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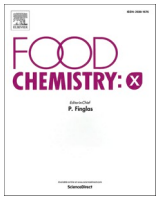

Assessment of hypolipidemic potential of cholesteryl esterase inhibitory peptides in different probiotic fermented milk through in vitro, *in silico*, and molecular docking studies

Feyisola Fisayo Ajayi [a], Fatimah AlShebli [a], Pei-Gee Yap [b], Chee-Yuen Gan [b], Sajid Maqsood [a,c,**], Priti Mudgil [a,*]

[a] Department of Food Science, College of Agriculture and Veterinary Medicine, United Arab Emirates University, Al-Ain 15551, United Arab Emirates
[b] Analytical Biochemistry Research Centre (ABrC), Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
[c] Zayed Centre of Health Science, United Arab Emirates University, Al-Ain 15551, United Arab Emirates

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ABSTRACT

Fermented milk (FM) is well-known to confer health-promoting benefits, particularly for managing chronic metabolic diseases. However, the specific cholesterol esterase (CE) inhibitory activities of FM produced from different animal milk sources have not been extensively explored. This study for the first time investigates the CE inhibition potential of FM derived from bovine (F_BM), camel (F_CM), sheep (F_SM), and goat milk (F_GM), each fermented with five different probiotic strains and stored for 14 days under refrigeration. Further, peptides identification was performed and *in silico* approaches were used to dock potent peptides with CE enzyme (PDB: 1AQL) to decipher mechanism of enzyme inhibition. Comprehensive approach of this study combined CE inhibition assays, peptide identification, and *in silico* molecular docking with the CE enzyme (PDB: 1AQL) to elucidate mechanisms underlying enzyme inhibition. Upon fermentation improvements in CE-inhibition (lower IC_{50} values) were observed compared to non-fermented counterparts. Moreover, the CE-inhibition potency of the FM varies significantly among the milk types and probiotic strain ($p < 0.05$). Regardless of probiotic strains, CE-inhibition was more evident for F_GM followed by F_CM. Peptide sequencing and molecular docking studies revealed APSFSDIPNPIGSENSEKTTMPLW from F_BM showed potent binding to CE's active site, while peptides from F_CM, F_SM, and F_GM showed indirect CE-inhibitory mechanisms. These findings suggest potential anti-hypercholesteremic effects of bovine and non-bovine fermented milk, indicating their potential use in developing novel dairy products with hypolipidemic activities.

1. Introduction

In the past decade, fermented dairy products have attracted more attention on the account of their nutritional and health promoting properties. Fermented milk products have increasingly received consumer's popularity as a functional food owing to their vast contribution towards combating the incidence of metabolic diseases. Milk fermentation by lactic acid bacteria strains under controlled conditions is the most practical and economical way of modifying milk beyond traditional nutrients to become "functional food". In general, the release of peptides to confer several bioactivities originates from the activity of proteolytic starter bacteria to hydrolyze milk proteins (caseins and whey) and release peptides encrypted in the proteins (Nguyen et al., 2020).

Lactic acid bacteria (LAB), a significant group of probiotic organisms are the most extensively used organisms in the fermentation of dairy products (Colombo et al., 2018). In the food industry, genera *Lactobacillus, Streptococcus, Lactococcus, Pediococcus, Bifidobacterium,* and *Leuconostoc* are most utilized. The proteolytic system of LAB is associated with the degradation of milk proteins and synthesis of essential amino acids (AAs) required for their cell growth (Kieliszek et al., 2021). Likewise, it also plays an important role in the organoleptic properties of

* Corresponding author.
** Corresponding author at: Department of Food Science, College of Agriculture and Veterinary Medicine, United Arab Emirates University, Al-Ain 15551, United Arab Emirates.
E-mail addresses: Sajid.m@uaeu.ac.ae (S. Maqsood), priti.d@uaeu.ac.ae (P. Mudgil).

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fermented milk products. This proteolytic system of LAB comprises of cell wall-cleaved proteinases, peptidases (intracellular) necessary for degrading larger peptides into smaller peptides and AAs, and then transport systems which transfer peptides and AAs across the cytoplasmic membrane (Kieliszek et al., 2021). Notably, proteinases are the main actors that generate BAPs as a result of the hydrolysis of milk protein.

The conceivable physiological effect of milk protein-generated BAPs on biomarkers associated with metabolic syndrome is one of the interesting areas in health and wellness. Over the past decades, there have been many studies demonstrating the biological activities linked with fermented milk-derived BAPs, such as antioxidant, anti-inflammatory, anti-hypertensive, antidiabetic, and immunomodulatory (Alu'datt et al., 2021; Loghman et al., 2022; Yuan et al., 2022; Zhao et al., 2019). Moreover, the pronounced positive potentials of BAPs released during milk fermentation through the proteolytic system of different *Lactobacillus* spp. were intensively investigated, particularly the *in vivo*, *in vitro* and *in silico* effects (Chaudhary et al., 2021; Parmar et al., 2018). Concurring the important health benefits conferred by fermented milk-derived BAPs, it could be an interesting option to manage the chronic metabolic diseases as an alternative to synthetic drugs.

Increased lipid levels and blood cholesterol, termed hyperlipidemia, is a significant major risk factors for stroke, heart diseases and other problems, is a growing public health problem. One of the most prevalent approaches for mitigating hyperlipidemia involves inhibiting metabolic enzyme cholesterol esterase (CE)- a polymeric enzyme synthesized in the pancreas that regulates the bioavailability of cholesterol from dietary cholesterol esters. The inhibition of CE enzyme, consequently, serves to regulate the release of cholesterol in the blood by blocking cholesterol production in the liver. Several CE inhibitors, such as niacin, statins, and fibrates are currently used as clinical cholesterol-lowering drugs (Lunagariya et al., 2014; Sirtori et al., 2020). Nonetheless, synthetic drugs therapy in hyperlipidemia treatments can pose adverse metabolic side effects (Sirtori et al., 2020). As a result, it is utmost to explore natural products that can be used to develop safer and multifunctional cholesterol-lowering drugs with little or no side effects.

Recently, food-derived peptides with CE inhibitory activities have been widely reported from several plant and animal protein sources. It is worth noting that no studies exist on the anticholesterolemic potentials of BAPs released from fermented milk by proteolytic LAB. To the best of our knowledge, most of the studies concerning cholesterol-lowering or anticholesterolemic effect of fermented milk reported so far, have concerned their probiotic functionality. Moreover, Gil-Rodríguez and Beresford (2020) and Mudgil et al. (2024), investigated the pancreatic lipase inhibitory activity of fermented milk with the aim of isolating fermentate that could decrease body mass in subjects suffering from obesity/overweight. A better knowledge of the CE-inhibitory activities of BAPs derived from fermented bovine milk and different non-bovine milk types would help to fully understand their potential anti-lipidemia health benefits. Thus, this approach may contribute to the development of diverse novel fermented milk products with great anti-lipidemia potential.

Therefore, this study aimed to; (i) assess the CE-inhibitory activities released from bovine, camel, sheep, and goat milk following fermentation; (ii) explore the capacity of five indigenous probiotic strains to generate bioactive peptides with potential CE-inhibitory activities. We hypothesized that different CE-inhibitions would ensue among the milk sources based on the differences in their physiochemical properties, and amino acid sequences of the generated peptides. Also, the probiotic bacteria would release different anti-lipidemia peptides because the proteolytic activity of LAB varies among strains and species. For this study, liquid chromatography mass spectrometer-quadrupole time-of-flight (LCMS QTOF) techniques were used to identify peptides and we attempted to understand the possible peptides interaction with CE enzyme using *in silico* analysis and molecular docking.

2. Materials and methods

2.1. Chemical and reagents

The probiotic species *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) used in this study were previously isolated in our laboratory from raw camel milk. Cholesterol esterase enzyme (sourced from porcine pancreas, EC 3.3.3.13), *p*-nitrophenyl butyrate as substrate were procured from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Other chemicals and solvents of analytical grade used in the study were also obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Probiotic Milk fermentation

Raw milk samples from bovine, camel, sheep, and goats were collected from local dairy farms in Al-Ain, United Arab Emirates. The milk samples were promptly delivered to the laboratory in a sterile glass container covered with ice packs, and the experiment commenced immediately by skimming at 10,000 × g for 15 min (Beckman Coulter, Allegra X-30R). Fermented milks (**FM**) were then prepared using the procedure described by Mudgil, Yuen Gan, et al. (2023). Herein, milk samples were pasteurized at 72 °C for 15 s, and allowed to cool to 37 °C. The experiment was designed to generate 24 experimental **FM** samples. Each pasteurized milk was divided into six batches per milk source; five batches were kept for the fermentation process with individual probiotic strain, while one batch for control (non-fermented milk) was also considered. The milks were then inoculated with 18 h old culture of the five probiotic strains used in this study at a concentration of 5.0 log CFU/mL. The inoculated milk samples were then incubated at 37 °C for 24 h, and afterwards stored for two weeks at 4 °C with interval sampling at 0 day, 7 day, and 14 days, respectively. Each **FM** sample was prepared with a single bacteria strain to give five batches of fermented bovine milk (**F_BM**), Fermented camel milk (**F_CM**), fermented sheep milk (**F_SM**), and fermented goat milk (**F_GM**). For each fermented milk, 3 different batches per milk type were produced which served as triplicates.

2.3. Peptide profiling of different fermented milks with five probiotic species

Following fermentation, milk samples were characterized for their peptide profile using reverse-phase ultra-performance liquid chromatography RP-UPLC based on the differential hydrophobic interactions between peptides and the stationary phase of the column. (Thermoscientific, Germering, Germany) as described earlier (Mudgil, Redha, et al., 2023).

2.4. Cholesterol esterase (CE) inhibitory activity

The CE-inhibitory activities was determined using a previous study with slight modifications (Baba, Mudgil, et al., 2021). Briefly, fermented milk samples were suspended into a 96-well microtiter plate with substrate (50 µL) containing *p*-nitrophenyl butyrate (5 m*M*) in sodium phosphate buffer (100 m*M*), and 100 m*M* NaCl. Subsequently, 50 µL of porcine pancreatic CE (5 µg/mL) was added to each well and then incubated at 37 °C for 30 min. Then, the absorbance of *p*-nitrophenol released from enzymatic hydrolysis of *p*-nitrophenyl butyrate was measured using a UV/Vis microplate spectrophotometer at a wavelength of 405 nm (Thermo Scientific Multiskan SkyHigh Microplate Spectrophotometer). The extent of inhibition was calculated using Eq. 1:

$$CE\ Inhibition\ (\%) = \frac{C - D}{A - B} \times 100 \quad (1)$$

where A = Enzyme, substrate and buffer (control); B = substrate and buffer (control blank); C = enzyme, substrate and test sample (reaction); D = substrate and test sample only (reaction blank).

The value of half-maximal inhibitory concentration ($\mathbf{IC_{50}}$) was measured based on the equation obtained from the curve of percentage inhibition as a function of the concentration of test compound. In sum, the value of $\mathbf{IC_{50}}$ is described as the amount of fermentate protein equivalent required to inhibit 50 % of CE activity and expressed in μg (equivalent protein)/mL.

2.5. Peptide identification using LC-MS QTOF and peptide analysis using bioinformatics

The characterization of peptide generated from bovine, camel, sheep, and goat milk was performed on Agilent LC-MS QTOF (Thermo Scientific, GER, model: 6520) based on a previously described method (Sarah et al., 2016). Subsequently, peptides were subjected to novelty check using a database search software (BIOPEP-UWM) available at https://www.uwm.edu.pl/biochemia/index.php/en/biopep. The list of sequenced peptides from the milk samples were analyzed using Peptide Ranker web server available at http://distilldeep.ucd.ie/PeptideRanker/. This *in silico* analysis reveals peptides bioactive potential by analyzing shared characteristics among various functional categories of peptides, utilizing data from the BIOPEP database. Peptides with peptide ranker score of greater than 0.80 were designated as potentially bioactive peptides.

2.6. Molecular binding and hotspot interaction of peptides with CE using Pepsite 2.0

To predict the identified peptides inhibitory potentials against CE and their enzyme inhibition molecular mechanism, we performed *in silico* molecular interaction on a web server-based program PepSite2 using the method described by Mudgil et al. (2022). Purposely, crystal structure of CE [Protein Data Bank (**PDB**) code: 1AQL: chain B] were downloaded from RCB PDB. The peptides predicted to be the most potent having highest CE-inhibition activities were selected based on their significance level of binding ($p < 0.05$) and number of potential binding sites.

2.7. Molecular docking analysis to elucidate structure–activity relationship (SAR)

The 3D structure of CE (PDB; ***1AQL: chain B***) from RCSB Protein Data Bank (http://www.rcsb.org) was downloaded and peptide structure was generated using the Pep-Fold 3 server (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/#overview). Consequently, the peptide docking into the enzyme structure was completed using the High Ambiguity Driven protein-protein DOCKing (HADDOCK) server (http://wenmr.science.un.nl/haddock2.4/) as per the methodology described by (Honorato et al., 2021). Thereafter, CE interactions with selected peptides were investigated using docking analysis. The HADDOCK web server predicts protein-protein interactions by integrating biochemical and structural information. It utilizes ambiguous distance restraints from experimental data to guide the docking process, allowing for flexible modeling of protein conformations upon binding. A scoring function evaluates the quality of docking poses based on energy terms, and the process involves iterative refinement to optimize binding modes. Furthermore, HADDOCK enables visualization and analysis of the docked complexes, providing valuable insights into the molecular recognition processes essential for understanding biological interactions.

2.8. Statistical analysis

All the fermentation experiments were conducted and analyzed in triplicates, and the results are presented as the mean value ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's New Multiple Range Test, utilizing SPSS version 29.0 (SPSS Inc., Chicago, IL, USA), with significance set at $P < 0.05$.

3. Results and discussions

3.1. Peptide profiling of different fermented milks (FM) with five probiotic species

Peptide profiling is an essential step for gaining deeper insights into the hydrolysis process of intact proteins via fermentation using distinctive probiotic strains. Fig. 1 (A-E) illustrates the peptide profiles obtained from unfermented milk as control and FMs from each milk types fermented using different probiotics strains. As shown in Fig. 1A, intact proteins from all milk types eluted major protein peaks consistently between 50 and 80 min, However, different peak profile between all milk types indicates a varying protein composition across different milk samples. These differences in protein composition likely stem from the inherent variations in protein structures among different animal milks. These results are in line with other researchers, reporting that content and composition of various protein vary greatly between different animal milks. For instance, camel milk is devoid of any β-lactoglobulin and contain almost 1/10th of κ-casein in comparison to bovine milk proteins while is higher in content of α-lactalbumin (Wang et al., 2017; Yang et al., 2013). Similar results are reported by El-Hatmi et al. (2015), where a comparative analysis between whey protein profile of human, camel, donkey, goat and bovine milk revealed significant differences between composition of whey protein fractions.

Upon fermentation, a significant portion of these intact proteins underwent hydrolysis causing the formation of a diverse array of peptides with varying compositions and molecular weight, which were eluted between 8 and 50 min. Results showed that **FMs** produced using diverse probiotic strains exhibited varying pattern of peptides (Fig. 1 B-E) suggesting that the hydrolysis of milk proteins by five different probiotic strains is indeed dependent on specific proteolytic activity of bacterial strains as well as on the milk types used for fermentation. These chromatographic findings provide valuable insights into the dynamics of protein hydrolysis during fermentation via different probiotic strains. These observations corroborated well with the study of Aspri et al. (2018) who found out that the extent of protein hydrolysis is dependent on the strain used among other factors. Similarly, the versatile proteolytic activity and the strain-specific hydrolysis of proteins by probiotic strains has been exhaustively investigated (Daliri et al., 2018; Lim et al., 2019; Nielsen et al., 2022; Raveschot et al., 2018).

A comparative analysis between the ability of same probiotic strain in degrading different milk types also revealed significant differences. Several peaks with varied intensity could be observed between 8 and 50 min of elution indicating varied level of hydrophobicity among generated peptides. When comparing different probiotics for single milk types it can be noticed that a *PP-957* produced clearly distinctive peaks from goat and bovine milk proteins in comparison to other probiotics Fig. 1E. Therefore, we hypothesize that probiotic strains can produce different peptides from different milk types. The results also indicated that proteolytic activity of different probiotics was not only strain-dependent but also protein-dependent. However, these results are in agreement with those obtained by Ye et al. (2021), where fermentation of bovine milk using five strains of (Three from *L. delbrueckii ssp. bulgaricus* strains, DXJLHTS2M2, DQHXNS8L6, and 2038 and two from *L. delbrueckii ssp. lactis* strains, D11M188 and ATCC123150, showed subspecies level variations in the peptidomic profile of fermented bovine milk. The results also showed variation in the protein degradation patterns among

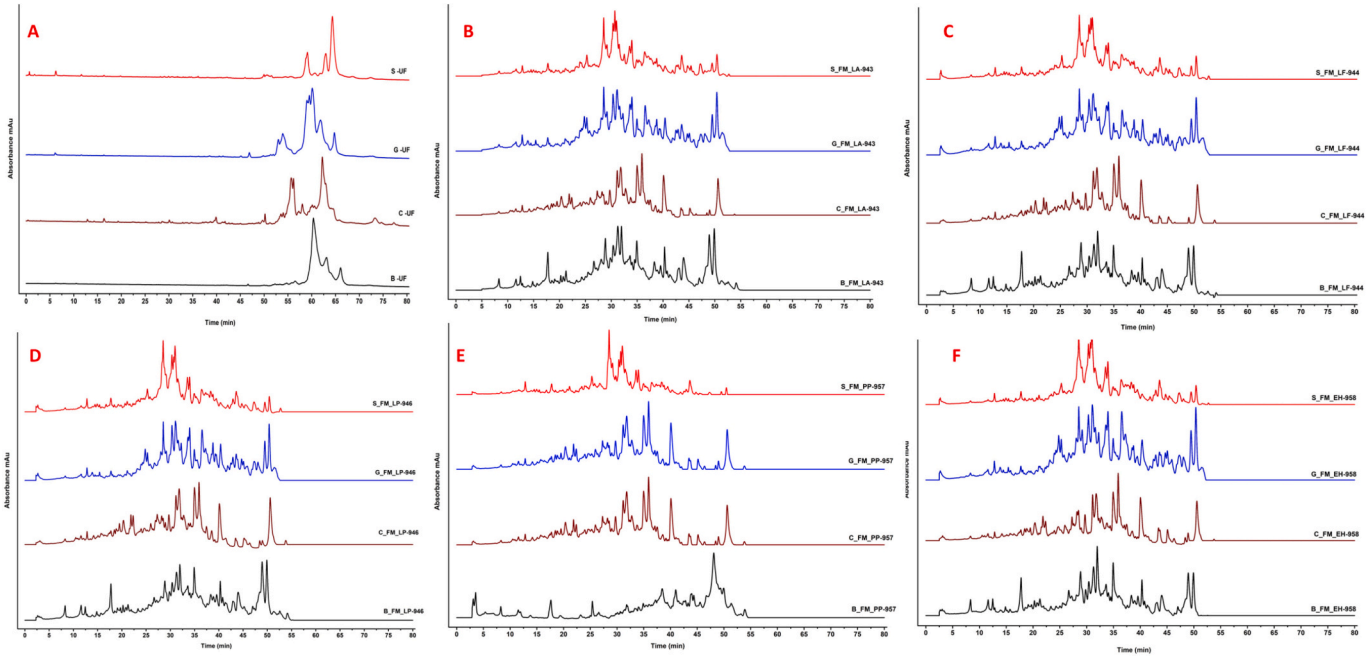

Fig. 1. Peptide profile of unfermented and fermented milk from bovine, camel, sheep and goat produced using five different probiotic strains.
Keynotes unfermented milk (A), LA-943: *Lactiplantibacillus argentoratensis* MF000943 (B), LF-944: *Limosilactobacillus fermentum* MF000944 (C), LP-946: *Lactiplantibacillus pentosus* MF000946 (D), PP-957: *Pediococcus pentosaceus* MF000957 (E), and EH-958: *Enterococcus hirae* MF000958 (F).

different strains, with strains from subspecies bulgaricus deriving the majority of their peptides from β-casein, while peptides from subspecies lactis were predominantly derived from αs1-casein and αs2-casein. Similar results, were also described by Isık et al. (2023), where fermentation by *L. helveticus* DPC 4571 and *L. casei* ATCC 334 showed more intense peptide profile in comparison to *S. thermophilus* 20S4.

3.2. Cholesterol esterase inhibitory activity

An effective strategy for managing hyperlipidemia and hypercholesterolemia is by preventing cholesterol absorption through the inhibition of cholesterol esterase (CE) enzyme. The obtained data related to the CE-inhibitory activities of **F_BM**, **F_CM**, **F_SM** and **F_GM** produced using different probiotic strains in terms of IC_{50} values (μg/mL protein equivalent) are presented in Table 1. Overall, results clearly revealed that the fermented milks exhibited higher CE-inhibitory activities than the non-fermented milk (i.e., lower IC_{50} values) demonstrating that the **FM** with probiotic strains effectively released bioactive peptides with improved CE-inhibitory activities. This was explained mainly due to diverse metabolic activities of lactic acid bacteria during the fermentation of milk (Undhad Trupti et al., 2021). Indeed, the fermentation of milk with different probiotic strains at 0, 7, and 14 days revealed significant ($p < 0.05$) differences in their CE-inhibitory activities.

At 0 day of storage after fermentation, among all **F_BM**, the minimum IC_{50} values of 53.7 ± 2.88 μg/mL indicating strong CE-inhibitory activity was displayed with *PP-957* derived fermented milk; moreover, it was not significantly different ($p > 0.05$) from the IC_{50} values observed in bovine milk fermented by *LF-944* and *LP-946*. The weakest inhibition of CE from F_BM was recorded with milk fermented by *EH-958* with an IC_{50} value of 137.17 ± 5.58 μg/mL. Among **F_CM** and **F_SM,** *PP-957* probiotic strain displayed the maximum CE inhibition with IC_{50} values of 42.2 ± 4.60 μg/mL and 34.3 ± 9.54 μg/mL, respectively. Contrarily to what we observed for other **FM**, goat milk fermented by *LF-944* showed the highest CE-inhibitory activity with IC_{50} values of 5.04 ± 0.17 μg/mL, among the probiotic strains followed by *LA-943 F_GM* at an IC_{50} value of 12.4 μg/mL. The weakest inhibition of CE among F_GM was observed for *LP-946* strains with an CE IC_{50} value of 24.1 μg/mL. Overall, results indicate that F_GM displays strongest inhibition of CE in comparison to FM obtained from other species at day 0 of milk fermentation. These variations among CE inhibitory profiles of different FMs could be ascribed to the differences in the ability of probiotic strains used for production of bioactive peptides with different inhibitory strengths. While there is no direct literature on the variability of cholesterol esterase (CE) inhibition in fermented milks resulting from probiotic fermentation, previous studies indicate that the angiotensin-converting enzyme (ACE) inhibitory activity in fermented milk samples is primarily influenced by the proteolytic efficiency of different probiotic bacteria and the properties of the milk proteins themselves. These findings provide a useful reference for understanding the potential variations in CE inhibition by fermented milk from different livestock species (Ashokbhai et al., 2022; Mudgil, Gan, et al., 2023; Rubak et al., 2022).

As milk fermentation progressed to 7 days, lower IC_{50} values were observed in both non-fermented and **FM** samples among the probiotic strains, suggesting improved CE-inhibition with the progress of fermentation. Generally, probiotic strains resulted in different CE-inhibition IC_{50} values after 7 days fermentation. The highest CE-inhibitory activity was observed in all the milk types fermented with *PP-957* strain. However, some strains i.e., *LP-943*, *LF-944*, *LP-946, and PP-957* showed no significant effect ($p > 0.05$) to inhibit CE in both **F_BM** and **F_CM**. For all the **FM** derived at 14 days of fermentation period, similar tendency pertaining to the CE-inhibition was apparent, where the **FM** maximum inhibitory performances (lowest IC_{50} values) were recorded for *PP-957* strain, except **F_CM.** In brief, the results showed that **FM** by *PP-957* showed the most potent inhibitory activity against CE compared to other strains. In general, CE-inhibitory activities in all the **FM** types considerably increased with prolonged fermentation. As per the best of our knowledge, the present study is the first where the CE-inhibitory potentials of different milk types fermented with different strains of LAB has been assessed. Although in line with the current findings, another researcher reported that the fermentation of bovine milk with two selected cultures of *LF-944* strains produced peptides which displayed anti-obesity functions (Kinariwala et al., 2020). In another, Wang et al. (2024), demonstrated the CE inhibitory activity of

Table 1
Cholesterol esterase IC_{50} inhibitory values (μg/mL) of fermented milk from bovine, camel, sheep, and goat produced using five different probiotic species.

		F_BM	F_CM	F_GM	F_SM
0 D	UC	290.18 ± 7.40^{dC}	152.62 ± 2.29^{dA}	276.84 ± 13.6^{dC}	179. ± 7.34^{dB}
	LA-943	86.1 ± 5.71^{bC}	56.3 ± 3.93^{bB}	96.7 ± 2.61^{cD}	12.4 ± 0.25^{bA}
	LF-944	59.1 ± 3.53^{aB}	54.9 ± 5.05^{abB}	76.3 ± 2.01^{bC}	5.04 ± 0.17^{aA}
	LP-946	64.9 ± 2.87^{aB}	89.5 ± 7.82^{cC}	83.0 ± 3.77^{bcC}	24.1 ± 0.54^{cA}
	PP-957	53.7 ± 2.88^{aC}	42.2 ± 4.60a^{BC}	34.3 ± 9.54^{aB}	16.3 ± 1.49^{bA}
	EH-958	137.17 ± 5.58^{cD}	56.3 ± 2.86^{bC}	43.4 ± 5.57^{aB}	16.3 ± 3.76^{bA}
7 D	UC	232.83 ± 0.82^{cC}	112.0 ± 3.35^{cA}	225.86 ± 17.1^{dC}	162. ± 4.16^{cB}
	LA-943	40.9 ± 0.57^{aB}	13.6 ± 0.75^{aA}	29.1 ± 9.16^{bB}	8.41 ± 0.44^{bA}
	LF-944	47.1 ± 1.86^{aC}	14.2 ± 4.23a^{AB}	17.6 ± 2.31^{abB}	8.08 ± 0.07^{bA}
	LP-946	43.0 ± 2.04^{aC}	10.2 ± 0.14^{aA}	20.3 ± 1.48^{abB}	9.22 ± 1.21^{bA}
	PP-957	42.5 ± 6.24^{aC}	10.4 ± 0.55a^{AB}	16.1 ± 0.04^{aB}	5.23 ± 0.50^{aA}
	EH-958	84.6 ± 0.77^{bD}	36.3 ± 0.39^{bB}	41.1 ± 0.25^{cC}	7.42 ± 0.73^{bA}
14 D	UC	187.37 ± 3.68^{dC}	95.6 ± 0.38^{cA}	210.12 ± 11.4^{dD}	122.77 ± 0.69^{dB}
	LA-943	47.7 ± 15.6b^{cB}	8.21 ± 1.26^{aA}	34.6 ± 2.83^{abB}	5.80 ± 0.51^{abA}
	LF-944	46.8 ± 2.50b^{cD}	13.7 ± 3.27^{bB}	40.1 ± 0.91^{bC}	6.34 ± 0.15^{bA}
	LP-946	30.8 ± 7.84^{abB}	11.9 ± 1.29^{abA}	37.5 ± 3.25^{bB}	9.29 ± 0.24^{cA}
	PP-957	23.1 ± 0.26^{aC}	12.8 ± 2.19^{abB}	29.2 ± 2.69^{aD}	5.47 ± 0.16^{aA}
	EH-958	59.0 ± 1.26^{cD}	13.1 ± 0.02^{abB}	52.3 ± 1.67^{cC}	5.41 ± 0.35^{aA}

Data represents mean ± SD. Different small and capital alphabets in the table represents significant difference between and across the samples, respectively. UC is represented as un-fermented milks, F_BM, F_CM, F_GM and F_SM represent fermented bovine, camel, goat and sheep milk, respectively.
Keynotes: D represents days of fermentation period, 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.

yak milk cheese and identitied some peptides with potent CE inhibitory activity. The study demonstrated that peptides RK7 (RPKHPIK), KQ7 (KVLPVPQ), QP13 (QEPVLGPVRGPFP), and VN10 (VYPFPGPIPN) exhibited inhibitory activity on CE with IC_{50} values of 8.16×10^{-7} mol/L, 8.10×10^{-7} mol/L, 4.63×10^{-7} mol/L, and 7.97×10^{-7} mol/L. In another study on fermentation of sea buckthorn juice by probiotic *Lactobacillus plantarum* Lp10211, 29.54 % to 84.68 %.

As presented in Table 1, **F_GM** demonstrated the highest CE-inhibitory activity (lower IC_{50} values) compared to other milk types at 0-, 7-, and 14- days of fermentation. Consequently, **F_GM** obtained using any of the probiotic strains showed enhanced bioactive functionality due to the highest CE-inhibitory activities compared with other milk types. This observations with **F_GM** may be attributed to the nature of bioactive peptides inherent in **F_GM**, indicating that a good correlation was observed between the CE-inhibitory activity and DH particularly for **F_GM**. Therefore, it is reasonable to believe that goat milk proteins served as a more suitable substrate for proteolytic enzymes secreted by the probiotic strains used, which facilitated the generation of shorter peptides with more potent bioactivity.

Our findings are also supported by the findings of Zhang et al. (2015) who reported that higher cholesterol-lowering effects were observed from cultured goat milk in comparison to bovine milk. They attributed it to the higher proteolytic activity in goat milk mainly due to its primary configuration and structural differences in the proteins. Another study showed that goat milk produced maximum bioactive peptides, as compared with bovine milk (Shu et al., 2018). Moreover, the most potent CE-inhibitory activities of **F_GM** (those with lowest IC_{50} values) were displayed by using *LF-944* at 0 day, and *PP-957* strain at 7 and 14 days of fermentation, respectively. Taken together, these results present strong evidence of the potential of **FM** in inhibiting enzymatic markers related to hypercholesterolemic, thus, regulating lipid metabolism. However, the mechanism whereby these **FM** inhibit these enzymes is unclear, thus, requires further investigations.

3.3. Molecular interaction of fermented milk types derived BAPs with cholesterol esterase

Peptides were identified from *PP-957* fermented bovine milk (**F_BM**), camel milk (**F_CM**), sheep milk (**F_SM**), and goat milk (**F_GM**) using LCMS-QTOF. The results showed that a total of 47, 37, 45, and 44 peptides were identified in **F_BM**, **F_CM**, **F_SM**, and **F_GM**, respectively (Appendix A-Tables 1-4). Peptides sequenced were further screened based on average local confidence (ALC) value of at least 80 % and peptides with ranker score of >0.80 were represented as potentially biologically active peptides (BAPs). The binding mechanism of BAPs with the target enzyme (cholesterol esterase) was examined with a computational prediction software (Pepsite2). The peptide sequence, *p*-value, and reactive residue in the identified peptides from **F_BM**, **F_CM**, **F_SM**, and **F_GM** with significant binding ($p < 0.05$) are shown in Table 2.

The cholesterol esterase (CE), a single domain α/β protein, is well-known to utilize a serine esterase catalytic mechanism. The dimers are formed in which the active sites gorge from the two subunits forming a mirror image with the catalytic triad containing Ser194-His435-Asp320. During catalysis, the tetrahedral transition state of the substrate is stabilized by a putative oxyanion hole containing the backbone amide groups of the residues Gly107 and Ala108 along-with amide group of Ala195 near the catalytic site (Alblooshi et al., 2023). Nonetheless, a "flap" or "lid" covers the active sites thereby regulates substrate binding, and also enhance the capacity of His435 in close proximity with several partly buried acidic residues (Asp434, Asp437, Asp438 and Glu193) to shuttle protons during catalytic function (Mudgil et al., 2022). Therefore, binding potentials of peptides to any of these binding sites; active sites (catalytic triad) and oxyanion hole is predicted to induce CE-inhibitory effects.

The number of amino acids residues in these peptides identified from milk types varied from 4 to 24, which is well within the range of previously reported potent hypocholesterolemia peptides from cumin seeds (Siow et al., 2016). Further, Alnuaimi et al. (2023) a potent CE-inhibitory peptide (ATSLDFPALWLLKLSAQYGSLRK) from soybean with up to 23 AA residues. Similarly, Mudgil et al. (2022) and Baba, Mudgil, et al. (2021) reported various CE inhibitory peptides with a length of 2–9 and 2–15 amino acids long, respectively.

In total,10, 7, 6, and 6 peptides were identified in **F_BM**, **F_CM**, **F_SM**, and **F_GM,** respectively based on their significance ($p < 0.05$) to bind CE residues (Table 2). Surprisingly, among all these peptides, only one peptide from **F_BM** (APSFSDIPNPIGSENSEKTTMPLW) was found to significantly bind ($p < 0.05$) to the important catalytic sites of CE i.e., Ala108, Ser194, Trp227, Phe324, and His435. Whereas all the peptides identified in **F_CM**, **F_SM**, and **F_GM** could not bind to any residues of the important active sites of CE. However, they only showed interactions with amino acids of enzymes. These results made it difficult to establish a direct relationship between IC_{50} CE-inhibitory activities of these milk types and binding ability of identified peptides to important catalytic residues of CE, as the peptides binding potentials were uncertain.

Even though we observed that three peptides in **F_BM** (YQEPVLGPVRGPFPIIV, LLYQEPVLGPVRGPFPIIV, LYQEPVLGPVRGPFPIIV), two peptides (RPPPPVAM and QMCNPVPK) in **F_CM**, four peptides (LYQEPVLGPVRGPFPILV, VPQRDMPIQA, RSPK, and

Table 2
Sequence of identified bioactive peptides derived from different milk types upon their fermentation with *PP-957* and their interaction with cholesterol esterase (CE) (PDB code: 1AQL-chain: B) binding sites.

MILK TYPE	PEPTIDE	P-VALUE	BOUND PEPTIDE RESIDUES	BOUND CHOLESTEROL ESTERASAE RESIDUES	HPEPDOCK SCORE
F_BM	RELEELNVPGE	0.02684	Arg-1, Glu-2, Leu-3, Glu-4, Glu-5, Val-8, Pro-9, Gly-10, Glu-11	Tyr7, Phe12, Phe235, Trp236, His283, Tyr284	−255.685
	HIQKEDVPSER	0.04167	His-1, Ile-2, Gln-3, Lys-4, Pro-8, Ser-9, Glu-10, Arg-11	Tyr7, Phe12	−207.474
	APSFSDIPNPIGSENSEKTTMPLW	0.0402	Ala-1, Pro-2, Pro-8, Asn-9, Ile-11, Gly-12, Ser-13, Met-21, Pro-22, Leu-23, Trp-24	Ala108*, Ser194*, Trp227*, Phe235, Trp236, Arg239, Glu278, Tyr279, His283, Tyr284, Leu285, Phe324*, Phe393, His435*	−166.163
	MMLM	0.005942	Met-1, Met-2, Leu-3, Met-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−191.406
	MMFL	0.01099	Met-1, Met-2, Phe-3, Leu-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−169.206
	WHLTY	0.03576	Trp-1, His-2, Leu-3, Thr-4	Phe235, Trp236, Glu278, Tyr279, His283	−235.805
	MMLF	0.01099	Met-1, Met-2, Leu-3, Phe-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−172.057
	MFSQ	0.006979	Met-1, Phe-2, Ser-3, Gln-4	Tyr7, Phe12	−196.346
	PAAY	0.02355	Pro-1, Ala-2, Ala-3	Phe235, Trp236, His283, Tyr284	−195.828
	YPPA	0.0155	Pro-2, Pro-3, Ala-4	Phe235, Trp236, His283, Tyr284	−156.661
F_CM	IEEQQQTEDEQQDK	0.0215	Glu-2, Glu-3, Gln-4, Gln-5, Gln-6, Glu-8, Glu-10, Gln-11, Gln-12, Lys-14	Tyr7, Phe12	−152.752
	IMEQQQTEDEQQDK	0.01126	Met-2, Glu-3, Gln-4, Gln-5, Gln-6, Glu-8, Glu-10, Gln-11, Gln-12, Lys-14	Tyr7, Phe12	−243.699
	FMLM	0.01099	Phe-1, Met-2, Leu-3, Met-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−175.942
	RLER	0.04779	Arg-1, Leu-2, Glu-3, Arg-4	Tyr7, Phe12	−239.363
	APLY	0.03861	Ala-1, Pro-2, Leu-3	Phe235, Trp236, Tyr279, His283	−149.293
	MMPY	0.004489	Met-1, Met-2, Pro-3, Tyr-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−172.902
	CQGR	0.001799	Cys-1, Gln-2, Gly-3, Arg-4	Tyr7, Phe12	−196.346
F_GM	FESEEQQQTEDELQDK	0.03797	Glu-4, Glu-5, Gln-6, Gln-7, Gln-8, Glu-12, Leu-13, Gln-14, Lys-15	Tyr7, Phe12	−160.578
	MAQY	0.03825	Met-1, Ala-2, Gln-3	Tyr7, Phe12	−166.423
	KASW	0.02586	Lys-1, Ala-2, Ser-3, Trp-4	Phe235, Trp236, Glu278, Tyr279, His283	−156.513
	MMLM	0.005942	Met-1, Met-2, Leu-3, Met-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−166.163
	QVLAPLSGNAVQ	0.04671	Gln-1, Val-2, Leu-3, Ala-4, Ala-10, Val-11, Gln-12	Tyr7, Phe12	−257.715
	MMLF	0.01099	Met-1, Met-2, Leu-3, Phe-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−215.703
F_SM	MPFPK	0.007865	Met-1, Pro-2, Phe-3, Lys-5	Tyr7, Phe12	−215.744
	DQHQKAMKPWTQPK	0.01498	Gln-2, His-3, Gln-4, Lys-5, Ala-6, Trp-11, Thr-12, Gln-13, Lys-15	Tyr7, Phe12	−260.810
	TPQH	0.001047	Thr-1, Pro-2, Gln-3, His-4	Tyr7, Phe12	−202.305
	FMLM	0.01099	Phe-1, Met-2, Leu-3, Met-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−162.476
	MMLF	0.01099	Met-1, Met-2, Leu-3, Phe-4	Phe235, Trp236, Arg239, Pro273, His283, Tyr284	−187.102
	WSGN	0.01397	Trp-1, Ser-2, Gly-3, Asn-4	Phe235, Trp236, Glu278, Tyr279, His283	−192.611
	MTPY	0.04846	Met-1, Thr-2, Pro-3	Phe235, Trp236, Arg239, His283, Tyr284	−144.477

* Hotspots of cholesterol esterase which were reported to be important for the inhibitory activity. For keynotes: Please see keynotes for Table 1.

Table 3
Molecular docking interactions between *PP-957* derived F_BM peptide with cholesterol esterase (CE) (PDB code: 1AQL-chain: B).

Source	Peptide	Binding affinity (kcal/mol)	Binding interaction with CE		
			Hydrogen bond	Hydrophobic interaction	Salt bridge
F_BM	APSFSDIPNPIGSENSEKTTMPLW	−11.4	Arg63, Asn118, His435*	Arg63, Gln66, Tyr75, Gly106, Gly107*, Ala108*, Met111, Gly112, Ala117, Asn118, Leu120, Ser121, Leu124, Tyr125, Glu193, Ser194*, Trp227*, Leu272, Leu274, Leu285, Leu323, Phe324*, Met327, His435*, Ala436, Leu439	–

RPGPNLTVY) in **F_GM,** and five peptides (LYQEPVLGPVRGPFPILV, YQEPVLGPVRGPFPILV,YYQEPVLGPVRGPFPILT, REQEELNVVGET-VESLSSSEESITHINK, and ESGGSVGTQ) in **F_SM** which showed suitable binding sites with CE active sites, however, their binding potentials were not significant ($p > 0.05$) as shown in Appendix A: supplementary data. This suggests that these peptides might not have the ability to inhibit CE enzyme because strong CE-inhibitory peptides are related to their binding significance level. Here, this observation with identified food-derived peptides is not the first, as the results were consistent with recent study reported by Alnuaimi et al. (2023) who reported peptides with higher binding sites but the binding potential was not significant.

The indirect CE-inhibitory mechanisms involving other mode of interactions which have been demonstrated in many studies, where the collective conclusion was that CE inhibitors does not only act by binding to the substrate binding sites (in this case cholesterol esterase) in a competitive inhibition mode, but also by non-competitive inhibition by binding to nearby hotspot causing a stearic hinderance. For example, Dorsilurin-F, a standard inhibitor of CE acts in a non-competitive way by binding to the residues away from active sites such as Glu 193, Gly106, Leu329, Gly112, Tyr125, Trp227, Met281, Val285, Leu282, Phe324, Ile323 and Ile327 (Sivashanmugam et al., 2013). Like Dorsilurin-F, the remaining nine peptides in **F_BM**, all eight peptides in **F_CM**, and all six peptides in **F_SM** and **F_GM**, respectively, displayed interactions with amino acid residues Tyr7, Ser220, Phe12, Phe235, His283, Trp236, Tyr284, Tyr279, and His345 on CE enzyme. Indeed, this indicated that most of the identified peptides from this study are potent CE-inhibitors, but their binding events would likely occur outside the active sites (with or without the substrate present) thus demonstrating non-competitive

mode of inhibition. The inhibition of drugs and peptides via various modes of interaction have been previously reported (Baba, Mudgil, et al., 2021; Mudgil et al., 2022). Our findings uphold the already existing knowledge that all CE inhibitors do not only act in a competitive inhibition mode.

Again, the majority of non-competitive peptides against CE produced from the milk types had higher docking scores, indicating that the identification results of CE-inhibitory peptides was very reliable (Table 2). For instance, RELEELNVPGE, HIQKEDVPSER, and WHLTY from **F_BM** showed higher docking scores of −255.685, −255.685, and − 235.805, respectively against CE. Two peptides from **F_CM** (IMEQQQTEDEQQDK and RLER), showed a HPEPDOCK score of −243.699 and − 239.363, respectively. Among the six peptides from **F_GM**, peptides MPFPK, DQHQKAMKPWTQPK, and TPQH had maximum docking score. Peptides QVLAPLSGNAVQ and MMLF from **F_SM** in this study appeared to be strong CE-inhibitors as they showed high docking scores of −257.715 and − 215.703, respectively. These results implied that high CE-inhibitory activity of these peptides, specifically from F_GM, might be related to these high docking score values.

Apart from peptides binding capabilities to catalytic sites and/or amino acid residues of CE, certain structural characteristics of candidate peptides are known to make a significant contribution to their CE-inhibitory potentials. The type and position of amino acids residues in the peptide may be the key factors for its inhibitory activity. Baba, Baby, et al. (2021), reported that the presence of hydrophobic residues at the N-terminal was an important feature of CE-inhibitory peptides. Correspondingly, sequence analysis of milk CE-inhibitory peptides fragments in our work showed that leucine (L), proline (P), Meth (M), isoleucine (I), and/or phenylalanine (F) were at the first or second position of N-terminus. Peptides rich in hydrophobic AAs that are located at the N-terminal position is proposed to favor their binding to the active sites of CE. For instance, we observed two hydrophobic residues at the first and second terminal Ala/Pro in peptide APSFSDIPNPIGSENSEKTTMPLW identified from **F_BM**, which may be an important feature for peptides with hypocholesterolaemic activity.

Besides, it could be observed that hydrophobic amino acids such as Pro-Leu-Tyr (i.e., P/L/W) located at the same peptide C-terminal region seemed to have played an important role in hypocholesterolemia activity. We hypothesize that hydrophobic AAs head- and -tail distribution pattern in a peptide may facilitate their attachment to the enzyme interface, form a cluster on protein components at active site location, and inhibit CE. As such, the presence of hydrophobic residues is a feature of cholesterol inhibitor, as they provided higher affinity towards the active site of CE. Detailed results revealing the contribution of hydrophobic amino acids to CE-inhibition has been previously reported (Ajayi et al., 2021; Alnuaimi et al., 2023; Baba, Baby, et al., 2021; Ngoh et al., 2017). Taken together, peptides identified in the milk types used in this study demonstrated potential inhibition of CE, however, peptide APSFSDIPNPIGSENSEKTTMPLW from **F_BM** competitively inhibits CE by binding to the enzyme active sites. In this study, we found novel peptide sequences from **FMs** that can inhibit the activity of CE, thus, can potentially serve as anti-hypercholesterolemic inhibitor.

3.4. *Molecular docking of peptide APSFSDIPNPIGSENSEKTTMPLW with CE*

The interaction between inhibitory peptides and targeted enzymes can be understood in detail at the domain of atomic level using molecular docking analysis. Hence, molecular docking is one of the most important processes for understanding of inter-atomic processes and interactions. Therefore, following, molecular interaction studies, peptide APSFSDIPNPIGSEM KETTMPLW was selected to be docked with cholesteryl esterase. Data obtained is presented in Table 3 along with Fig. 2, which unveil the nature of bond formation by revealing peptide residues involved in the case, including hydrogen bonds and hydrophobic interactions.

Through formation of hydrogen bonds between the peptide and enzyme residues such as Arg63, Asn118, and predominantly His435*, an evident picture of the recognition and binding of the peptide in the enzyme's catalytic site is displayed. As discussed above as His435* is part of the catalytic triad and is an active hotspot for CE, which implies that the peptide APSFSDIPNPIGSEM KETTMPLW may restrict the

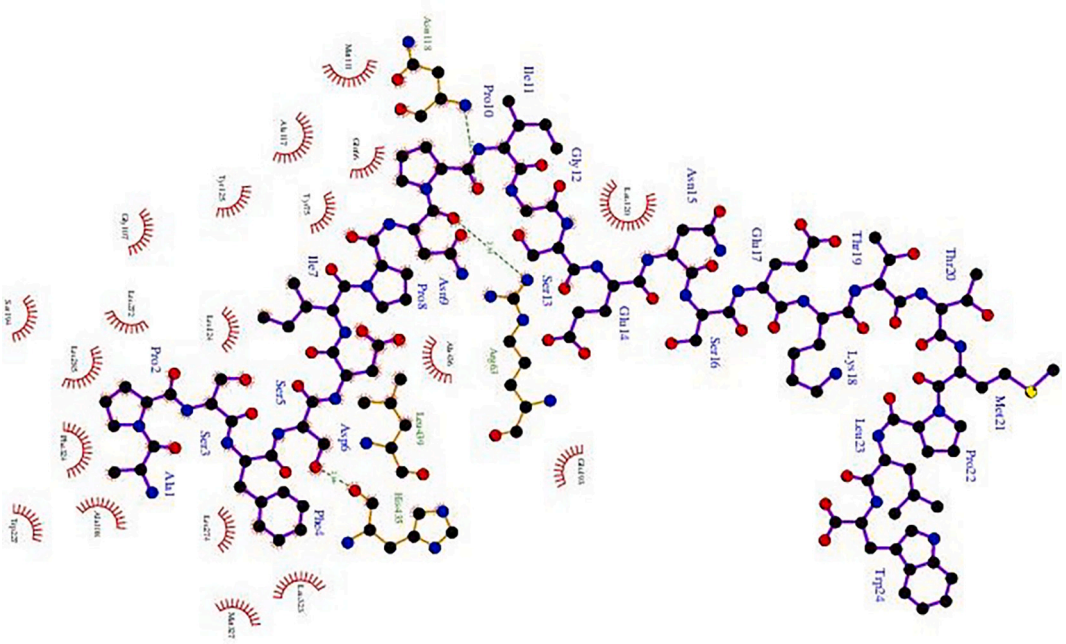

Fig. 2. Binding interaction between CE and APSFSDIPNPIGSENSEKTTMPLW.
Note: Peptide residues are joined through purple line whereas enzyme residues are joined through brown lines. The red, blue, black and yellow dots indicate oxygen, nitrogen, carbon and Sulphur atoms, respectively. Green dotted line indicates hydrogen bond whereas red dotted line indicates salt bridge. Brick red eyelashes indicate hydrophobic interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enzyme's function causing this enzyme's inhibition.

Moreover, the remarkable hydrophobic interactions between the peptide and Gly107*, Ala108*,

Ser194*, the substrate binding sites also indicate that peptide may competitively inhibit the enzyme. Moreover, further hydrophobic interaction with CE hotspots Trp227*, Phe324*, His435*, and other residues are believed to provide additional stabilization of the peptide-enzyme complex. Further, it was evident that peptide also showed interaction with Leu272, Leu274, Leu285, a part of the integral coiled-coil loop in the N-terminal catalytic domain of CE that serves as the pathway for releasing catalysed products from the enzyme. These interactions at the different regions of the CE enzyme, in a way seems to suggest presence of multiple binding sites and a high affinity between the peptide and the enzyme. These broad spectra of binding sites such as hydrogen bonding and multiple hydrophobic interactions on the CE enzyme, enhances the potential of this peptide as a therapeutic agent.

Further, the binding energy value of −11.4 kcal/mol further indicates favourable and stronger binding between the peptide and cholesteryl esterase as a low binding energy means a complex formation that is more stable and relatively conjugating with cholesteryl esterase inhibition mechanism. These results are in accordance with those obtained by other researchers, for instance, four peptides (FLF, IYF and QIF) identified from tea protein displayed a binding energy of −8.9, 7.4, and 7.8 kcal/mol, respectively with CE and the bound catalytic hotspots such as Ser194, Asp320, His435, Gly107, Ala108 and Ala195 (Ye et al., 2023). Similarly, Zhao et al. (2024) identified CE inhibitory peptides LGGLDSSLLPH, FDTGSSFYNKPAG, IWVGGSGMDM, IFNNDPNNHP, and YLQGFGKNIL from adzuki beans. Similar to the results of our study, they also found that LLGGLDSSLLPH, FDTGSSFYNKPAG, and IWVGGSGMDM bound to amino acid residues in both the catalytic and substrate-binding sites. However, the peptide YLQGFGKNIL bound only to the catalytic site, while IFNNDPNNHP bound exclusively to the substrate-binding sites. In another study, peptides from yak milk cheese demonstrated similar interaction with CE enzymes including hydrogen bonding, hydrophobic interaction and Vander wall interactions (Wang et al., 2024). However, the seven different peptides investigated in this study had minimal interaction with CE's major hotspots, in comparison to the peptide APSFSDIPNPIGSEMKETTMPLW which bound to six different hotspot residues.

Overall, in the present investigation, peptide APSFSDIPNPIGSEMKETTMPLW appears to be peptide that could effectively stop the CE enzyme from further action either by occupying the catalytic and substrate-binding sites or obstructing the release channel, thereby impeding its activity. Therefore, these results imply the specific importance of APSFSDIPNPIGSEMKETT-MPLW peptide as an efficient antilipidemic medication. Which could be an innovative strategy in battling against hypercholesterolemia and atherosclerosis, disorders associated with dyslipidaemia.

4. Conclusions

In conclusion, this study systematically demonstrated that milk fermentation with various probiotic strains significantly enhanced the antilipidemic action of fermented milk derived from bovine, camel, sheep, and goat milks via cholesterol esterase (CE) inhibition. Overall, each strain showed distinct proteolytic activity and were able to generate diverse bioactive peptides with varied biological activities, with goat milk fermentations yielding the highest proteolytic efficiency, as indicated by elevated peptide profiles and lower IC_{50} values for CE inhibition. Subsequently, it was observed that CE inhibition varied based on both the probiotic strain and the specific milk protein substrate. Upon peptide identification, *in silico* studies and molecular docking analysis, peptides derived from camel, sheep, and goat milk displayed indirect CE-inhibitory mechanisms, likely involving alternative modes of interaction with the enzyme. However, the peptide APSFSDIPNPIGSENSEKTTMPLW from fermented bovine milk was identified as a potent competitive CE inhibitor, marking its potential as an anti-hypercholesteremic agent. Overall, this research provides comprehensive insights into the inhibitory potential of fermented milks from different animal sources, underscoring their relevance in developing functional dairy products to target hypercholesterolemia.

CRediT authorship contribution statement

Feyisola Fisayo Ajayi: Writing – review & editing, Writing – original draft, Methodology. **Fatimah AlShebli:** Writing – original draft, Methodology. **Pei-Gee Yap:** Writing – review & editing, Software, Investigation. **Chee-Yuen Gan:** Writing – review & editing, Supervision, Software, Investigation. **Sajid Maqsood:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Priti Mudgil:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101998.

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