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Unlocking the hypolipidemic potential of bioactive peptides derived from probiotic fermented cattle, camel, goat, and sheep milk: a comprehensive investigation through *in vitro*, *in silico*, and molecular docking studies

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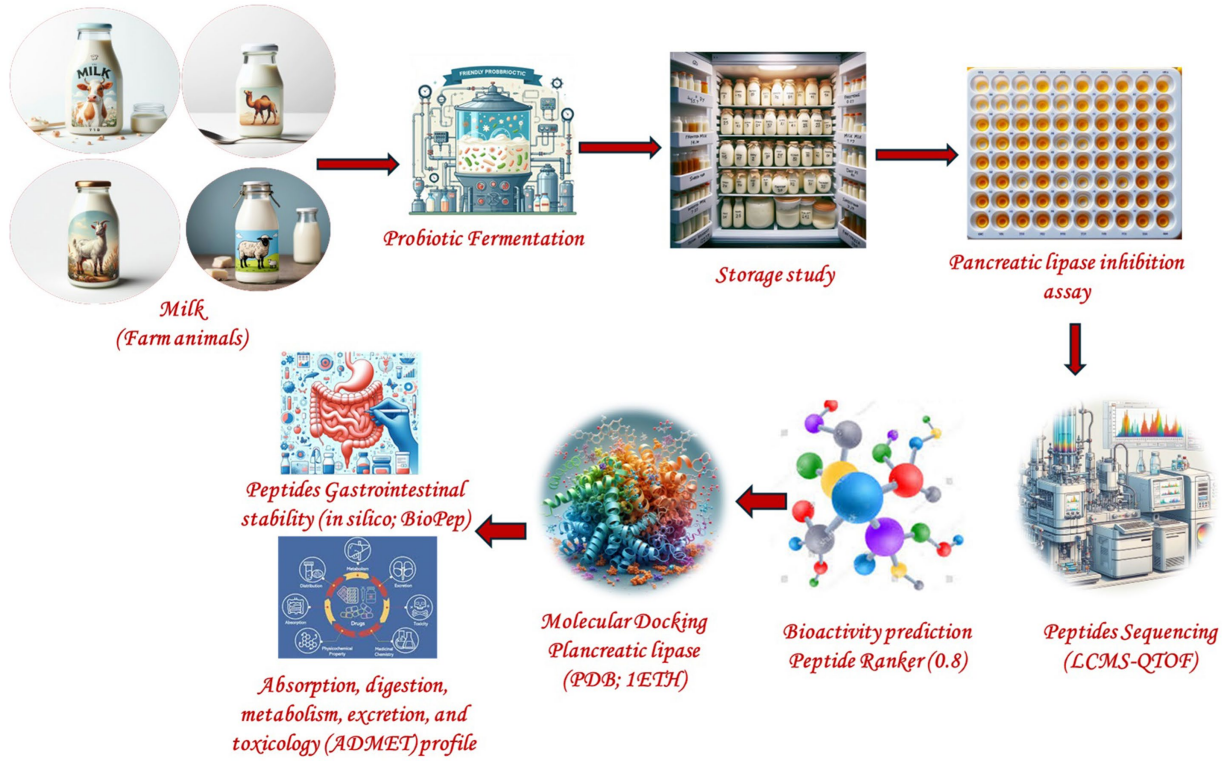
With hyperlipidemia posing a significant cardiovascular risk, innovative strategies are essential to unlock new therapeutic possibilities. Probiotic fermentation of milk proteins offers a natural and effective means to produce peptides with hypolipidemic properties, providing a promising approach to lowering lipid levels and reducing cardiovascular risk. In this study, fermented cattle milk (FCTM), fermented camel milk (FCM), fermented goat milk (FGM), and fermented sheep milk (FSM) were produced using a total of five probiotic bacterial strains to investigate the release of bioactive peptides (BAPs) with hypolipidemic potential via *in vitro* inhibitory activity toward pancreatic lipase (PL) during a 14-day refrigerated storage study. The PL inhibitory activities of these fermented milk (FM) varied according to the types of probiotic strains and milk types used. Overall, the *Pediococcus pentosaceus MF000957 (PP-957)* strain showed the highest PL inhibitory activity spanning across all milk types, and therefore, PP-957-derived fermented samples were analyzed for BAP identification by LCMS-QTOF. The identified BAPs were further analyzed using in silico and bioinformatics approaches for bioactivity prediction, molecular docking, and drug pharmacokinetic studies. Overall, four peptides derived from FCTM, one from FCM, and two peptides common in FGM and FSM were predicted as active PL inhibitors based on their binding energy and number of binding sites on the PL enzyme. All peptides were non-toxic, non-carcinogenic, and had appropriate drug-like properties. The outcomes of this study suggest that FM-derived peptides from animal milk are anticipated to be useful for combating hypercholesterolemia.

KEYWORDS

probiotic fermentation, pancreatic lipase inhibition, milk proteins, dairy animals, bioactive peptides



GRAPHICAL ABSTRACT

1 Introduction

The present world is contending with the escalating epidemic of obesity affecting millions of people, which, if not tackled, will have a long-term detrimental impact (Manzanarez-Quín et al., 2021). In general, obesity is strongly associated with hyperlipidemia, which acts as a risk factor for multiple health complications. Normally, dietary fats consumed in excess need to be hydrolyzed into smaller molecules by lipases in order to be absorbed. Over the past decades, Pancreatic lipase (PL) a carboxylesterase enzyme secreted by pancreatic acinar cells, has become the most important of these enzymes that play a vital digestive function for fat digestion and absorption in the small intestine (Liu et al., 2020; Kumar and Chauhan, 2021). PL is also one of the two key digestive enzymes responsible for triglyceride hydrolysis into glycerol and fatty acids, and the extreme accumulation of hydrolyzed fatty acids in the blood would lead to hyperlipidemia, hence increasing obesity occurrences. Inhibition of lipid metabolizing enzymes, specifically PL, is particularly prominent among the strategies for managing hyperlipidemia, as this could reduce dietary fat absorption and in turn the incidence of obesity (Birari and Bhutani, 2007; Liu et al., 2020; Kumar and Chauhan, 2021).

Using natural PL inhibitory compounds with no or little side effects has been encouraged because synthetic PL inhibitors have presented serious side effects (Birari and Bhutani, 2007; Mudgil et al., 2019). In response to the growing health concerns of consumers, functional foods or natural products are considered a promising approach to reducing obesity and its associated risks since they do not present any negative effects (Sridhar et al., 2019). The potential anti-hyperlipidemia influence of functional foods can act through (i) decreasing the bioavailability of nutrients or inhibition of lipase (Manzanarez-Quín et al., 2021), (ii) stimulation of energy expenditure, (iii) modulation of gut microbiota, or (iv) inhibition of adipocyte proliferation and differentiation (Trigueros et al., 2013; Mohamed et al., 2014). Thorough investigations on the anti-obesogenic effect of plant extracts, other legumes, and edible plants via PL inhibition are available. However, studies on the PL inhibitory potentials of food-derived BAPs are still in their infancy. Some reports do exist on the PL inhibitory activity of peptides from food proteins, including cow and camel casein hydrolysates (Mudgil et al., 2022), amaranth protein hydrolysates (Ajayi et al., 2021), camel whey hydrolysates (Baba et al., 2021), and brewer's spent grain peptides (Garzón et al., 2020).

Fermented dairy products have become one of the most acclaimed raw materials for the development of functional foods due to their broad range of biologically active compounds (García-Burgos et al., 2020). BAPs with diverse activities have been generated during the fermentation of milk proteins with bacterial strains that produce physiologically active metabolites (Granato et al., 2010). Several authors have demonstrated the health-promoting benefits of FM besides their nutritional benefits, and thus they have been considered potential candidates for novel and functional foods to improve health (Khakhariya et al., 2023; Shukla et al., 2023; Pipaliya et al., 2024).

Previous studies have documented that FM contains anti-obesity, hyperlipidemia, and hypercholesterolemia properties (Cheng et al., 2015; Yoda et al., 2015; Pothuraju et al., 2016; Park et al., 2018; Tiss et al., 2020). However, in the literature, there is not enough information about the potential of using different probiotics for releasing peptides with PL inhibitory attributes. Only a few studies have demonstrated the lipase inhibitory activity of FM, which directly

influences the management of obesity and increased body weight (Gil-Rodríguez and Beresford, 2019, 2020, 2021; Pipaliya et al., 2024); however, the identification of peptides that are released due to the fermentation of milk from different farm animals is still not being carried out. This research gap is crucial to be filled through the investigation of the fermentation of milk from different farm animals and their potential to release PL inhibitory peptides. To the best of our knowledge, there is no research available concerning the PL inhibitory potentials of FM sourced from different species using different probiotic species. Therefore, this present study aimed to investigate five different probiotic microorganisms, i.e., *Lactiplantibacillus argentoratensis MF000943 (LA-943)*, *Limosilactobacillus fermentum MF000944 (LF-944)*, *Lactiplantibacillus pentosus MF000946 (LP-946)*, *Pediococcus pentosaceus MF000957 (PP-957)*, *and Enterococcus hirae MF000958 (EH-958)*, for their potential to produce FM from cattle, camel, goat, and sheep milk with high PL inhibitory activities and the impact of 14-day refrigerated storage. In the present study, the hypothesis was that proteins from the milk of individual animal species (cattle, camel, goat, and sheep) might influence their PL inhibitory properties due to their diverse structural, compositional, and genetic variability. Furthermore, different probiotic species can exert and produce a diverse range of BAPs that could demonstrate diverse biochemical properties (Ganatsios et al., 2021). The objective was to demonstrate the application potential of FM from different farm animals in the management of obesity and elucidate its relevance for the research community as well as the industry that is exploring novel functional foods.

2 Materials and methods

2.1 Chemicals, enzymes, and reagents

Enzyme PL (EC 3.4.23.1, source: porcine pancreas; L3126: 100–500 units/mg protein) was purchased from Sigma Aldrich (St. Louis, MO, United States). Chemicals and solvents of analytical grades, including *o*-phthaldialdehyde (OPA), *p*-nitrophenyl butyrate, formic acid, methanol (HPLC grade), acetonitrile (HPLC grade), β-mercaptoethanol, sodium tetra-borate, Trizma base, and SDS, were purchased from Sigma Aldrich (St. Louis, MO). The five probiotic bacterial strains, *Lactiplantibacillus argentoratensis MF000943 (LA-943)*, *Limosilactobacillus fermentum MF000944 (LF-944)*, *Lactiplantibacillus pentosus MF000946 (LP-946)*, *Pediococcus pentosaceus MF000957 (PP-957)*, and *Enterococcus hirae MF000958 (EH-958)*, were previously isolated from raw camel milk in our laboratory (Ahmad et al., 2019). Culture media for microbial growth [De Man, Rogosa, and Sharpe (MRS)] and additional chemicals were purchased from BDH Middle East (Dubai, United Arab Emirates).

2.2 Milk samples collection

Raw cattle (*Holstein Friesian*), camel (*Camelus dromedarius*, local breed), goat, and SM used in this study were procured from local dairy farms located in the Al-Ain region of Abu Dhabi Emirate, UAE. The raw milk samples obtained were transferred to the laboratory under chilled conditions and immediately stored in the refrigerator.

2.3 Probiotic fermentation of milk samples

As explained in our previous publication (Mudgil et al., 2023), FM samples were obtained by inoculating pasteurized skim milk samples (cattle, camel, goat, and sheep) with each probiotic bacterial strain in its early stationary phase at an inoculum level of 5.0 log CFU/mL. The incubation was carried out at 37°C for 24 h under microaerophilic conditions. Thereafter, the batches of FM samples were cooled down to 15°C in ice water and then stored at 4°C for 14-day refrigerated storage with a sampling period of 0, 7, and 14 days. The FM produced from cattle, camels, goats, and sheep were referred to as FCTM, FCM, FGM, and FSM, respectively. Overall, at the end of this storage period experiment, a total of 60 FM were produced, and non-FM samples at each storage period and from each milk type were kept as controls.

2.4 Determination of degree of hydrolysis of milk proteins

The estimation of DH% of milk proteins upon fermentation by various probiotic bacteria was conducted as per the original method of Church et al. (1983) using OPA method with slight modifications as already described in our previous publication (Mudgil et al., 2023). The details of the method can be assessed in Supplementary material S1.1.

2.5 Pancreatic lipase inhibition assay

A method previously described by Baba et al. (2021) was used to analyze PL inhibitory activity. For detailed methodology, please refer to Supplementary material S1.2. The percent PL inhibition was calculated using the equation below:

$$\%PL\ inhibition = \left\{ 1 - \left[\frac{(C-D)}{(A-B)} \right] \right\} X\ 100$$

Here, A is the absorbance of the control (E + Su-Sa), B is the absorbance of the control blank (only Su), C is the absorbance of the test reaction (E + Su + Sa), and D is the absorbance of the sample blank (Su + Sa), respectively. The IC_{50} values were calculated from the slope of the inhibition curve obtained by varying the peptide concentration of the sample.

2.6 Peptide sequencing and identification of bioactive peptides

The amino acid sequences of peptides from FM obtained through PP-957 strain fermentation were analyzed using liquid chromatography-mass spectrometry quadrupole time-of-flight (LCMS-QTOF) system (Agilent, CA, United States) according to the method of Sarah et al. (2016). The detailed methodology has been described in Supplementary material S1.3. The list of peptides was subsequently screened using the Peptide Ranker web server, available at http://distilldeep.ucd.ie/PeptideRanker/. Purposefully, average local confidence (ALC) was set above 80%, and peptides with a pepsite

score above 0.80 were designated as potentially BAPs. Peptides novelty was checked against various databases such as BIOPEP-UWM,[1] EROP-Moscow,[2] PeptideAtlas,[3] and PepBank.[4] Additionally, a flexible peptide–protein docking server, HPEPDOCK 2.0, was used to calculate the docking score of selected peptides with PL enzyme (PBD ID:1ETH) (Zhou et al., 2018) and molecular interactions between each peptide and PL enzyme were explored using pepsite 2.0 web-based server available at http://pepsite2.russelllab.org/ (Trabuco et al., 2012).

2.7 Molecular docking

2.7.1 Preparation of peptides and enzyme structures

Triacylglycerol lipase, a human PL protein (PBD ID:1ETH), was downloaded in its three-dimensional (3D) form from the RCSB database.[5] Any organic matter, attached ligands, and removal of water molecules from enzyme structure were performed by PyMOL software.[6] *De novo* peptide structures were generated using PEPFOLD 3 software[7] (Lamiable et al., 2016). The peptide structures were then optimized for geometrical stability as per the methodology described by Fadimu et al. (2023).

2.7.2 Peptide docking

The molecular interaction between selected FM-derived peptides and 1ETH was then generated using biomolecular interaction web portal high ambiguity-driven protein–protein docking (HADDOCK 2.4) (Honorato et al., 2021), an information-driven flexible docking approach freely available at https://wenmr.science.uu.nl/haddock2.4/. Upon docking, the binding affinity as kcal/mol, molecular interaction, and 2-D visualization were generated to understand peptide structure and activity relationship with lipase enzyme.

2.8 *In silico* analysis of selected peptides

An *in silico* evaluation of the various physicochemical properties such as molecular weight, charge, isoelectric point (Ip), and toxicity was predicted by the online web server ToxinPred, available at http://crdd.osdd.net/raghava/toxinpred/ (Gupta et al., 2013). Solubility predictions were made using http://pepcalc.com/ppc.php, carcinogenicity, and other drug-like properties such as parameters associated with absorption, distribution, metabolism, and excretion (ADME) were carried out using online tools ADMETlab 2.0 and ADMETlab 3.0 available at https://admetmesh.scbdd.com/ and https://admetlab3.scbdd.com/, respectively (Xiong et al., 2021; Fu et al., 2024). Furthermore, selected peptides underwent *in silico* simulated gastrointestinal digestion using enzyme activity for pepsin, trypsin, and chymotrypsin embedded inside BIOPEP-UWM: https://biochemia.uwm.edu.pl/biopep-uwm/ (Minkiewicz et al., 2019) to predict their stability to gastrointestinal digestion.

2.9 Statistical analysis

All FM prepared from five probiotic strains were produced in three batches, representing triplicates. Mean significant differences were calculated using multiple comparison test/two-way ANOVA, and mean significant letters were determined using the compact letter display feature that displays the results of multiple pairwise comparisons embedded within GraphPad prism 10.2.3 (Boston, MA, United States) at $p < 0.05$.

3 Results and discussion

3.1 Degree of hydrolysis

The DH of FM produced using different probiotic strains was investigated and presented in our previous publications (Mudgil et al., 2023, 2024). In summary, the DH and proteolytic activity of various probiotic bacterial strains in FM were investigated, demonstrating significant variations among strains and milk types ($p < 0.05$). Higher proteolytic activity was observed in FM compared to non-FM, attributed to the strains' ability to effectively hydrolyze milk proteins and release small peptides (Pihlanto et al., 2010). The detailed discussion of these results can be assessed in our previous publications (Mudgil et al., 2023, 2024). In brief, the key findings summary indicated that over the storage period, increased proteolysis was observed, with its peak at 7 and 14 days, regardless of milk type or probiotic strains. Furthermore, among FCTM, LP-946 showed the highest activity after 14 days. In FCM, LF-944 was the most active. EH-958 and LA-943 exhibited higher proteolysis in FGM and FSM, respectively, suggesting substrate specificity in proteolytic behavior. Moreover, a comparative analysis between single strain activity across different milk types showed significant variability with more active hydrolysis of goat milk (GM) proteins. Overall, strain LA-943 was particularly active in GM and SM protein hydrolysis. Similarly, LF-944 also showed a preference for GM proteins over cattle milk (CTM) and SM proteins. In general, all probiotic strains remained active in hydrolyzing proteins throughout the 14 days of refrigerated storage. Interestingly, upon storage, FCTM exhibited higher proteolytic activity compared to FGM, FCM, and FSM. The reported findings are supported by the study of other researchers (Soleymanzadeh et al., 2016; Patel and Hati, 2018) indicating variability in proteolytic activity among lactic acid bacteria due to factors such as enzymatic conditions, cell envelope proteins, and genetic factors. Overall, LP-946 and EH-958 were the most effective strains in protein hydrolysis, particularly in FCTM and FGM, respectively. These insights are crucial for selecting specific probiotic strains for targeted milk fermentation processes.

3.2 Determination of pancreatic lipase inhibitory activities

In the current investigation, we assessed the inhibitory potencies of FM on PL enzymes associated with hyperlipidemia. The PL

1 https://biochemia.uwm.edu.pl/biopep-uwm/
2 http://erop.inbi.ras.ru/
3 https://peptideatlas.org/
4 http://pepbank.mgh.harvard.edu/
5 https://www.rcsb.org/structure/1ETH
6 https://pymol.org/
7 https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/

inhibitory activity of FM obtained using probiotic strains is reported as IC_{50} (protein concentration required to inhibit 50% of the original PL activity) in Figure 1. The IC_{50} inhibitory values demonstrating the FM potencies as PL inhibitors (i.e., lower IC_{50} value infers more potency at inhibiting PL) varied among the strains and milk types. The PL inhibitory activity of non-FM in this study for cattle, camel, goat, and sheep milk was 195.91, 114.82, 106.22, and 119.35 μg/mL, respectively. According to statistical results, the IC_{50} value for non-FM was higher in comparison to the values for FM inoculated with different probiotic strains for all four milk sources at 0, 7, and 14 days of fermentation. Consequently, increased PL inhibitory potency was recorded after the inoculation of probiotic strains, reaching an average of 5-fold increase for FCTM and FCM and a 2-fold increase for FGM and FSM, respectively, at the same fermentation period. This result confirmed the inhibitory potentials of FM against the PL enzymatic marker. The observations from this study were confirmed by a previous study, which already demonstrated that milk fermentation with *Lactobacillus* species maximized their potential to inhibit PL (Gil-Rodríguez and Beresford, 2019).

According to Figure 1, all the strains used in this study significantly decreased the IC_{50} value of FM, which varied at different fermentation periods of 0, 7, and 14 days. It is interesting to note that all the probiotic strains demonstrated high PL inhibitory activities at 0 days. These results suggest that the fermentation in the pre-culture has the potential to produce PL inhibitory peptides, as already demonstrated by Gonzalez-Gonzalez et al. (2011), after ACE-inhibition activity was performed on probiotic FM at 24 h. In FCTM, the *PP-957* strain showed the most potent PL inhibitory activity (IC_{50}: 20.2 μg/mL); however, no significant difference was observed in the IC_{50} values for samples fermented with *LF-944* and *LP-946* strains ($p < 0.05$), and the least potent strain was found to be *EH-958* (IC_{50}: 56.2 μg/mL). Similarly, CM and SM fermented by *PP-957* strain showed significantly higher PL inhibitory activity with lower IC_{50} values of 14.2 and 7.21 μg/mL, respectively. Moreover, it is interesting to note that most of the milk fermented with the *PP-957* strain developed the most potent PL inhibitory activity, except for FGM.

A further progression of the milk fermentation period to 7 days resulted in an imperative increase in PL inhibition ($p < 0.05$). As the fermentation duration increased, the PL inhibitory IC_{50} values of non-fermented and FM decreased, thus exhibiting higher PL inhibitory activity. The highest PL inhibitory activity was obtained in cattle milk fermented with *PP-957* strain (IC_{50}: 8.70 μg/mL at 7 days). On the other hand, camel milk fermented by the *LA-943* strain produced the highest PL inhibitory activity (IC_{50}: 3.01 μg/mL). The IC_{50} value for all GM fractions fermented with *LA-943, LF-944*, *LP-946*, and *PP-957* strains showed no significant difference in the PL inhibitory activities ($p > 0.05$). Unexpectedly, FSM fermented by *PP-957* strain also showed high PL inhibitory activity (IC_{50}: 9.33 μg/

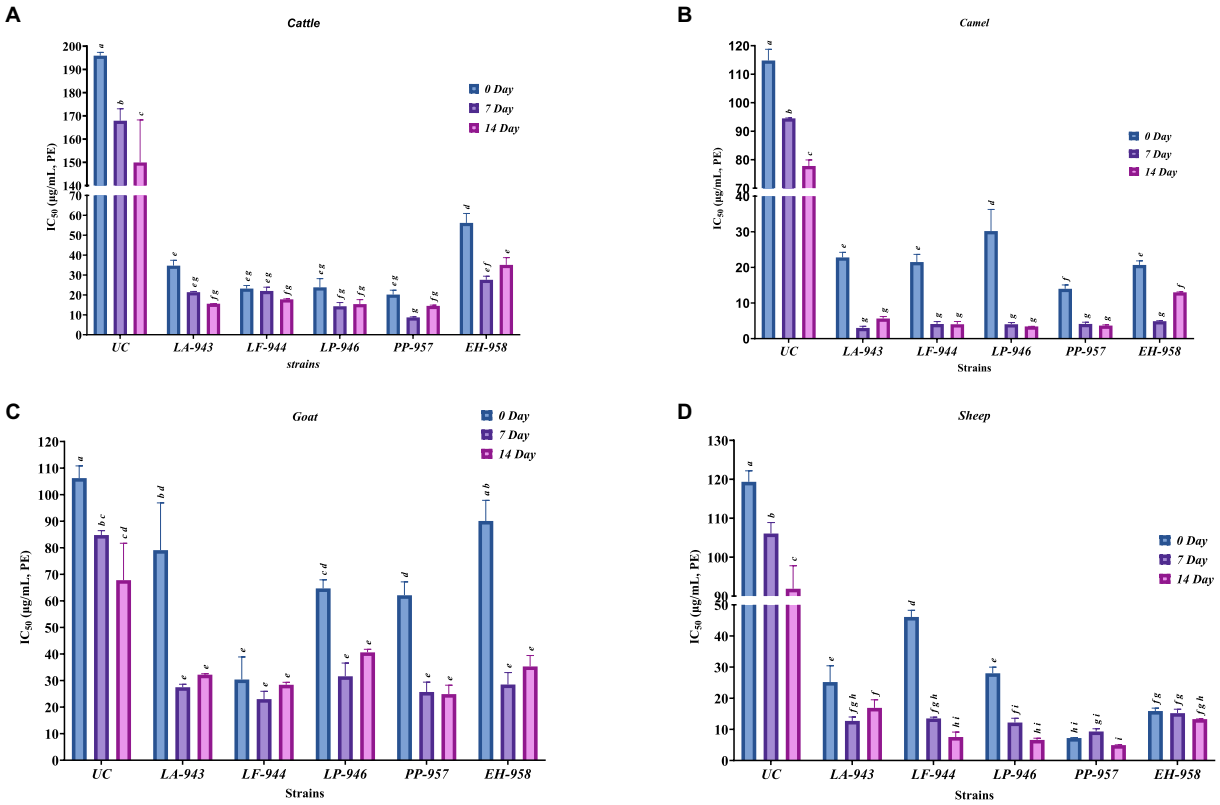


FIGURE 1
Pancreatic lipase IC_{50} inhibitory values (μg/mL) of fermented milk derived from cattle **(A)**, camel **(B)**, goat **(C)**, and sheep **(D)** milk following fermentation with five distinct probiotic microorganisms during refrigerated storage for 14 days. Data represent mean ± SD. Different small alphabets on each bar represent significant differences between the samples. UC, Non-fermented milk; LA-943, *Lactiplantibacillus argentoratensis* MF000943; LF-944, *Limosilactobacillus fermentum* MF000944; LP-946, *Lactiplantibacillus pentosus* MF000946; PP-957, *Pediococcus pentosaceus* MF000957; and EH-958, *Enterococcus hirae* MF000958.

mL). Moreover, the *EH-958* probiotic strain produced FM with the least potent PL inhibitory activity among all the probiotic strains after 7 days of fermentation.

Furthermore, after 14 days of fermentation, the PL inhibitory activity of FM showed varied activities. The *PP-957* strain displayed a similar tendency of higher PL inhibitory activity in all the FM when compared to other strains such as *LF-944* and *LP-946*, while the FM inoculated with *EH-958* exhibited the least potent PL inhibitory activities. It was clear that the PL inhibitory activity recorded among the different FM depends on the probiotic strain type used. From this study, all the probiotic strains exhibited a different ability to produce PL inhibitions during fermentation. This observation was confirmed by previous studies of Gil-Rodríguez and Beresford (2019) and Kinariwala et al. (2020), who reported that the PL inhibitory activity of FM had a greater dependence on the strain of starter cultures. Gonzalez-Gonzalez et al. (2011) reported that strain-dependent potentials might be due to different proteases and peptidase encryption in different bacteria displaying greater specificity for those peptide sequences in FM. Overall, the different proteolytic systems existing in specific bacteria influence their enzyme inhibitory activity.

Although some probiotic strains (e.g., *LA-943* and *LF-944*) showed higher levels of PL inhibition during the fermentation periods, FM produced by *PP-957* showed higher potencies to produce peptides from different milk proteins that could strongly inhibit PL. This could also indicate that this probiotic strain possesses a more potent proteolytic system than that of the other strains used in this study. Interestingly, *P. pentosaceus* strains have been previously reported to influence cholesterol metabolism (Jiang et al., 2021). Other studies have also reported the strong cholesterol-lowering ability of *P. pentosaceus* in both *in vitro* and *in vivo* experiments compared to other LABs (Damodharan et al., 2015; Lim et al., 2019). Taken together, FM produced by the *PP-957* probiotic strain were confirmed to demonstrate more effective PL inhibitory properties, suggesting its superior anti-obesity potential.

Furthermore, here we reported for the first time that the source or nature of milk may also influence the PL inhibitory activity of FM despite being inoculated with the same probiotic strain. As shown in Figure 1, notable significant differences ($p < 0.05$) were observed in the PL inhibitory activity of FM sourced from different farm animals (cattle vs. camel vs. goat vs. SM). FGM exhibited much lower PL inhibitory capacities at 0, 7, and 14 days of fermentation, as indicated by high IC_{50} values. Contrarily, FGM using *L. fermentum* at 0 days showed similar PL inhibitory activity with FCTM and FCM counterparts ($p > 0.05$). Higher PL inhibitory activity was shown in FCM among all the FM samples throughout the fermentation periods. In line with these results, Elayan et al. (2010) have earlier reported the hypocholesterolemic validity of FCM in diet-induced hypercholesterolemic rats. Moreover, all FSM presented superior PL inhibitory activities than their FCTM counterpart. Therefore, according to these observations, inhibition of PL enzyme is not only governed by the strain type but also by the nature or composition of milk. The outcomes strongly suggested that both strains and milk sources are significant factors governing the PL inhibitory activities of peptides derived from FM. Overall, FCM was found to demonstrate higher PL inhibitory activity, irrespective of the type of probiotic strain used.

3.3 Characterization and identification of bioactive peptides with anti-hyperlipidemic activity from milk types

The FM (FCTM, FCM, FGM, and FSM) obtained after fermentation with *PP-957* were subjected to LCMS-QTOF analysis for the characterization of peptide sequences. The interaction of the identified peptide sequences with the PL (1ETH) is presented based on their *p* value, potential binding sites, and docking scores, as shown in Table 1.

According to the Peptide Ranker score (>0.80), a total of 47, 37, 44, and 45 peptides were identified as BAPs in *PP-957*-derived FCTM, FCM, FGM, and FSM, respectively (Supplementary Tables S1–S4). Among these, only 22 FCTM peptides, 14 FCM peptides, 16 FGM peptides, and 10 FSM peptides were identified as potent PL inhibitors with a significance level of <0.05, as presented in Table 1. It is well-known that PL plays a part in the hydrolysis and breakdown of dietary fats, thus yielding fatty acids as end products. The activation of PL occurs at the sites where residues Cys238 and Cys262 with the ability to form surface loops (lid) are removed, hence blocking substrate accessibility to active binding sites (Haque and Prabhu, 2016; Chia et al., 2023). PL has a catalytic triad consisting of six major amino acid residues Ser 153, Asp 177, and His 264, as part of the catalytic triad in the active site, and Phe78, His152, and Phe216 as part of the oxyanion hole that holds the substrate within the active site for hydrolysis. Therefore, binding of peptides with these six amino acids present in catalytic triad or oxyanion hole is of particular interest, as binding with any of these amino acids on PL enzyme could destabilize the transition state intermediates, causing inhibition of the enzyme's hydrolytic action and eventually limiting the hydrolysis and absorption of lipids (Chia et al., 2023).

From the results obtained, all the peptides from all milk types were predicted to be potent PL inhibitors as they could bind to the important above-mentioned residues on the active site of PL. Regarding FCTM, all the identified peptides were found to bind strongly onto 4–5 potential residues of the PL enzyme catalytic triad (i.e., Phe78, His152, Ser153, Phe216, and His264), with docking scores ranging from −109.161 to −215.313 (Table 1). Interestingly, long peptides consisting of 9–24 amino acids (LLYQEPVLGPVRGPFPIIV, LYQEPVLGPVRGPFPIIV, IPNPIGSENSEKTTMPLW, PIGSENSEKTTMPLW, IGSENSEKTTMPLW, SEKTTMPLW, APSFSDIPNPIGSENSEKTTMPLW, and SDIPNPIGSENSEKTTMPLW) in FCTM appeared to be stronger PL inhibitors than short peptides as they significantly ($p < 0.05$) bind all five of the above-mentioned PL enzyme active sites. On the other hand, tetra-peptides (NRAM, NHTW, MMLF, MFSQ, PAAY, and YPPA) bind to four of these PL residues. Moreover, among the identified peptides LLYQEPVLGPVRGPFPIIV, PIGSENSE
KTTMPLW, SDIPNPIGSENSEKTTMPLW, and NRAM were predicted to be the most potent, as they exhibited maximum docking energy with −200.002, −195.614, −215.313, and −192.193, respectively, against PL.

All the significant BAPs ($p < 0.05$) identified in FCM appear as potential PL inhibitors as they could bind 4–5 of these hot spots, except for peptide CQGR, which could not bind to any hot spot residues of PL (Table 1). Among FCM-derived peptides, YDLF was predicted to be a very potent PL inhibitor having the ability to bind onto five significant binding sites (i.e., His264, Phe78, and Ser 153, Asp 177, His152, and

TABLE 1 Bioactive peptides derived from cattle, camel, goat, and sheep milk proteins and their interaction with pancreatic lipase (PL) (1ETH) as elucidated by pepsite 2.0.

Pepsite2 analysis for PL-1ETH					
Milk type	Peptide sequence	*p* value	Bound peptide residues	Bound residues on lipase (1ETH)	HPEPDOCK docking score
Cattle	LLYQEPVLGPVRGPFPIIV	0.03503	Tyr-3, Gln-4, Leu-8, Pro-10, Val-11, Phe-15, Pro-16, Ile-17, Ile-18	Gly77, Phe78*, Asp80, Lys81, Glu84, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Ala179, Pro181, Phe216*, Trp253, Arg257, His264*	−200.002
	LYQEPVLGPVRGPFPIIV	0.03503	Tyr-2, Pro-5, Val-6, Leu-7, Val-10, Phe-14, Pro-15, Ile-16, Ile-17	Gly77, Phe78*, Asp80, Glu84, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, Arg257, His264*	−192.74
	IPNPIGSENSEKTTMPLW	0.04759	Ile-1, Pro-2, Asn-3, Asn-9, Thr-14, Met-15, Pro-16, Leu-17, Trp-18	Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, His264*	−174.616
	PIGSENSEKTTMPLW	0.01934	Pro-1, Ile-2, Gly-3, Thr-11, Met-12, Pro-13, Leu-14, Trp-15	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−195.614
	IGSENSEKTTMPLW	0.01934	Gly-2, Ser-3, Asn-5, Ser-6, Thr-10, Met-11, Pro-12, Leu-13, Trp-14	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−109.161
	SEKTTMPLW	0.04759	Thr-5, Met-6, Pro-7, Leu-8, Trp-9	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−121.145
	APSFSDIPNPIGSENSEKTTMPLW	0.01934	Ala-1, Pro-2, Ser-3, Phe-4, Asn-9, Gly-12, Ser-13, Asn-15, Ser-16, Thr-20, Met-21, Pro-22, Leu-23, Trp-24	Gly77, Phe78*, Lys81, Glu84, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, His264*	−121.956
	SDIPNPIGSENSEKTTMPLW	0.04891	Asn-5, Pro-6, Ile-7, Gly-8, Asn-11, Met-17, Pro-18, Leu-19, Trp-20	Gly77, Phe78*, Lys81, Glu84, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Glu180, Pro181, Phe216*, Trp253, His264*	−215.313
	MMLM	0.006827	Met-1, Met-2, Leu-3, Met-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Ala261, His264*	−147.838
	NRAM	0.02771	Asn-1, Arg-2, Ala-3, Met-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, His264*	−161.827
	MMFL	0.002787	Met-1, Met-2, Phe-3, Leu-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−156.065
	NHTW	0.02983	Asn-1, His-2, Thr-3, Trp-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, His264*	−179.657
	NRAM	0.02771	Asn-1, Arg-2, Ala-3, Met-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, His264*	−192.193
	WRPLN	0.0131	Trp-1, Pro-3, Leu-4, Asn-5	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Glu180, Pro181, Phe216*, His264*	−153.683
	MMLF	0.002787	Met-1, Met-2, Leu-3, Phe-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−175.11
	NRAM	0.02771	Asn-1, Arg-2, Ala-3, Met-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, His264*	−143.319
	MFSQ	0.01303	Met-1, Phe-2, Ser-3, Gln-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, Ala261, His264*	−173.336
	HDHLLF	0.01803	His-1, His-3, Leu-4, Leu-5, Phe-6	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−184.759
	CCVMLNPLW	0.04456	Met-4, Leu-5, Asn-6, Pro-7, Trp-9	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Glu180, Pro181, Phe216*, His264*	−129.242
	PAAY	0.01683	Pro-1, Ala-2, Ala-3	Phe78*, Tyr115, Ser153*, Ala179, Glu180, Pro181, Phe216*, His264*	−150.141
	YPPA	0.002314	Tyr-1, Pro-2, Pro-3, Ala-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Pro181, Phe216*, His264*	−156.769
	MPAAASR	0.02605	Met-1, Pro-2, Ala-3, Ala-5	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−146.06

(Continued)

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TABLE 1 (Continued)

Pepsite2 analysis for PL-1ETH					
Milk type	Peptide sequence	*p* value	Bound peptide residues	Bound residues on lipase (1ETH)	HPEPDOCK docking score
Camel	DVPKTKETIIPK	0.04995	Asp-1, Val-2, Pro-3, Lys-4, Ile-9, Ile-10, Pro-11, Lys-12	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−150.638
	ETIIPK	0.04995	Ile-3, Ile-4, Pro-5, Lys-6	Phe78*, Tyr115, Ser153*, Pro181, Phe216*, His264*	−157.577
	PKLLHPVPQESSF	0.0492	Lys-2, Leu-4, His-5, Pro-6, Val-7, Pro-8, Gln-9, Glu-10, Ser-11	Gly77, Phe78*, Ile79, Tyr115, Ser153*, Leu154, Ala179, Pro181, Phe216*, Ala261, His264*	−158.979
	FMLM	0.002787	Phe-1, Met-2, Leu-3, Met-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−157.147
	YDLF	0.04172	Tyr-1, Asp-2, Leu-3, Phe-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−195.349
	APLY	0.003134	Ala-1, Pro-2, Leu-3, Tyr-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Pro181, Ile210, Phe216*, His264*	−146.803
	MMPY	0.002151	Met-1, Met-2, Pro-3, Tyr-4	Gly77, Phe78*, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−134.714
	CQGR	0.03343	Cys-1, Gln-2, Gly-3, Arg-4	Lys81, Glu84, Trp253	−160.34
	RPPPPVAM	0.04684	Pro-4, Pro-5, Ala-7, Met-8	Phe78*, Tyr115, Ser153*, Pro181, Phe216*, His264*	−176.972
	PMAVY	0.01609	Met-2, Ala-3, Val-4, Tyr-5	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Glu180, Pro181, Phe216*, His264*	−161.346
	QMCNPVPK	0.01885	Gln-1, Met-2, Cys-3, Pro-7, Lys-8	Phe78*, Lys81, Glu84, Tyr115, Ser153*, Phe216*, Trp253, His264*	−97.488
	PTHLW	0.04465	Pro-1, Thr-2, His-3, Trp-5	Phe78*, Tyr115, Ser153*, Phe216*, His264*	−158.567
	FAEAC	0.04561	Phe-1, Ala-2, Ala-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Pro181, Phe216*, His264*	−139.133
	FDELLF	0.02141	Phe-1, Glu-3, Leu-4, Leu-5, Phe-6	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−149.625
Goat	LYQEPVLGPVRGPFPILV	0.03342	Tyr-2, Pro-5, Val-6, Leu-7, Val-10, Phe-14, Pro-15, Ile-16, Leu-17	Gly77, Phe78*, Asp80, Glu84, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Phe216*, Trp253, Arg257, His264*	−170.068
	MPFPK	0.001084	Met-1, Pro-2, Phe-3, Pro-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Phe216*, His264*	−160.65
	IHPFAQAQS	0.02298	Ile-1, His-2, Pro-3, Phe-4, Gln-8	Gly77, Phe78*, Lys81, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, His264*	−209.141
	KIHPFAQAQS	0.00603	Lys-1, Ile-2, His-3, Pro-4, Phe-5, Gln-9	Gly77, Phe78*, Lys81, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, His264*	−189.594
	KPWTQPKTNAIP	0.02128	Lys-1, Trp-3, Thr-4, Pro-6, Lys-7, Thr-8, Asn-9, Ala-10, Ile-11	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Pro181, Phe216*, His264*	−148.891
	DMESTEVFTKK	0.02254	Asp-1, Met-2, Ser-4, Thr-5, Val-7, Phe-8, Thr-9, Lys-10	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Ala261, His264*	−190.132
	TPQH	0.004182	Thr-1, Pro-2, Gln-3, His-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Ala261, His264*	−135.685
	VPEH	0.02401	Val-1, Pro-2, Glu-3, His-4	Phe78*, Tyr115, Ser153*, Pro181, Phe216*	−178.229
	FLDY	0.04172	Phe-1, Leu-2, Asp-3, Tyr-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−158.411

(Continued)

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TABLE 1 (Continued)

Pepsite2 analysis for PL-1ETH					
Milk type	Peptide sequence	*p* value	Bound peptide residues	Bound residues on lipase (1ETH)	HPEPDOCK docking score
	NRAM	0.02771	Asn-1, Arg-2, Ala-3, Met-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, His264*	−135.968
	FMLM	0.002787	Phe-1, Met-2, Leu-3, Met-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−138.547
	MMLF	0.002787	Met-1, Met-2, Leu-3, Phe-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−141.011
	RAPRW	0.02105	Arg-1, Ala-2, Pro-3, Trp-5	Phe78*, Tyr115, Ser153*, Ala179, Glu180, Pro181, Phe216*, His264*	−164.878
	FDVVPK	0.01266	Asp-2, Val-3, Val-4, Pro-5, Lys-6	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Phe216*, His264*	−174.97
	LYQEPVLGPVRGPFPILV	0.03342	Tyr-2, Pro-5, Val-6, Leu-7, Val-10, Phe-14, Pro-15, Ile-16, Leu-17	Gly77, Phe78*, Asp80, Glu84, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Phe216*, Trp253, Arg257, His264*	−170.068
Sheep	MPFPK	0.001084	Met-1, Pro-2, Phe-3, Pro-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Phe216*, His264*	−160.65
	IHPFAQAQS	0.02298	Ile-1, His-2, Pro-3, Phe-4, Gln-8	Gly77, Phe78*, Lys81, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, His264*	−209.141
	KIHPFAQAQS	0.00603	Lys-1, Ile-2, His-3, Pro-4, Phe-5, Gln-9	Gly77, Phe78*, Lys81, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Trp253, His264*	−189.594
	KPWTQPKTNAIP	0.02128	Lys-1, Trp-3, Thr-4, Pro-6, Lys-7, Thr-8, Asn-9, Ala-10, Ile-11	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Pro181, Phe216*, His264*	−148.891
	DMESTEVFTKK	0.02254	Asp-1, Met-2, Ser-4, Thr-5, Val-7, Phe-8, Thr-9, Lys-10	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Ala261, His264*	−190.132
	TPQH	0.004182	Thr-1, Pro-2, Gln-3, His-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, Ala261, His264*	−135.685
	VPEH	0.02401	Val-1, Pro-2, Glu-3, His-4	Phe78*, Tyr115, Ser153*, Pro181, Phe216*	−178.229
	FLDY	0.04172	Phe-1, Leu-2, Asp-3, Tyr-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−158.411
	NRAM	0.02771	Asn-1, Arg-2, Ala-3, Met-4	Phe78*, Tyr115, Ser153*, Leu154, Ala179, Phe216*, His264*	−135.968
	FMLM	0.002787	Phe-1, Met-2, Leu-3, Met-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−138.547
	MMLF	0.002787	Met-1, Met-2, Leu-3, Phe-4	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Pro181, Phe216*, His264*	−141.011
	RAPRW	0.02105	Arg-1, Ala-2, Pro-3, Trp-5	Phe78*, Tyr115, Ser153*, Ala179, Glu180, Pro181, Phe216*, His264*	−164.878
	FDVVPK	0.01266	Asp-2, Val-3, Val-4, Pro-5, Lys-6	Gly77, Phe78*, Tyr115, His152*, Ser153*, Leu154, Gly155, Ala179, Phe216*, His264*	−174.97
	MTPY	0.004759	Met-1, Thr-2, Pro-3, Tyr-4	Phe78*, Tyr115, Ser153*, Pro181, Phe216*	−201.876
	QLALTY	0.02859	Leu-2, Ala-3, Leu-4, Thr-5, Tyr-6	Gly77, Phe78*, Tyr115, Ser153*, Leu154, Ala179, Pro181, Phe216*, His264*	−185.198

*Catalytic hotspots of PL, HPEPDOCK score represents the binding energy.

(Continued)

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TABLE 2 Projected binding energies and molecular interactions as obtained by molecular docking of selected fermentation-derived peptides with PL-1ETH.

Source	Peptide	Binding affinity (kcal/mol)	Binding interaction with PL-1ETH		
			Hydrogen bond	Hydrophobic interaction	Salt bridge
Cattle	LLYQEPVLGPVRGPFPIIV	−9.9	Leu25, Cys182	Val21, Gln22, Arg23, Pro24, Leu25, Ile27, Ile79, Asp80, Arg112, Tyr115, Thr116, Glu180, Pro181, Cys182, Gln184, Ile210, Ile211, Pro212, Phe216*, Val260	-
	LYQEPVLGPVRGPFPIIV	−9.0	-	Val21, Pro24, Phe78*, Asp80, Tyr115, His152*, Ser153*, Ile210, Ile211, Leu214, Phe216*, Phe259, Val260, Ala261, His264*, Leu265	Asp80
	PIGSENSEKTTMPLW	−10.3	Ile79, Asp80, Lys81, Glu84	Asn49, Tyr50, Phe78*, Ile79, Asp80, Lys81, Glu83, Glu84, Asp85, Arg112, Phe216*, Trp253, Arg257, Val260, Ala261, Leu265	Asp85
	SDIPNPIGSENSEKTTMPLW	−11.4	-	Phe78*, Tyr115, Thr116, Pro212, Asn213, Leu214, Phe216*, Cys238, Gln239, Lys240, Ile242, Trp253, Phe259, Val260, Asn263	-
Camel	YDLF	−6.9	His152*	Phe78*, Ile79, Tyr115, His152*, Ser153*, Ile210, Leu214, Phe216*, Val260, Ala261, His264*, Leu265	-
Goat and sheep	IHPFAQAQS	−8.8	-	Phe78*, Ile79, Tyr115, Ser153*, Leu154, Pro181, Ile210, Ile211, Phe216*, Val260, Ala261, His264*, Leu265	-
	DMESTEVFTKK	−10.6	Leu25	Arg23, Pro24, Leu25, Ile27, Phe78*, Ile79, Tyr115, Thr116, Gln120, Ser153*, Ala179, Pro181, Cys182, Ile211, Phe216*, Val260, His264*	-

*Catalytic hotspots of PL-1ETH.

Phe216), as represented by a maximum docking score of −195.349. Baba et al. (2021) have also previously reported YDLF from camel whey protein hydrolysates as an important lipase-inhibiting peptide. Similarly, other peptides such as DVPKTKETIIPK, FMLM, MMPY, PMAVY, and FDELLF were also predicted to be potential PL inhibitors as they showed the ability to bind to five active site residues on PL. Concerning peptides derived from FGM, most of the BAPs identified were predicted to successfully bind to five active sites residues of PL. Five peptides from FGM (KPWTQPKTNAIP, NRAM, RAPRW, FDVVPK, and QLALTY) were able to bind to four active sites, and only two peptides (VPEH and MTPY) could bind three of the amino acid residues in the catalytic triad (Table 1). However, we presumed that peptides IHPFAQAQS and DMESTEVFTKK were qualified as the most effective lipase inhibitors due to their high docking scores of −209.141 and −190.132, respectively, from FGM and their ability to bind to five important PL active sites.

Moreover, it was found that seven peptides (LYQEPVLGPV RGPFPILV, IHPFAQAQS, MSQF, FMPY, FMLF, MMLM, and MMLF) generated from FSM could potentially bind to five hot spot residues on the active site of PL. Nonetheless, other peptides such as KTLVPQ, YTVAFE, and KASW were able to bind four and three of the reported sites, respectively, hence, considered potent lipase inhibitors (Table 1). In this study, peptides LYQEPVLGPVRGPFPILV and IHPFAQAQS, which were previously sequenced in FGM, were also identified in FSM, exhibiting similar binding patterns to the lipolytic sites of PL. Notably, the peptide HPFAQAQS also showed the highest docking scores in both FGM and FSM of −209.141 and −241.724, respectively, indicating satisfactory PL inhibitory activity. In addition, most of the peptides were also found to interact with bound residues of PL (i.e., Ala179, Pro181, Ile210, Ala261, and Try115) apart from binding with the characteristic catalytic triad, suggesting possible stearic inhibition of lipase enzyme (Mudgil et al., 2022). The above-discussed binding tendencies of potent peptides derived from different milk types to PL active sites were also previously reported by Mudgil et al. (2019, 2022) where they reported binding of PL-inhibiting peptides to various active hot spots on PL from enzyme-hydrolyzed cow and camel milk. It was also interesting to observe that despite higher inhibition (lower IC50 values) of PL by FCM, only one peptide (YDLF) met the necessary activity threshold for docking, and this deviation could be attributed to several factors such as synergistic effects, sequence specificity, or peptide length (Shao et al., 2023).

It is thought that the hydrophobicity and differences in the composition of protein's primary structure could influence the variation in PL inhibition demonstrated by BAPs (Urbizo-Reyes et al., 2022). The majority of the selected peptides identified in all the milk types had an exceptionally high content of hydrophobic amino acids, such as proline, leucine, isoleucine, and phenylalanine, which might explain their higher PL inhibitory activity. In fact, the inhibition of lipase by hydrophobic amino acids could be seen in their capacity to bind easily to active sites of lipophilic enzymes. These results were similar to those reported by Baba et al. (2021), who reported the presence of hydrophobic amino acid residues, i.e., leucine and proline, in most of the lipase-inhibiting peptides from camel whey protein hydrolysates. More importantly, the presence of other hydrophobic amino acids such as Met (M) and Tyr (Y), whereas hydrophilic amino acids such as Ser (S) and His (H), is a common feature among lipid-lowering peptides that bind to the active site of lipase enzyme via electrostatic interactions and hydrogen bonding (Martinez-Villaluenga et al., 2010). Therefore, we assumed that both hydrophobic and hydrophilic amino acids present in the isolated

peptides contributed to PL enzyme inhibition. Ajayi et al. (2021) reported peptides with excellent PL inhibitory activity from amaranth protein, which contained hydrophilic amino acid residues. Overall, we found out that the identified peptides exhibited higher binding ability to important catalytic residues and, thus, can be useful for combating hyperlipidemia.

3.4 Molecular docking analysis

The peptides with relatively higher HPEPDOCK docking scores i.e. LLYQEPVLGPVRGPFPIIV, LYQEPVLGPVRGPFPIIV, PIGSENSEKTTMPLW, and SDIPNPIGSENSEKTTMPLW from FCTM, YDLF from FCM, and IHPFAQAQS and DMESTEVFTKK from FGM and FSM, were screened and analyzed Using molecular docking techniques. On the whole, the weakest binding energy of −6.9 kcal/mol was observed for YDLF from FCM and the strongest binding energy of −11.4 kcal/mol for SDIPNPIGSENSEKTTMPLW from FCTM (Table 2). Peptides DMESTEVFTKK from FGM and FSM showed a binding energy of −10.6 kcal/mol, while another peptide from FCTM, i.e., PIGSENSEKTTMPLW, showed a binding energy of −10.3 kcal/mol, indicating strong binding force and, in turn, a robust binding effect. As indicated by Chen et al. (2024), binding energy lower than −7 kcal/mol suggests strong binding affinities. Hence, all these peptides suggest a stronger affinity of binding with PL enzyme, which in turn predict stronger inhibitions. These results indicated that the peptides have strong contacts with the receptor enzyme 1ETH. The visualization of binding poses in their 2-D form is presented in Figure 2, and their molecular interactions are presented in Table 2.

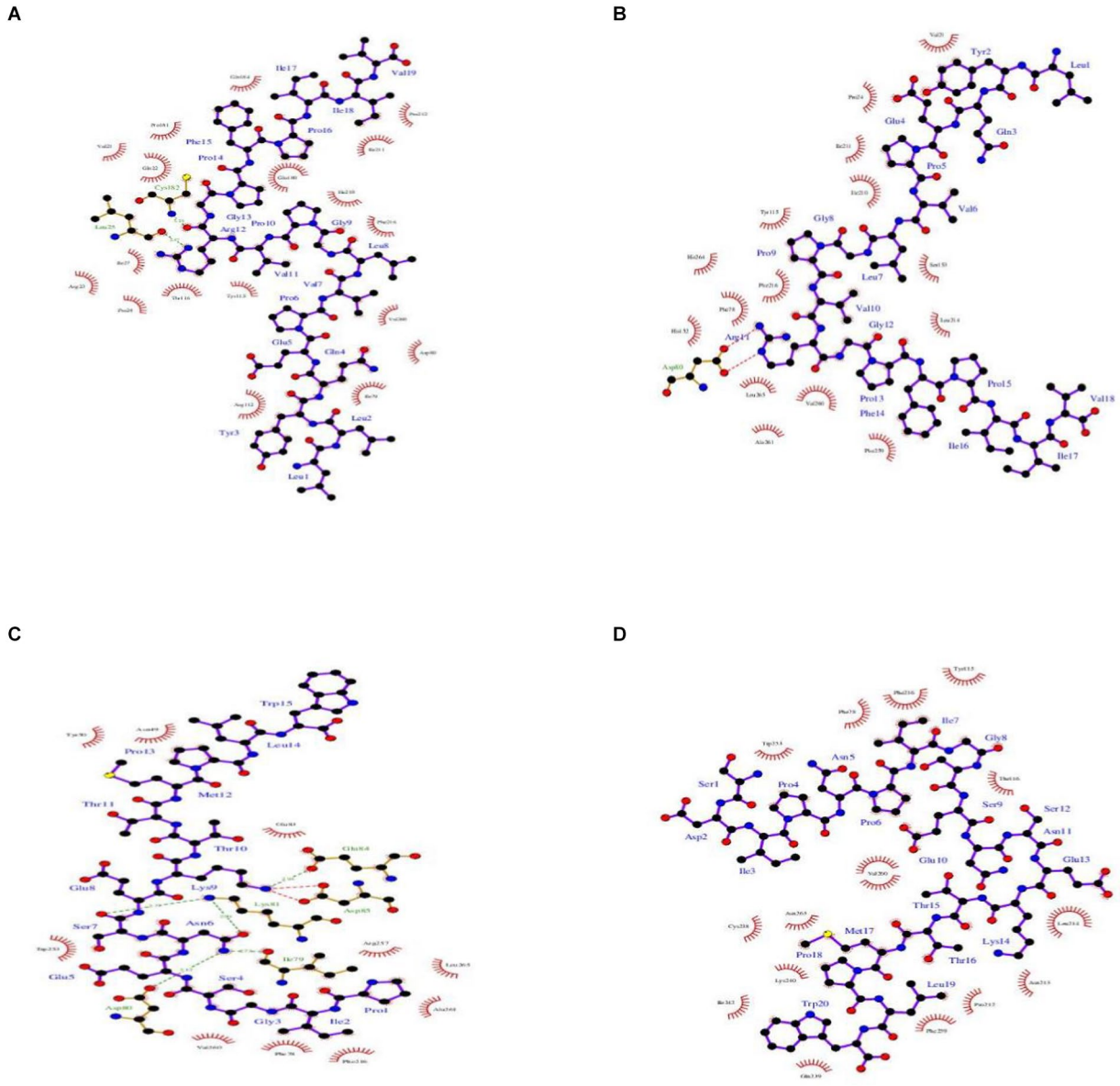

FIGURE 2 (continued)

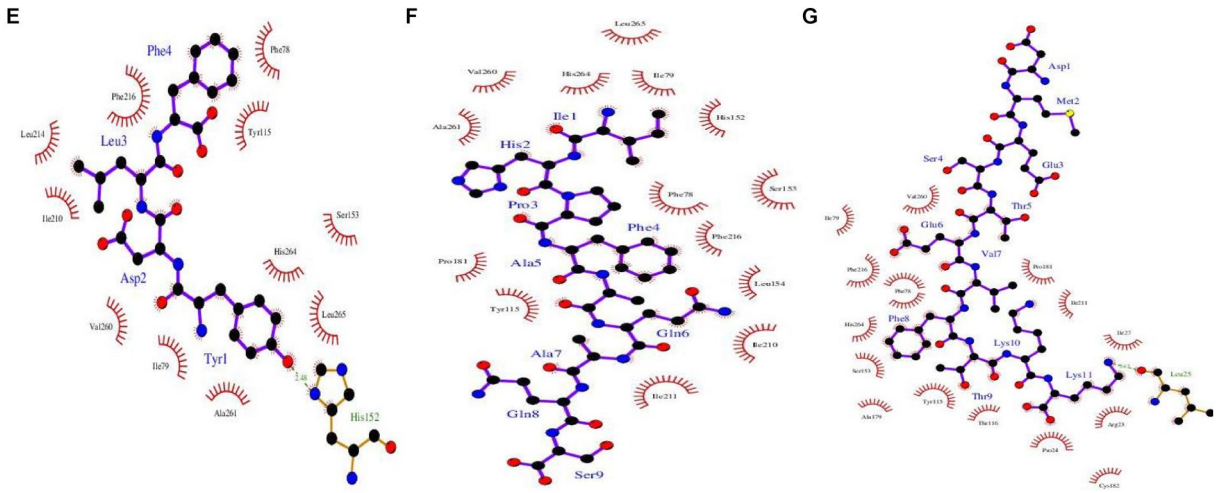

FIGURE 2
Binding poses and interactions between PL-1ETH with **(A)** LLYQEPVLGPVRGPFPIIV, **(B)** LYQEPVLGPVRGPFPIIV, **(C)** PIGSENSEKTTMPLW, **(D)** SDIPNPIGSENSEKTTMPLW, **(E)** YDLF, **(F)** IHPFAQAQS, and **(G)** DMESTEVFTKK. Peptide residues are joined through purple lines, whereas enzyme residues are joined through brown lines. The red, blue, black, and yellow dots indicate oxygen, nitrogen, carbon, and sulfur atoms, respectively. Green dotted line indicates a hydrogen bond, whereas the red dotted line indicates a salt bridge. Brick red eyelashes indicate hydrophobic interaction.

PIGSENSEKTTMPLW formed four hydrogen bonds with the amino acid residues Ile79, Asp80, Lys81, and Glu84 and a salt bridge with amino acid Asp85, whereas, hydrophobic interactions with 13 amino acids of 1ETH protein molecule were demonstrated. Peptides, YDLF showed hydrogen bonding with His152, a part of the oxyanion hole at the active site of PL. Whereas LLYQEPVLGPVRGPFPIIV showed two hydrogen bonds with Leu25 and Cys182, and peptide DMESTEVFTKK showed hydrogen bonding with Leu25. Another salt bridge was observed with Asp80 by LYQEPVLGPVRGPFPIIV peptide. Overall, it was observed that FCM-derived peptide YDLF and FCTM-derived peptide LYQEPVLGPVRGPFPIIV could bind to five of the six important active site residues (Ser 153, His 264, Phe78, His152, and Phe216). Similarly, FGM- and FSM-derived peptides IHPFAQAQS and DMESTEVFTKK could bind to four of these active site residues, indicating that these two peptides strongly interacted with the active cavity of PL, confirming their high hypolipidemic potential. Previous studies have also identified YDLF as a promising anti-obesity peptide; however, other peptides identified in this study have not been reported earlier for their PL inhibitory actions (Baba et al., 2021). Therefore, it can be suggested that probiotic fermentation can be used as an effective strategy for the production of novel anti-obesity peptides with stronger hypolipidemic potential.

3.5 *In silico* analysis of selected peptides

Absorption, distribution, metabolism, and excretion of various compounds, including BAPs, with the likelihood of being used as a nutraceutical and therapeutic agent have been very useful for the prediction of their drug-like characteristics. ADMETlab 2.0 and 3.0 are online prediction tools for pharmacokinetics assessment, including their medicinal chemistry (Xiong et al., 2021; Fu et al., 2024). These tools are an alternative to laborious and expensive experimental methods, along with being cost-effective (Ngo et al., 2023). Various physicochemical characteristics such as molecular weight (MWt); Ip, the logarithm of aqueous solubility value (LogS), the logarithm of the n-octanol/water distribution coefficient [Log P (Crippen method)], the number of hydrogen bond acceptors (HBAs), the number of hydrogen bond donors (HBDs), topological polar surface area (TPSA), human intestinal absorption (HIA), blood–brain barrier (BBB), metabolism as P450 CYP3A4 inhibitor (MB), clearance rate as a measure of excretion, mL/min/kg (CL), drug likeliness (Pfizer rule), toxicity, and carcinogenicity analysis were assessed, and the results are presented in Table 3. As seen in Table 3, all seven peptides that were selected qualified based on their drug likeliness as per the Pfizer rule. Solubility analysis as revealed by Log S and Log P indicated that four peptides, i.e., PIGSENSEKTTMPLW, SDIPNPIGSENSEKTTMPLW from FCTM; YDLF from FCM and DMESTEVFTKK from FGM and FSM, respectively, showed good solubility. Furthermore, as can be seen from LogS (water solubility) values, the values were well within the optimal range of −4 to 0.5 log mol/L described by the server as optimal. However, only two peptides, i.e., LLYQEPVLGPVRGPFPIIV (2.284), SDIPNPIGSENSEKTTMPLW (0.251), and DMESTEVFTKK (2.284), showed an optimal range of LogP values between 0 and 3 log mol/L. TPSA values were found to be higher than the optimal value of 0–140, based on the Veber rule. Similar to the results obtained by Wang et al. (2023), HIA and BBB values in all peptides indicated excellent values between 0 and 0.3. HIA rate is known to significantly influence the bioavailability of bioactive compounds across the intestine; similarly, BBB is crucial for maintaining the stability of the compound in the brain's environment. Both systems permit the passage of selective nutrients and other macromolecules required while blocking harmful substances and eliminating metabolic waste. The possession of higher or recommended values for HIA and BBB indicates superior bioavailability of these peptides and is consistent with those obtained by Wang et al. (2023). Furthermore, based on the drug-like soft rule, all peptides except YDLF showed more than the optimal number of HBA and HBD. The bioavailability radar for these seven peptides,

TABLE 3 *In silico* physicochemical properties and absorption, distribution, metabolism, excretion (ADME) profile of fermented milk-derived bioactive peptides from cattle, camel, goat, and sheep milk.

Source	Peptide sequence	MWt.	Ip	Charge	Solubility	LogS	Log P (Crippen method)	HBA	HBD
Cattle	LLYQEPVLGPVRGPFPIIV	2107.574	6.81	0	Poor	−2.71	2.284	24	22
	LYQEPVLGPVRGPFPIIV	1994.414	6.81	0	Poor	−1.823	−3.132	23	21
	PIGSENSEKTTMPLW	1689.91	4.15	−1	Good	−1.471	−4.183	25	24
	SDIPNPIGSENSEKTTMPLW	2216.457	3.69	−2	Good	−1.939	0.251	33	30
Camel	YDLF	556.616	0.76	−1	Good	−1.715	−2.762	7	7
Goat and sheep	IHPFAQAQS	998.109	7.82	0.1	Poor	−0.833	−4.087	14	13
	DMESTEVFTKK	1314.481	4.32	−1	Good	−2.71	2.284	21	20
Orlistat		495.73	nd	nd	Good	−7.053	8.817	6	1
	Peptide sequence	**TPSA**	**BBB**	**HIA**	**MB**	**CL**	**Toxicity prediction**	**Drug likeness (Pfizer rule)**	**Carcinogenicity**
Cattle	LLYQEPVLGPVRGPFPIIV	714.48	0.038	0.221	0.885	−0.878	Non-Toxin	Accepted	Nil
	LYQEPVLGPVRGPFPIIV	685.38	0.038	0.221	0.885	−0.918	Non-Toxin	Accepted	Nil
	PIGSENSEKTTMPLW	688.36	0.032	0.24	0.9	−1.061	Non-Toxin	Accepted	Nil
	SDIPNPIGSENSEKTTMPLW	930.89	0.038	0.24	0.907	−1.061	Non-Toxin	Accepted	Nil
Camel	YDLF	208.15	0.69	0.412	0.303	0.924	Non-Toxin	Accepted	Nil
Goat and sheep	IHPFAQAQS	422.42	0.039	0.244	0.849	0.429	Non-Toxin	Accepted	Nil
	DMESTEVFTKK	578.95	0.145	0.293	0.721	−0.968	Non-Toxin	Accepted	Nil
Orlistat		81.7	0.0002	0.996	0.998	4.847	Non-Toxin	Accepted	Nil

MWt, Molecular weight; Ip, Isoelectric point; LogS, The logarithm of aqueous solubility value; Log P (Crippen method), The logarithm of the n-octanol/water distribution coefficient; HBA, Number of hydrogen bond acceptors; HBD, Number of hydrogen bond donors; TPSA, Topological polar surface area; HIA, Human intestinal absorption; BBB, Blood–brain barrier; MB, Metabolism as P450 CYP3A4 inhibitor, CL, Clearance rate; mL/min/kg.

TABLE 4 Peptides stability upon *in silico* gastrointestinal digestion as predicted through *BIOPEP-UWM*.

Source	Peptide	Fragmentation by gastric enzyme	Fragmentation by gastrointestinal enzymes	DHt [%]
Cattle	LLYQEPVLGPVRGPFPIIV	L - L - YQEPVL - GPVRGPF - PIIV	L - L - Y - QEPVL - GPVR - GPF - PIIV	33.33
	LYQEPVLGPVRGPFPIIV	L - YQEPVL - GPVRGPF - PIIV	L - Y - QEPVL - GPVR - GPF - PIIV	29.41
	PIGSENSEKTTMPLW	PIGSENSEKTTMPL - W	PIGSEN - SEK - TTM - PL - W	28.57
	SDIPNPIGSENSEKTTMPLW	SDIPNPIGSENSEKTTMPL - W	SDIPN - PIGSEN - SEK - TTM - PL - W	26.32
Camel	YDLF	YDL - F	Y - DL -F	66.67
Goat and Sheep	IHPFAQAQS	IHPF - AQAQS	IH - PF - AQAQS	25.00
	DMESTEVFTKK	DMESTEVF - TKK	DM - ESTEVF - TK - K	30.00

DH_t, Theoretical degree of hydrolysis; Gastric enzyme-pepsin EC 3.4.23.1; Gastrointestinal enzyme-pepsin EC 3.4.23.1; Chymotrypsin (A) EC 3.4.21.1; and Trypsin EC 3.4.21.4.

along with commercial drug orlistat, is shown in Supplementary Figure S1.

Further computational methods, such as BioPep UWM, were used, which provide insights into the stability of BAPs under a simulated gastrointestinal digestive environment, together with providing in-depth information into degradation sites, products generated, their sequence, and composition. The results obtained from *in silico* hydrolysis of selected seven peptides and their possible fragments upon gastric and gastrointestinal digestion are presented in Table 4. DH_t values showed that peptide YDLF showed the highest value of 66.67%, followed by LLYQEPVLGPVRGPFPIIV with a DH_t of 33.33%. The rest of the five peptides showed DH_t values in the range of 26.32–30.00%. Overall, all peptides showed hydrolysis under optimum

TABLE 5 Absorption, distribution, drug likeliness metabolism, excretion (ADME), and drug likeliness profile of after *in silico* gastrointestinal digestion through BIOPEP-UWM.

Source	Active fragment sequence	Peptide ranker score	Lipophilicity	Drug Likeliness	Pharmacokinetics				Carcinogenicity	Drug likeness by Pfizer rule
			Log P Crippen method	Bioavailability score	HIA	BBB	MB	CL		
Cattle	QEPVL	0.19	−2.152	0.11	0.938	0.067	0.01	2.131	0.073	Accepted
	GPVR	0.48	−2.471	0.17	0.537	0.281	0.005	1.979	0.035	Accepted
	GPF	0.98	−0.748	0.55	0.844	0.715	0.014	2.493	0.032	Accepted
	PIIV	0.23	1.078	0.55	0.8	0.07	0.018	3.058	0.161	Accepted
	PIGSEN	0.14	−3.644	0.11	0.929	0.114	0.002	1.612	0.217	Accepted
	SEK	0.04	−3.876	0.17	0.918	0.081	0.006	1.877	0.03	Accepted
	TTM	0.13	−1.769	0.55	0.028	0.212	0.005	1.679	0.038	Accepted
	PL	0.81	−0.706	0.55	0.244	0.262	0.011	3.53	0.133	Accepted
	SDIPN	0.39	−3.035	0.11	0.841	0.119	0.003	1.817	0.117	Accepted
Camel	DL	0.32	−1.859	0.56	0.02	0.28	0.017	4.688	0.058	Accepted
Goat and Sheep	IH	0.21	−1.108	0.55	0.017	0.923	0.184	3.538	0.017	Accepted
	PF	0.99	−0.951	0.55	0.431	0.377	0.013	2.751	0.252	Accepted
	AQAQS	0.08	−3.791	0.17	0.03	0.238	0.001	1.645	0.026	Accepted
	DM	0.6	−2.52	0.11	0.016	0.188	0.011	3.272	0.154	Accepted
	ESTEVF	0.11	−2.646	0.11	0.922	0.066	0.007	1.572	0.27	Accepted
	TK	0.03	−3.48	0.55	0.441	0.237	0.004	3.707	0.132	Accepted

Log P (Crippen method), the logarithm of the n-octanol/water distribution coefficient; HBA, Number of hydrogen bond acceptors; HBD, Number of hydrogen bond donors; TPSA, Topological polar surface area; HIA, Human intestinal absorption; BBB, Blood–brain barrier; MB, Metabolism as P450 CYP3A4 inhibitor, CL, Clearance rate; mL/min/k.

conditions of enzymatic action. The higher stability of peptide PIGSENSEKTTMPLW to digestion could be attributed to the presence of proline residues at the C-terminal and N-terminal, which is known to provide resistance to hydrolysis. The results are in accordance with those suggested by other researchers (Nielsen et al., 2017; Wang et al., 2023). Results as obtained from ADMET analysis for peptides obtained after *in silico* digestion are shown in the Supplementary material (Table 5). The bioactivity scoring for these hydrolyzed fragments using Peptide Ranker showed that only three peptides' fractions (GPF, PL, and PF) showed higher values for bioactivity than the threshold of 0.8, with peptide ranking scores of 0.98, 0.81, and 0.99, respectively. These results indicated that these seven peptides, even after digestion, could produce BAPs that can further exert biological function even after digestion. Overall, the in-depth *in silico* analysis of the peptides provided valuable insights into the drug-like characteristics and bioavailability of the seven selected peptides, with several showing promising solubility and bioactivity post-digestion. These findings also underscore the potential of these peptides as effective nutraceuticals and therapeutic agents.

4 Conclusion

The findings from the present study suggested that the five different probiotic strains used for producing FM from cattle, camel, goat, and SM resulted in the generation of BAPs with hypercholesterolemic activity. FM produced by *Pediococcus pentosaceus MF000957 (PP-957)* probiotic strain was most effective in inhibiting PL, which appeared to be strain-dependent and substrate-dependent. Novel peptides identified from the different milk types have shown their ability to inhibit PL activities due to their structural features, hydrophobic amino acid compositions, interactions with catalytic residues, and their ability to bind to important PL active sites. However, more specific studies are needed to establish their mechanisms of action and efficacy. Importantly, most of these sequences have not yet been reported, suggesting that fermentation-derived peptides have the potential to produce new types of PL inhibitory peptides for preventing or reducing hyperlipidemia. Moreover, further studies can be undertaken for in-depth *in vivo* investigations into these selected peptides.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PM: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal

analysis, Data curation, Conceptualization. FA: Writing – review & editing, Writing – original draft, Methodology, Investigation. AA: Writing – original draft, Methodology, Investigation. P-GY: Writing – review & editing, Software, Methodology, Investigation. C-YG: Writing – review & editing, Supervision, Software, Methodology, Investigation, Formal analysis. SM: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Formal analysis, Data curation, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2024.1443708/full#supplementary-material

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