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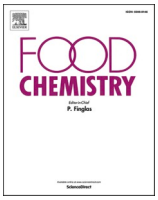

Novel casein antimicrobial peptides for the inhibition of oral pathogenic bacteria

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ABSTRACT

Milk casein is a rich source of antimicrobial peptides (AMPs) and the most common way to produce AMPs is enzymatic hydrolysis *in vitro*. In this study, active casein antimicrobial peptide (CAMPs) mixtures were generated by optimized proteolytic cleavage of milk casein. These natural-safe CAMPs mixtures exhibited high activity in the inhibition of *Streptococcus mutans* and *Porphyromonas gingivalis*. Morphological characterization suggested the pathogenic bacteria presented incomplete or irregular collapsed membrane surface after the treatment with active CAMPs mixtures. The CAMPs inhibition activity was also effective in the attachment and development of microbial biofilm. Potential CAMPs sequences were unambiguously determined by unbiased proteomic analysis and 301 potential CAMPs were obtained. The activity of 4 novel CAMPs was successfully confirmed by using synthetic standards. This study provides a promising milk CAMPs resource for the development of safe agents in oral bacteria inhibition and functional foods.

1. Introduction

Oral diseases are the sixth non-communicable disease reported by the World Health Organization (WHO). More than 3.5 billion people worldwide suffer from dental diseases, including dental caries, periodontal disease, and oral cancer (Benzian et al., 2021; Benzian & Beltràn-Aguilar, 2021). Oral infections and inflammation are among the most common infections with impact on the development of Alzheimer's disease, colorectal carcinoma, diabetes, and cardiovascular diseases (Holmes et al., 2009; Lockhart et al., 2012; Chapple & Genco, 2013; Daly et al., 2018; Sansores-España, Carrillo-Avila, Melgar-Rodriguez, Díaz-Zuñiga, & Martínez-Aguilar, 2021; Wang et al., 2021). Mechanical debridement and antimicrobial therapy are the main treatment options for oral inflammatory diseases. Nevertheless, billions of people could not afford oral surgery therapeutics even essential oral healthcare. Moreover, due to the COVID-19 pandemic, clinical dental services were limited (Benzian & Beltràn-Aguilar, 2021). Therefore, traditional antibiotic treatment is the most feasible approach at present. However, conventional antibiotics are effective against metabolically active bacteria and less effective on the dormant bacteria encapsulated in biofilms that lead to the destruction of gingival tissue and tooth or alveolar bone loss (Ten Cate, 2006). In addition, side effects and bacterial resistance to antibiotics result in the deterioration of the treatment effect. An alternative approach might be the usage of antimicrobial peptides (AMPs) and proteins, which have bactericidal and inflammatory against effects and could block the inflammatory influence of bacterial toxins (Gorr & Abdolhosseini, 2011; Browne et al., 2020).

AMPs are a class of peptides with strong and broad-spectrum antimicrobial activity against bacteria, viruses, parasites, and fungi (Michael Zasloff, 2002). Most of AMPs possess positive charges and antipathy that enable them could dissolve in an aqueous environment and penetrate lipid rich membranes (Izadpanah & Gallo, 2005). More than 5000 AMPs have been discovered or synthesized up to date (X. Zhao et al., 2013). Owning to their ability to avoid drug resistance, great interest in AMPs

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Table 1
Model summary statistics of the quadratic response surface.

Responses	Model		
	F-value	P	R^2
Y_1	5.77	0.0218	0.9058
Y_2	3.89	0.0434	0.8969

has spread to medical, pharmaceutical and food industries (Fry, 2018; Thery, Lynch, & Arendt, 2019; Browne et al., 2020). AMPs derived from natural sources have gained widespread attention.

Milk protein can not only supply nutrients to human body but also produce a variety of biologically active compounds with important physiological functions (Haque et al., 2009). As a low-cost and harmless source, milk AMPs have incontestable application potential in the food and pharmacy industry. Casein is the main component of milk proteins as well as an important derivation source of bioactive peptides including AMPs, which can be released and activated during gastrointestinal digestion and food processing by *in vitro* enzymatic hydrolysis (Abdel-Hamid et al., 2016; Arruda et al., 2012; Fontenele et al., 2017; Haque et al., 2009; Sibel Akalın, 2014; Q. Zhao et al., 2020). However, there are few reports on the effects of casein-derived active peptides on oral diseases (Nielsen et al., 2017). The discovery of novel casein antimicrobial peptides (CAMPs) with high inhibition activity to pathogenic bacteria is still a challenging task. Conventional strategies for CAMPs screening usually include multiple-step isolation and extraction, followed by activity assay of purified components, which are time-consuming and troublesome (Tan et al., 2013; Tulini et al., 2014). The composition and activity of casein hydrolysate are dependent on the hydrolysis conditions. The interaction characteristics between preparation factors and inhibition rates on oral cariogenic bacteria are ambiguous. To obtain the desired bioactive products, it is necessary to systematically optimize the casein hydrolysis conditions. Furthermore, the composition of casein hydrolysate is complex and the antimicrobial activity is usually the result of a synergistic effect of various active peptides. Thus, to extend the understanding of the mechanism of antimicrobial activity, further analysis is needed to profile the components and sequences of potential active peptides. However, compared with the sodium dodecyl sulfate polyacrylamide gel electrophoresis and high-performance liquid chromatography approaches, the application of advanced proteomic strategy is less common for unbiased active peptide discovery in high throughput (Basilicata et al., 2018; Nath et al., 2021).

The primary objective of this study is to identify and discover novel CAMPs derived from casein hydrolysate through an unbiased proteomic analysis. Additionally, we will investigate their inhibition activity against oral cariogenic bacteria including *Streptococcus mutans* (*S. mutans*) and *Porphyromonas gingivalis* (*P. gingivalis*). We systematically optimized the generation of active CAMPs mixtures from milk casein by *in vitro* proteolytic cleavage. The exact sequences of polypeptides within the active candidate mixtures were determined unambiguously through unbiased proteomic analysis. Finally, potential CAMPs were screen out by matching them in the Milk Bioactive Peptide Database with greater than 70% sequence similarity, and the activity of novel CAMPs was confirmed by using synthetic standards. Therefore, our results could potentially play crucial roles in developing effective and natural-safe agents for the prevention of oral periodontitis.

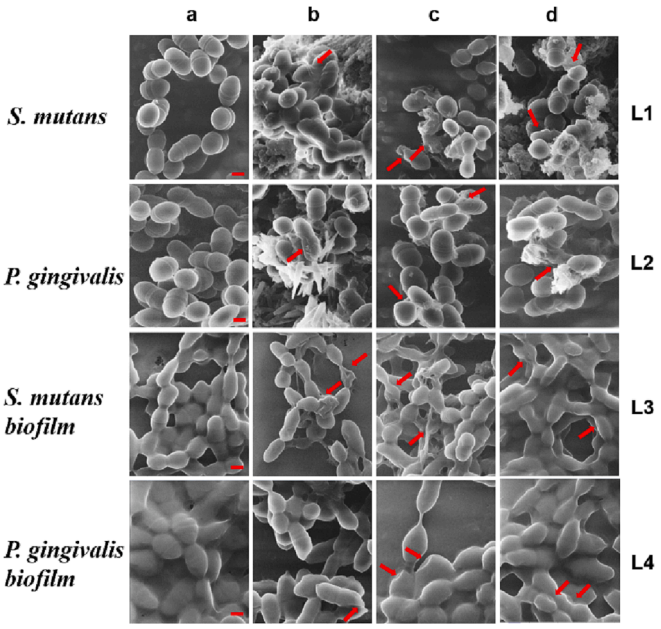

Fig. 2. Microarchitecture of microbial characterized by SEM for negative control (a), positive control (chlorhexidine treatment) (b), and active CAMP mixture 1 (c) and 2 (d) treatments Scale bar = 200 nm.

Table 2
The numbers of peptide sequences matched with antimicrobial peptides in MBPDB database with 100% and >70% sequence similarity.

Similarity	Active mixture 1	Active mixture 2	Total
100 %	10	10	11
>70 %	261	282	301

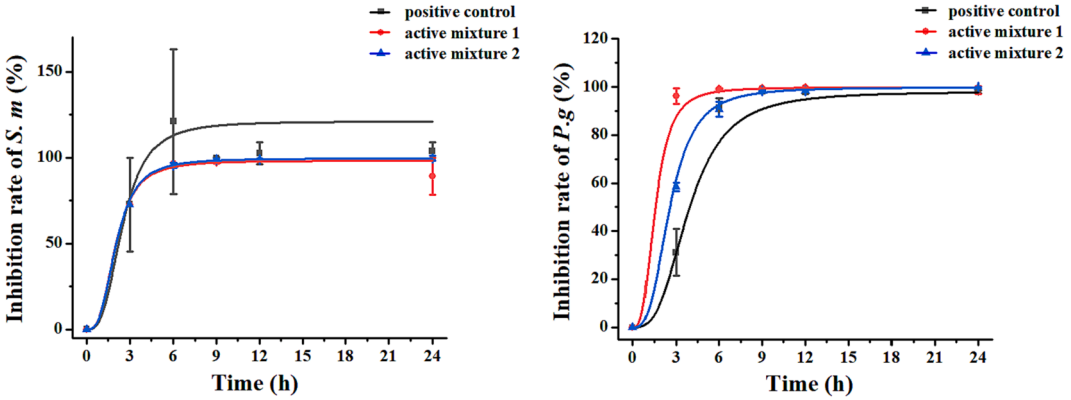

Fig. 1. The inhibition rates of CAMP active mixture 1 and 2 to *S. mutans* and *P. gingivalis*.

Table 3
The sequences of known CAMPs discovered in the active mixtures.

Peptide sequences	Protein ID	Activity Protein Fragment	Origin	Normalized MS intensity (%)
AASDISLLDAQSAPLR	P02754	β-lactoglobulin (41–56)	Active mixture 1	0.001
YQEPVLGPVRGPFPIIV	P02666	β-casein (208–224)	Active mixture 2	0.045
TPEVDDEALEK	P02754	β-lactoglobulin (141–151)	Both	0.002
HKEMPFPK	P02666	β-casein (121–128)	Both	0.002
VKEAMAPK	P02666	β-casein (113–120)	Both	0.003
LEQLLRLKKY	P02662	α-s_1-casein (110–119)	Both	0.139
RPKHPIKHQGLPQEVLNENLLRFF	P02662	α-s_1-casein (16–39)	Both	0.020
VLNENLLR	P02662	α-s_1-casein (30–37)	Both	0.008
TKKTKLTEEEKNRL	P02663	α-s_2-casein (163–176)	Both	0.031
TKVIPYVRYL	P02663	α-s_2-casein (213–222)	Both	0.016
YYQQKPVA	P02668	κ-casein (63–70)	Both	0.055

2. Materials and methods

2.1. Materials

Milk casein was purchased from Gansu Hualing Dairy Co. Ltd (protein greater than 90%, Gansu, China). The *Streptococcus mutans* ATCC 25175, *Porphyromonas gingivalis* ATCC 33277 were acquired from BeNa Culture Collection Co. Ltd (Beijing, China). Trypsin (USP grade, 130 u/mg) was obtained from Shanghai Maclin Biochemical Co. Ltd (Shanghai, China). Dispase (5×10^5 u/g), papain (1×10^6 u/g), trypticase soy broth medium (TSB), brain heart infusion broth chlorhematin (BHI), agar powder, vitamin K, and L-cysteine hydrochloride were procured from Beijing Solarbio Science & Technology Co. Ltd (Beijing, China). Columbia blood agar medium was obtained from Nanjing Quanlong Biotechnology Co. Ltd (Nanjing, China). Yeast extract was obtained from Beijing Bairdi Biotechnology Co. Ltd (Beijing, China). BCA protein quantitation kit was acquired from Shanghai Biyuntian Biotechnology Co. Ltd (Shanghai, China). HCl was purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). HPLC solvents were obtained from Merck (Darmstadt, Germany). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of active CAMPs mixtures

The casein aqueous solution was prepared with a concentration between 2 and 8% and an appropriate pH value (Table S1). Protease was added into the casein solution and incubated at the proper temperature for 5 h to release potential CAMPs candidates (Table S1). Then, the solution was heated to 100 ℃ for 10 min to inactivate the enzyme and centrifuged (10, 000g × 20 min, 4 °C). After neutralization to pH 7.0, the supernatant was filtered through a 0.22 μm membrane filter (Tianjin Jinteng Co., China) and a 10 kDa ultrafiltration cube (Millipore Co., USA) in sequence. Finally, the filtrate was lyophilized (Beijing Songyuan Huaxing Technology Development CO., China) and stored at −80 °C until usage.

2.3. Antimicrobial activity optimization by response surface methodology (RSM)

RSM, combined with Box-Behnken (BB) design, was used to measure the individual and interactive effects of the process variables. The experiment design and data analysis used design-expert 8.0.6 (Stat-Ease, Inc., Minneapolis, USA). The substrate concentration (X_1), the amount of enzyme (X_2), and enzymatic hydrolysis time (X_3) were chosen as independent variables, while the inhibition rates of *S. mutans* (Y_1) and *P. gingivalis* (Y_2) were selected as the dependent variable. The result was entered into an empirical second order polynomial model. Statistical analysis of the data was considered significant at $p < 0.05$ (Huang et al., 2008).

2.4. Antimicrobial activity assay

2.4.1. Antimicrobial activity assay by spectrophotometric determination

Antimicrobial activity was first measured by a modified antibacterial assay method (Tang et al., 2013). *S. mutans* was cultured in sterilized BHI medium and *P. gingivalis* was grown in TBS medium supplemented with 5 μg/mL hemin, 1 μg/mL menadione and 4 mg/mL L-Cysteine (TBSHM). The bacterial suspension was diluted to 10^6 CFU/mL and added 100 μL/well to a 96-well microtiter plate. The culture medium was the blank, and the negative control was CAMPs culture medium solution under test. The positive control consisted of bacterial suspension (10^6 CFU/mL). The mixture suspension was incubated in an anaerobic incubator with an atmosphere containing 80 % N_2, 10% H_2O, and 10% CO_2 at 37 ℃ for 0, 3, 6, 9, 12, and 24 h. The absorbance was measured at a wavelength of 600 nm. The inhibition rate of bacteria was calculated using Eq. (1):

$$\text{Inhibition rate } (\%) = 1 - (OD_1 - OD_2/OD_3 - OD_0) \times 100\% \quad (1)$$

Where: OD_0 represents the absorbance of the culture medium OD_1 is the absorbance of the test group, OD_2 and OD_3 are the absorbance of negative and positive control, respectively.

The MIC measurement was performed according to previous reports (Klančnik et al., 2010; Tang et al., 2013; Touch et al., 2003). Each test was performed three times.

2.4.2. Antimicrobial activity assay by agar diffusion

Agar diffusion assay was carried out in accordance with previous reports (Moscoso-Mujica et al., 2021; Q. Zhao et al., 2020). *S. mutans* (10^6 CFU/mL) were suspended in sterilized BHI medium culture containing 1.5% (w/w) agar at 50 ℃. Meanwhile, *P. gingivalis* suspension was uniformly plated on a Columbia blood agar medium (10^6 CFU/mL). Drilled wells and gradient concentrations of CAMPs aqueous solution (12.5, 25 and 50 mg/mL) were added to the well (20 μL/well). Chlorhexidine aqueous solution (4MIC, 80 μg/mL) and sterile water as the positive and negative control, respectively. The plate was incubated at 37 °C for 24 h in an anaerobic incubator with an atmosphere containing 80 % N_2, 10% H_2O, and 10% CO_2. Subsequently, the diameter of the bacterial growth inhibition zone was measured.

2.5. Scanning electron microscopy (SEM) characterization

The microstructural and morphology of microbial and biofilm with or without CAMPs treatment were observed with Helium ion microscope (NanoFeb, Carl Zeiss AG, Germany), by mounting on the metal stubs at a high vacuum and using an accelerated voltage of 2.0 kV.

The *S. mutans and P. gingivalis* (5×10^5 CFU/mL) were cultured in the medium at 37 °C for 18 h in an anaerobic incubator with an atmosphere containing 80 % N_2, 10% H_2O, and 10% CO_2. Each suspension of *S. mutans* and *P. gingivalis* was treated by CAMPs for 24 h at 37 °C, chlorhexidine (4 MIC, 80 μg/mL), and without treatment as positive and negative controls, respectively. The suspension was centrifuged at 2000*g*

for 5 min. After washing with PBS, the microbial were fixed overnight with 4% paraformaldehyde at 4 °C. Samples were rinsed three times with distilled water and dehydrated with a graded series of ethanol (30, 50, 70, 90 and 100%, v/v) for 15 min at each concentration. Then, the samples were lyophilized by using speed vacuum freeze dryer (RVT 5105, Thermo Scientific Co., USA).

To evaluate the effect of CAMPs on preformed biofilms, the *S. mutans* and *P. gingivalis* (5×10^5 CFU/mL) were added to the cell slide and cultured in medium at 37 °C for 48 h in an anaerobic incubator with an atmosphere containing 80 % N_2, 10% H_2O, and 10% CO_2. After discarding the supernatant, the microbial were treated with CAMPs (50 mg/mL) at 37 °C, and chlorhexidine (4MIC, 80 μg/mL) and without CAMPs treatment as positive and negative controls, respectively. After 24 h, the cell slides were removed from incubation and washed with PBS. Then, the cell slides were fixed overnight with 4% paraformaldehyde at 4 °C and operated as described above.

2.6. Potential CAMPs identification by proteomic analysis

2.6.1. LC-MS/MS analysis

The procedures of LC-MS/MS analysis were similar to our previous works (Gu et al., 2022; Jin et al., 2016; Sun et al., 2020). Proteolytic peptides of casein were dissolved in mobile phase A (1 mL/L FA in H_2O) and analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer coupled with a Vanquish UPLC (Thermo Fisher, San Jose, CA). The peptide samples were loaded onto a 3 cm × 200 μm i. d. trap column packed with C18 particles (5 μm, 120 Å) with Buffer A, and separated by 30 cm × 150 μm i. d. separation column packed with C18 particles (3 μm, 120 Å). The binary reversed phase separation gradient from 5 to 35% mobile phase B (1 mL/L FA in acetonitrile) for 90 min at a flow rate of 500 nL/min.

The MS datasets were acquired in a data dependent acquisition (DDA) mode with a survey scan from 375 to 1650 *m/z* and a mass resolution of 60,000. The temperature of the ion transport capillary was 250 °C and the spray voltage was 2 kV. The fragment ions (MS/MS) were performed for the top 20 intense precursor ions with charge states 2–7 by high energy collision dissociation (HCD) mode with a mass resolution of 15, 000. The dynamic exclusion was enabled with an exclusive duration of 60 s. Each experiment was performed three times.

2.6.2. Database searching

The MS datasets were searched against the casein database (https://www.uniprot.org/) by using MaxQuant (Version 1.6.7.0) software. The database searching parameters were as follows: non-enzymatic digestion mode. The variable modifications included oxidation of methionine (+15.9949 Da), lysine acetylation (+42.011 Da), phosphorylation (+79.966 Da), and no fixed modification. Label-free quantification mode of MaxQuant was selected. The mass tolerance of the fragment ion is 0.5 Da, and the precursor ion in the first and main search is 20 and 4.5 ppm, respectively. The false discovery rates (FDR) of identified peptides and proteins were<1% and other parameters were the default values of MaxQuant (Chen et al., 2019). Extract matched antimicrobial peptides were searched from the Milk Bioactive Peptide Database (https://mbpdb.nws.oregonstate.edu) (Nielsen et al., 2017).

2.7. Statistical analysis

The statistic difference analysis was conducted by ANOVA and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. The generation and optimization of active CAMPs mixtures

Suitable protease is crucial for casein cleavage to release potential CAMPs. Previous studies have demonstrated that trypsin can be used to prepared bioactive peptides from milk, including antimicrobial peptides isolated from camel and cow milk by Wang et al. and bioactive peptides from liquid milk by Nath et al. (Pellegrini et al., 2004; Wang et al., 2020; Nath et al., 2021). In our results, we tested three types of casein proteolytic mixtures with dispase, papain, and trypsin cleavage and found that the tryptic peptide mixture displayed highest inhibition activity to oral cariogenic bacteria *S. mutans* and *P. gingivalis* (Fig. S1). Amphipathicity and charge are important feature of AMPs, as they determine solubility and affinity to lipid-rich membranes and electrostatic bonding with bacterial surfaces, respectively. Trypsin cleaves at the C-terminal of cationic arginine or lysine residues (Brogden, 2005), and prefers to generate potential CAMPs with cationic and amphipathic features. Therefore, trypsin was selected as the protease for further optimization of active CAMP mixtures.

Next, we investigated the proteolytic conditions influencing the antimicrobial activities of CAMP candidate mixtures. No significant difference in antimicrobial activity was found when the substrate concentration was under 2–5% (Fig. S2). The antimicrobial activity increased significantly along with the increase of enzyme amount (Fig. S2b and S3b). In contrast, there was no significant difference in antimicrobial activity when hydrolysis time was beyond 5 h (Fig. S2c and S3c).

Then, we fully optimized the preparation conditions of CAMP candidate mixture by RSM, which was suitable for multiple parameter optimization related to nonlinear data processing (Ferreira et al., 2007) (Table S2). After statistics analysis (ANOVA) of quadratic regression models (Table S3), the regression simulation equation of responses Y_1, Y_2 were established as:

$$Y_1 = 97.01 + 4.48X_1 + 21.36X_2 - 13.63X_3 - 8.10X_1X_2 - 1.89X_1X_3 + 19.00X_2X_3 - 1.73X_1^2 - 21.68X_2^2 - 2.71X_3^2 - 68.87X_1X_2X_3 \quad (2)$$

$$Y_2 = 94.07 + 0.22X_1 + 28.41X_2 - 7.77X_3 - 5.24X_1X_2 + 2.95\ X_1X_3 + 15.50\ X_2X_3 - 17.86\ X_1^2 - 21.92\ X_2^2 + 1.20X_3^2\text{-}85.85\ X_1X_2X_3 \quad (3)$$

where a "+" signified positive effect and "-" represent antagonistic effect.

The model equations of Y_1 and Y_2 were significant and adequate ($P < 0.05$, Table 1). The R^2 values of response Y_1 and Y_2 were more than or approach 0.9, indicating the fitted model could be used to explain the process under various conditions. The relationships between the responses and variables were illustrated by three-dimensional response diagrams (Fig. S4 and S5).

Overall, two optimized proteolytic conditions with similar antibacterial activity were obtained for the generation of active CAMP mixtures: substrate concentration 5.5%, enzyme amount 11, 000 u/g, reaction time 5 h for active mixture 1, and substrate concentration 4%, enzyme amount 10, 000 u/g, reaction time 3.2 h for active mixture 2. The active CAMP candidate mixtures prepared by optimal parameters had broad and desired antibacterial activity (Fig. 1 and Table S4). The yields of active mixture 1 and 2 were 49.0% and 38.0%, respectively. The MIC values (active mixture 1 and 2) against *S. mutans* and *P. gingivalis* were both about 3 and 6 μg/μL, respectively. The diameter of inhibition zones of *S. mutans* and *P. gingivalis* were 6.0, 5.5 and 5.8, 5.0 mm, respectively (Fig. S6).

As shown in Fig. 1, the two types of CAMP mixtures exhibited varying degrees of inhibition on different bacteria, which could be attributed to the of microorganisms' membrane and the physicochemical properties of peptides. The lipopolysaccharide (LPS) molecules, which serve as the primary target of the of the outer membrane of Gram-negative bacteria, are abundant in negative charge. The CAMPs with positive charge could destabilize the outer membrane by competition with divalent ions present on surface of the membrane. Subsequently, CAMP molecules could integrate into the cell membrane bilayer and ultimately inhibit cell proliferation or lead to cell death (Yin et al., 2020). On the other hand, the cell wall of Gram-positive bacteria lacks LPS and, instead, is composed of a thick layer of peptidoglycan and

teichoic acids. Similar to LPS, teichoic acids possess polyanionic properties, which are abundant in phosphate rich polymers and rely on divalent cations to maintain membrane stability (Neuhaus & Baddiley, 2003). Thus, both negative- and positive- charged CAMPs can damage the cell wall of Gram-positive bacteria. Therefore, two types of CAMP mixtures were employed in the subsequent experiments to characterize the morphological features of bacteria.

3.2. CAMPs activity evaluation by morphological characterization

The morphological of the *S. mutans* and *P. gingivalis* before and after the treatments of active CAMPs mixture 1 and 2 were characterized by SEM (Fig. 2). Generally, as shown in Fig. 2c and 2d, all kinds of oral pathogenic bacteria outside or within biofilms presented incomplete or irregular collapsed membrane surface after CAMPs mixture treatment (marked by red arrows). Compared with the positive control group with chlorhexidine drug treatment, the CAMPs groups presented similar alterations in pathogenic-bacteria surface topography (Fig. 2 L1 b-d and L2 b-d), indicating they had similar antimicrobial activity. Furthermore, the changes of bacteria surface topography after active mixture 1 treatment (Fig. 2c) were more significant than active mixture 2 (Fig. 2d), demonstrating active mixture 1 had higher activity in destroying the bacteria surfaces.

The formation of biofilm can significantly increase bacterial resistance to antibiotics and innate host defense, which is also one of the main virulence determinants in many bacterial infections. In our experiments, active CAMPs mixtures or chlorhexidine both exhibited inhibition effects on the attachment and development of biofilm (Fig. 2L3 and L4) and the morphology and membrane integrity of bacteria were also significantly changed. Overall, the optimized active CAMPs mixtures displayed desired antimicrobial activity to oral pathogenic bacteria even if within the biofilms. Therefore, the active CAMPs mixtures may be good alternatives to chlorhexidine drugs with better natural safety.

The morphology changes of membrane surface of pathogenic bacteria give a hint to explore the mechanism of antimicrobial activity of CAMPs (Ma et al., 2015; Mojsoska et al., 2017). As shown in Fig. 2, after treatment with CAMP mixture 1 and 2, the bacterial membrane was damaged and biofilm formation was inhibited. There were several small holes on the surface of the bacteria, indicating CAMPs might insert into the cell membrane bilayer and inhibit the bacterial proliferation.

3.3. Potential CAMPs identification by unbiased proteomic profiling

Based on the antimicrobial characteristics of casein hydrolyzed products, unbiased proteomic profiling was carried out to discovery the sequences of potential CAMPs from the active mixtures. After proteomic analysis and database searching, 1239 and 1291 distinct peptides were identified from the active CAMPs mixture 1 and 2. Furthermore, 1127 peptides were identified simultaneously from both samples, accounting for 80.3% of the total number of identified peptides. This indicated that peptides within the two active mixtures were highly similar but also had certain specificity. About 28% of these peptides were derived from α-s_1-casein, 25% from β-casein, and 22% from α-s_2-casein. The others were from κ-casein, β-lactoglobulin, α-lactalbumin, and lactotransferrin (Table S5). Compared with the sequences of bioactive peptides in the MBPDB database with a 100% match, 44 and 47 bioactive peptides, including ACE inhibition, antimicrobial, antioxidant, and immunomodulatory ones, could be fully matched with the identified results of active mixture 1 and 2, respectively (Table S6). Interestingly, 9 bioactive peptides with antimicrobial activity were identified in both active CAMPs mixtures (Tables 2 and 3). Most of these matched CAMPs (7/11) were positively charged with molecular weights (MWs) ranging from 800 to 3000 Da and Leu, Pro, Lys, Arg, Glu, and Val as predominant residues (Tables 2 and 3). As shown in Table 3, the relative abundances of antimicrobial peptides in total identified peptides were<0.3% in both active mixtures, while the relative abundances of bioactive peptides were no more than 2.1% (Table S6). Hence, it is usually the peptides with low abundances that exhibit bioactivity, which further demonstrates the advantage and necessity of large-scale proteomic profiling in potential CAMP discovery.

Compared with previous studies (Basilicata et al., 2018; Spada et al., 2021), we have identified more components and sequences of peptides from casein hydrolysate through the large-scale proteomic profiling, especially including the low-abundant ones as described earlier. By further matching against the MBPDB database with a sequence similarity of over 70%, 261 and 282 potential CAMPs were obtained from the active mixture 1 and 2, respectively. Overall, we have successfully generated a large number of bioactive peptides, which included 301 potential CAMPs in the active CAMP candidate mixtures through optimized proteolytic cleavage, further demonstrating their antimicrobial activity (Nielsen et al., 2017).

Based on the physicochemical properties of potential CAMPs, such as positive charged, MW between 1000 and 3000, and antipathy, we synthesized eight potential CAMPs identified from the active candidate mixtures. Then, their inhibition activities to oral pathogenic bacteria were investigated. As a result, 4 novel CAMPs HQGLPQEVLN, APKHKEMPFPK, ELQDKIHPF, and VEELKPTPEGD exhibited high inhibition activity to oral pathogenic bacteria (Table S7.).

Therefore, the peptide datasets of active CAMP mixtures provide valuable resources to discover more effective CAMPs for the inhibition of pathogenic bacteria.

4. Conclusion

Overall, we have successfully established an integrated strategy that combines *in vitro* proteolysis optimization, antimicrobial activity assay, unbiased proteomics profiling, and MBPDB database matching for the discovery of novel CAMPs to inhibit anaerobic oral pathogenic bacteria. Both the active CAMP mixtures and synthetic CAMP standards demonstrated high activity in damaging the bacteria membrane surfaces and inhibiting their growth, providing essential CAMP resources for the development of effective and safe antimicrobial reagents in oral periodontitis therapy. Furthermore, this powerful and high-throughput strategy is also suitable for discovery of novel AMPs to combat other pathogenic bacteria.

CRediT authorship contribution statement

Shizhe Qi: Investigation, Formal analysis, Writing – original draft. **Shan Zhao:** Investigation, Writing – review & editing. **Huiyan Zhang:** Investigation. **Shiwen Liu:** Validation. **Jiaxin Liu:** Formal analysis, Validation. **Jian Yang:** Conceptualization. **Yanxia Qi:** Conceptualization, Supervision, Writing – review & editing. **Qiancheng Zhao:** Conceptualization, Supervision. **Yan Jin:** Project administration, Supervision. **Fangjun Wang:** Project administration, Supervision, Writing – review & editing.

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.foodchem.2023.136454.

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