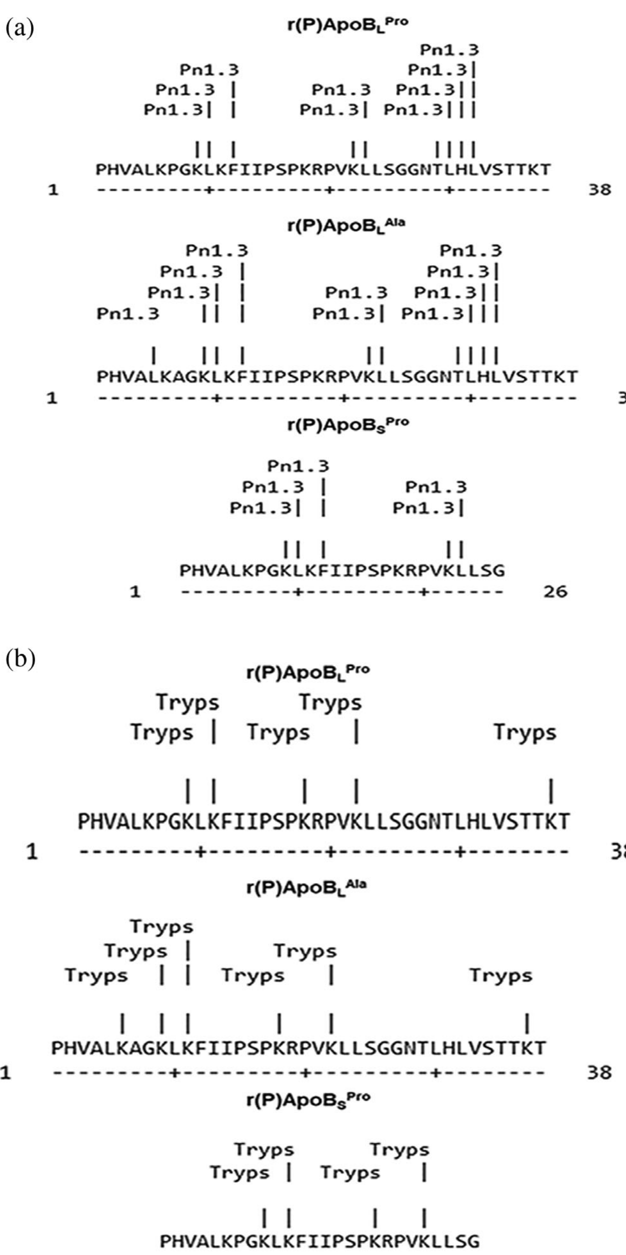

FIGURE 3 In silico identification of pepsin cleavage sites (a) and trypsin cleavage sites (b) in ApoB-derived peptides sequences. Analyses have been performed by using Peptide Cutter tool on ExSPAsy database. The total number and the position of pepsin and trypsin cleavage sites are shown in r(p)ApoB$_L$^{Pro}, r(p)ApoB$_L$^{Ala}, and r(P)ApoB$_S$^{Pro}.

3.4 | Analysis of ApoB-derived peptides in vitro digestion in simulated gastric and intestinal fluids

Allergenic proteins or peptides are generally more resistant to digestion with proteases with respect to nonallergenic ones.[38] Based on this, evaluation of protein stability in the gastro-intestinal tract is considered not only an indication of protein digestibility but also a putative indication of the absence of potential allergenic features.[39] For

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TABLE 2 PROSPER prediction of protease cleavage sites in ApoB-derived peptides.

Protease family	Protease name	Position	P4-P4' site	N-fragment (kDa)	C-fragment (kDa)	Cleavage score[a]
r(P)ApoB$_L^{Pro}$ PHVALKPGKLKFIIPSPKRPVKLLSGGNTLHLVSTTKT						
Cysteine	Cathepsin K	24	VKLL|SGGN	2.77	1.62	1.24
Metallo	Matrix Metallopeptidase-9	9	KPGK|LKFI	1.04	3.35	1.1
	Matrix Metallopeptidase-9	31	NTLH|LVST	3.67	0.73	1.06
	Matrix Metallopeptidase-3	17	IPSP|KRPV	1.93	2.46	1.02
Serine protease	Elastase-2	33	LHLV|STTK	3.88	0.51	1.04
	Elastase-2	21	KRPV|KLLS	2.41	1.98	1.01
	Cathepsin G	24	VKLL|SGGN	2.77	1.62	1.25
r(P)ApoB$_L^{Ala}$ PHVALKAGKLKFIIPSPKRPVKLLSGGNTLHLVSTTKT						
Cysteine	Cathepsin K	24	VKLL|SGGN	2.74	1.62	1.24
Metallo	Matrix Metallopeptidase-9	31	NTLH|LVST	3.64	0.73	1.06
	Matrix Metallopeptidase-3	17	IPSP|KRPV	1.91	2.46	1.02
Serine protease	Chymotrypsin A (cattle-type)	10	AGKL|KFII	1.13	3.24	1.08
	Elastase-2	33	LHLV|STTK	3.85	0.51	1.04
	Elastase-2	21	KRPV|KLLS	2.39	1.98	1.01
	Cathepsin G	24	VKLL|SGGN	2.74	1.62	1.25
r(P)ApoB$_S^{Pro}$ PHVALKPGKLKFIIPSPKRPVKLLSG						
Cysteine	Cathepsin k	24	VKLL|SG	2.77	0.26	1.24
Metallo	Matrix metallopeptidase-9	9	KPGK|LKFI	1.04	1.99	1.1
	Matrix metallopeptidase-3	17	IPSP|KRPV	1.93	1.09	1.02
Serine protease	Elastase-2	21	KRPV|KLLS	2.41	0.61	1.01
	Cathepsin G	24	VKLL|SG	2.77	0.26	1.25

[a]Cleavage score generated by machine learning methods depending on multiple features including local amino acid sequence profile, predicted secondary structure, solvent accessibility and predicted native disorder.

this reason, experiments were here performed to evaluate the stability of ApoB-derived peptides in Simulated Gastric Fluid (SGF) and Simulated Intestine Fluid (SIF)[40] by incubating peptides with pepsin (SGF) or trypsin (SIF) enzyme in 1:1 or 1:20 molar ratio, respectively. Once prepared, the mixtures have been incubated for 0, 10, 30, 60, 120, and 180 min at 37°C and then analyzed by SDS-page (18%) followed by densitometric analysis of full-length peptides bands. As shown in Figure 4a,b r(P)ApoB$_L^{Pro}$ was found to be significantly degraded by pepsin within 10 min of incubation (about 40% protein degradation) (Figure 4a), and degradation was found to be even more pronounced (about 80% protein degradation) upon treatment with trypsin (Figure 4b). Due to its shorter length, peptide r(P)ApoB$_S^{Pro}$ has been found to be more resistant to both pepsin and trypsin enzymes (Figure 4a,b). Only 40%–50% peptide degradation was detected upon incubation with pepsin or trypsin even after 180 min of incubation (Figure 4a,b). In the case of r(P)ApoB$_L^{Ala}$ peptide, about 50% degradation has been detected upon incubation with pepsin (Figure 4a), whereas the peptide was found to be much more sensitive to trypsin, with about 80% degradation detected already upon 10 min of incubation (Figure 4b). This appears in disagreement with previously reported data indicating the presence in r(P)ApoB$_L^{Ala}$ peptide of 6 cleavage sites recognized by trypsin and 10 cleavage sites recognized by pepsin (Figure 3a,b). However, it has to be considered that in SGF and SIF media peptide r(P)ApoB$_L^{Ala}$ might assume local conformations that might interfere with protease cleavage by masking specific sites.

4 | DISCUSSION

4.1 | Host defense peptides as novel food biopreservatives

Host Defense Peptides (HDPs), released upon proteolytic cleavage of precursor proteins, have gained great attention because of their broad-spectrum antimicrobial activity and anti-biofilm properties.[23] Furthermore, despite conventional antibiotics, HDPs general mechanism of action minimizes the rise of resistance phenotype development. This was found to be the case of antimicrobial ApoB-derived peptides that were found to be effective on food-borne pathogens while not inducing the development of resistance in bacterial strains generally causing skin infections.[21,23] ApoB-derived peptides were found to be effective on both *Salmonella enteriditis* 706 RIVM and *Salmonella typhimurium* ATCC® 14028 bacterial strains with MIC values ranging from 2.5 to 5 μM,[23] a value similar to those reported in the literature for different AMPs found to be effective on *Salmonella*

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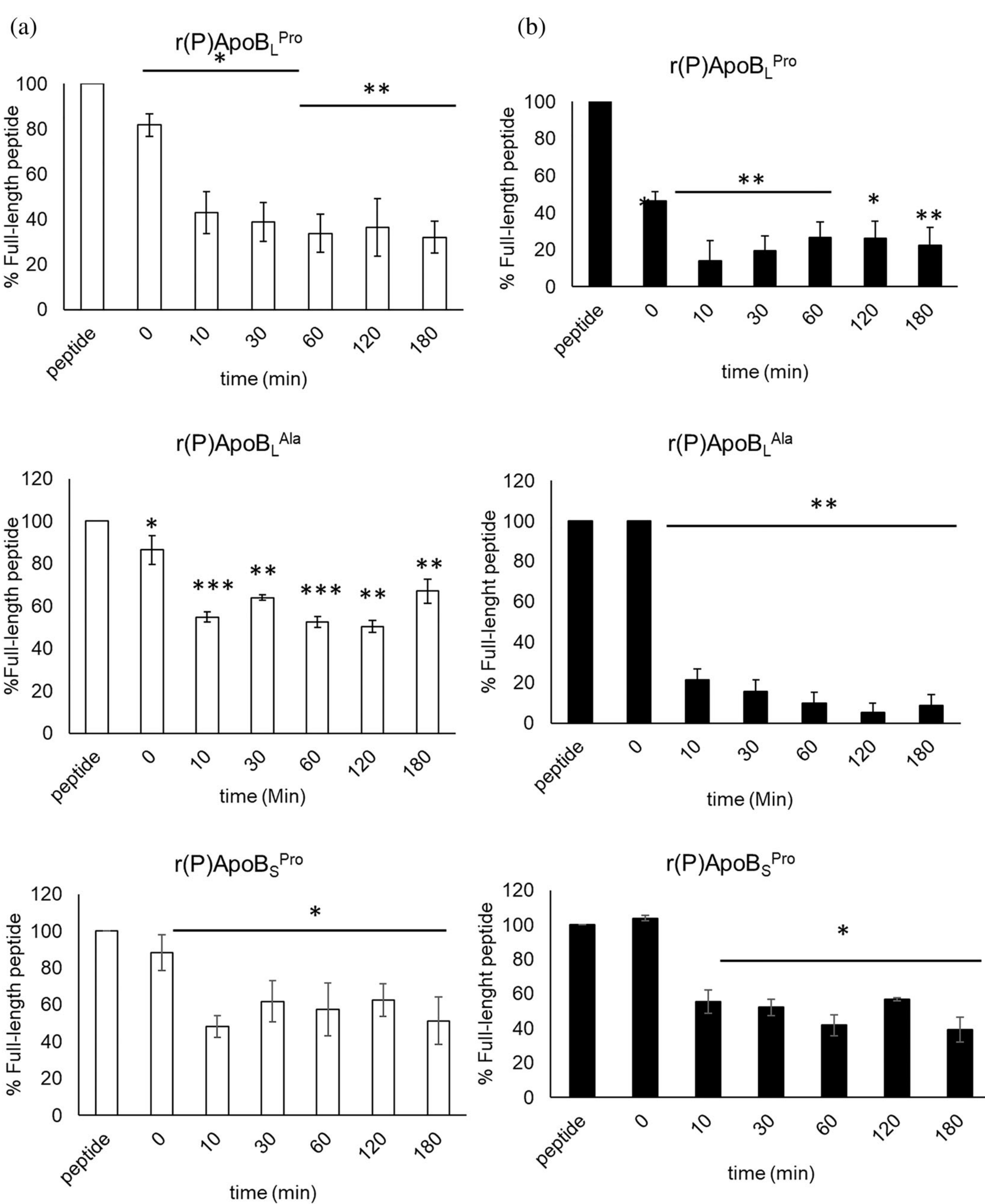

FIGURE 4 Analysis of ApoB-derived peptides hydrolysis in simulated gastric fluid (a, white bars) or in simulated intestine fluid (b, black bars). Graphs have been obtained upon SDS-PAGE and densitometric analyses of the intensity of the bands corresponding to full-length peptides in the absence or in the presence of pepsin (a) or trypsin (b). Experiments were performed in triplicate. Significant differences were indicated as * ($P < 0.05$), **($P < 0.01$) or ***($P < 0.001$) for treated versus untreated samples.

strains and proposed as effective tools to counteract infections caused by multidrug resistant strains.[41] Here, we also demonstrated that a prolonged exposure of *Salmonella* strains to r(P)ApoB$_L$^{Pro} peptide is not responsible for the development of resistance phenotype in contrast with what observed upon treatment with conventional antibiotic ciprofloxacin. ApoB-derived peptides were also found to be cytocompatible, since they were found not to affect the viability of human gastric carcinoma cells SNU-1 and of human colon-rectal adenocarcinoma cells HT-29, thus suggesting that they might not induce toxic effects upon consumption by humans. An example of antimicrobial peptide largely employed as a food preservative is represented by nisin, a peptide endowed with broad activity against Gram-positive bacteria and that was approved for human consumption by both US Food and Drug Administration (FDA) and by the European Food Safety Authority (EFSA) with its assigned E number being E 234.[42] Nisin has been reported to act by inhibiting bacterial growth, by generating pores in cell membrane and by interrupting cell-wall biosynthesis through specific lipid II interaction,[43] a mechanism similar to that reported for ApoB-derived peptides whose main target is represented by bacterial membranes. We demonstrated that

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ApoB-derived peptides antimicrobial activity is dependent from their interaction with specific components of bacterial surfaces, such as LPS or LTA, which induce peptides to form β-sheet-rich amyloid-like structures.[20,44] Starting from these elements, in the future it will be possible to design variants of the antimicrobial peptides under study even more effective in counteracting bacterial infections and in preventing food contamination and spoilage. It has also to be highlighted that ApoB-derived peptides under study present some advantages over peptides conventionally employed in food preservation as nisin. Indeed, it has been widely reported that nisin is less effective in dairy foods with a neutral pH,[45,46] probably due to a lack of peptide stability at neutral pH together with the possible development of resistance phenotype. Nisin stability and activity have been reported to be greatly affected by pH with a maximum of solubility and stability detected at pH 3,[47,48] an aspect that strongly influences nisin applicability. ApoB-derived peptides, instead, were found to exert maximal antimicrobial activity at neutral pH, to remain active at pH 9 an to be affected by acidic pH.[23] This might suggest their applicability and effectiveness to prevent bacterial contaminations in conditions where nisin is less effective. It has been also described the development of resistance to nisin in vitro in *L. monocytogenes*, *Listeria innocua*, *Clostridium botulinum*, *Streptococcus thermophilus*, *S. aureus*, and *S. bovis* strains.[49,50] In the case of ApoB-derived peptides, instead, no resistance development was detected both upon prolonged exposure of *Salmonella* strains to r(P)ApoB$_L^{Pro}$ peptide, as here reported, and upon prolonged exposure of *A. baumannii* ATCC 17878 and *S. epidermidis* ATCC 35984 bacterial cells to r(P)ApoB$_L^{Pro}$, r(P)ApoB$_L^{Ala}$, and r(P)ApoB$_S^{Pro}$ peptides, as previously described.[21] This is a further important aspect evidencing a hypothetical advantage of ApoB-derived peptides over peptides conventionally used for food preservation and that opens interesting perspectives to their future applicability.

4.2 | Evaluation of ApoB-derived peptides digestibility

To perform a further step towards ApoB-derived peptides applicability as food biopreservatives, we also performed experiments to evaluate peptides digestibility. To this purpose, we performed as first an in silico analysis by using Expasy[51] and Prosper[52] tools, a score-based and a feature-based tool, respectively. By using these tools, multiple cleavage sites were predicted in ApoB-derived peptides. In particular, Prosper bioinformatic tool allowed us to analyze peptides susceptibility to 24 proteases comprising Aspartic (A), Cysteine (C), Metallo (M) and Serine (S) proteases from MEROPS database.[53] In order to increase the performance, analyses were performed on the basis of local amino acid sequences predicted secondary structure, solvent accessibility and predicted native disorder.[52] By this way, in each peptide, cleavage sites for five proteases were identified, i.e., cathepsin K (cysteine proteases), metallopeptidase-3 and -9 (metalloproteases), elastase-2 and cathepsin G (serine proteases). These proteases are involved in key physiological processes, such as tissues remodeling (matrix metalloproteases),[54] regulation of bone resorption (cathepsin K largely expressed by osteoclasts),[55] epidermis skin barrier and digestion of extracellular matrix components and ingested bacteria (elastase-2 mainly produced by polymorphonuclear leukocytes and cathepsin G found in azurophil granules of neutrophils).[56–58] In the case of r(P)ApoB$_L^{Ala}$ peptide, it was also identified a cleavage site for chymotrypsin A, a serine protease contributing to the digestion of food proteins in mammalian intestine.[59] Cleavage scores were also generated to evaluate the accuracy of PROSPER prediction and determined values were found to be high for chymotrypsin A (88.5%), elastase-2 (82.9%), matrix metallopeptidase-9 (81.2%), cathepsin G (81.0%), matrix metallopeptidase-3 (79.9%) and cathepsin K (79.6%). To integrate bioinformatic data with experimental evidence, analyses were also performed to simulate the exposure of ApoB-derived peptides to gastrointestinal tract by using Simulated Gastric Fluid (SGF) and Simulated Intestine Fluid (SIF) models. By this way, we demonstrated that r(P)ApoB$_L^{Pro}$ peptide was hydrolysed within 10 min of incubation in both SGF (50% hydrolysis) and SIF (80% hydrolysis), whereas r(P)ApoB$_S^{Pro}$ was degraded after 180 min incubation in SGF (50% hydrolysis) and SIF (50% hydrolysis). r(P)ApoB$_L^{Ala}$ peptide was also found to be highly susceptible to degradation even if with some differences between SIF (50% degradation upon 10 min incubation) and SGC (80% degradation upon 10 min incubation). Similar analyses are reported in the literature for nisin peptide highly used as a food preservative.[60,61] It has been reported that nisin peptide is completely digested only when incubated in the small intestine simulated fluid, whereas, in oral and gastric digestion fluid, only 16% of the peptide is hydrolyzed.[60,61] When computer-aided designed peptides PepGAT and PepKAA have been analyzed in SGF and SIF, PepGAT peptide was found to be readily degraded by pepsin and trypsin, whereas PepKAA was found to be completely degraded by trypsin only after 2 h.[62] Hence, based on literature data, we can affirm that ApoB-derived peptides, once consumed by humans, should be rapidly digested, thus probably avoiding side effects. Even if some allergenic proteins have been found to be quickly degraded in the gastro-intestinal tract, probably because of specific environmental conditions that might alter proteases activity,[29] it has to be highlighted that ApoB-derived peptides are of human origin and have been found to be neither toxic nor haemolytic when tested on eukaryotic mammalian cells.

4.3 | Future perspectives

Based on the preliminary promising findings reported in the present manuscript, further experiments will be performed in the future to deepen on peptides' digestibility and allergenicity. Reported data appear to indicate peptides cytocompatibility, since no toxic effects were detected when peptides were analyzed on stomach and intestinal cell lines under the experimental conditions tested. Furthermore, despite conventional antibiotics, ApoB-derived peptides were found not to induce resistance phenotype even after prolonged incubation with *Salmonella* cells. Preliminary data on peptides digestibility were also collected by performing bioinformatic analyses that revealed the high susceptibility of peptides to several proteases, as also confirmed

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by experiments in simulated gastric and intestinal fluids, thus suggesting peptide fast degradation upon ingestion. Hence, ApoB-derived peptides appear promising candidates to be employed as novel food biopreservatives able to counteract bacterial infections without inducing resistance development. Based on this, future experiments will be performed by incorporating peptides in edible films used to wrap food samples[24,63] and to prevent their contamination.

5 | CONCLUSION

Finding natural antibacterial molecules rather than antibiotics or chemical additives to preserve food samples from contamination is desired to meet consumers' needs. Currently, nisin, produced by microbial fermentation of *Lactococcus lactis*, is the only antimicrobial peptide widely utilized for the preservation of food.[64] However, several antimicrobial peptides have been found to possess features that make them suitable as natural food biopreservatives.[64] Here, we demonstrated that ApoB-derived recombinant peptides are not toxic when tested on stomach and intestinal cell lines and do not induce resistance phenotype upon prolonged incubation with *Salmonella* cells. By bioinformatic analyses, the peptides were also found to be susceptible to several proteases, as confirmed by experiments in simulated gastric and intestinal fluids, indicating that peptides, once consumed by humans, should be rapidly digested, thus probably avoiding side effects. Altogether, these findings open interesting perspectives to the future applicability of ApoB-derived peptides in the food industry and to their employment in combination with other antimicrobial compounds, essential oils and polymeric nanoparticles to enhance the shelf-life of food samples.

AUTHOR CONTRIBUTIONS

Eliana Dell'Olmo: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review & editing. **Katia Pane**: Data curation, investigation, methodology, software, validation, writing – original draft. **Martina Schibeci**: Conceptualization, data curation, formal analysis, investigation, methodology. **Angela Cesaro**: Conceptualization, data curation, formal analysis, investigation, methodology. **Maria De Luca**: Conceptualization, data curation, formal analysis, investigation, methodology. **Shurooq Ismail**: Data curation, formal analysis, investigation, methodology. **Rosa Gaglione**: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing - original draft. **Angela Arciello**: Conceptualization, data curation, formal analysis, validation, funding acquisition, project administration, resources, supervision, writing - original draft, writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

All the used data are contained within the article.

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