Stimulatory Effect of Milk Protein Peptides on the Growth and Propagation of *Lacticaseibacillus casei*

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Abstract

Lacticaseibacillus casei has a slow growth rate in milk. The present study sought to assess the influence of peptides derived from milk on the growth and propagation of *L. casei*. The milk protein peptides were generated using flavourzyme (FPMP) or neutral protease (NPMP). The peptide concentrations of $FPMP_{20\%}$-supplemented fermented milk tended to decrease throughout the fermentation process, furthermore the free amino acid (FAA) concentrations in the $FPMP_{20\%}$ sample (409.96 mg/kg) were much higher than in the control fermented milk (FM, 62.48 mg/kg) and $NPMP_{20\%}$ samples (226.34 mg/kg), indicating that FPMP could be well utilized by *L. casei*. The $FPMP_{20\%}$ sample reached the pH end-point of 3.7 at 42 h, while $NPMP_{20\%}$ and FM reached the same pH end-point at 67 h and 90 h. Although NPMP promoted acidification in the early stage of fermentation, the propagation and survival of *L. casei* were impaired. The number of viable *L. casei* cells in the $FPMP_{20\%}$, $NPMP_{20\%}$, and FM at 72 h was 9.58, 8.61, and 8.85 log CFU/g, respectively. Moreover, the contents of critical flavour components in $FPMP_{20\%}$ were significantly higher than in FM and $NPMP_{20\%}$. The FPMP-supplemented milk fermented with *L. casei* showed higher cell numbers, reduced fermentation time, and increased abundance of flavour components.

Keywords: *Lacticaseibacillus casei*, milk fermentation, flavourzyme, **peptide**, growth

Introduction

Lacticaseibacillus casei, previously known as *Lactobacillus casei*, is traditionally recognized as a probiotic and has numerous applications in the food fermentation industry (Bellaver et al., 2024; Sultana et al., 2023). *L. casei* has been reported to show several health-promoting and nutritional functions, such as immune system regulation, intestinal pathogen inhibition, obesity treatment, cardiovascular disease prevention, and microbiota-gut-brain axis modulation (Balasubramanian et al., 2024; Hill et al., 2018; Ibrahim et al., 2023; Pimentel et al., 2021). These promising functions have contributed to a gradual increase in consumer demand for this probiotic. Fermented milk has been widely used to produce probiotic products due to its favourable taste and high nutritional value. However, *L. casei* strains have a slow growth rate in milk; thus, producing fermented dairy products using pure *L. casei* strains is time-consuming (Ma et al., 2015b; Zhang et al., 2020b). Therefore, determining and optimizing the fermentation conditions of probiotic *L. casei* in milk have important practical application value.

L. casei is one of the most commonly used lactic acid bacteria (LAB), a general term for bacteria that can ferment lactose to produce lactic acid, and they have similar physiological characteristics and metabolic pathways. LAB are typically auxotrophic to some amino acids and vitamins and cannot biosynthesize several nutrients (Koduru et al., 2022). Thus, LAB strains are nutritionally fastidious and require many FAA or peptides that are present in trace amounts in milk, which is insufficient to sustain growth

(Lin et al., 2021; Playford and Weiser, 2021). In the field of milk fermentation, the proteolytic system of LAB has gained considerable attention due to its ability to hydrolyze milk casein into peptides or amino acids, ensuring successful reproduction. The first step in milk casein utilization by LAB is performed by cell envelope proteases (CEP), as the production of amino acids depends on the extracellular protease activity (Koduru et al., 2022). Although *L. casei* and *Lacticaseibacillus paracasei* possess a complete proteolytic system like CEP, the protease activity is low (Satılmış et al., 2023; Solieri et al., 2018), which may be a limiting factor for culture in milk. Boulay et al. (2020) found that *Streptococcus thermophilus* CEP PrtS involved the proteolysis of soya proteins, and the deletion of PrtS gene from *S. thermophilus* resulted in slow acidification and low growth levels. In another study, peptides served as a potential nitrogen source for *Bifidobacterium animalis* ssp. *lactis* during fermentation (Zhang et al., 2024), which could be effectively utilized by different bifidobacterial strains through the oligopeptide transport systems (Cui et al., 2022). A previous study reporting the effect of enzymatic hydrolysis of milk proteins on the growth of *Lactobacillus gasseri* found that oligopeptides were the optimal nitrogen source rather than free amino acids or proteins (Arakawa et al., 2015). In summary, peptides play a key role in the fermentation process of LAB. Therefore, utilizing exogenous proteolytic enzymes to improve the growth and propagation of probiotic *L. casei* during milk fermentation has high significance.

Currently, research on peptides initially present in milk and their effect on the metabolism and propagation of *L. casei* is scarce. Therefore, this study aimed to obtain

milk protein peptides using different proteases (neutral protease and flavourzyme), determine the effects of these peptides on *L. casei*, evaluate the properties of fermented milk, and explore whether peptides may act as a growth-stimulatory factor for *L. casei*.

Materials and Methods

Bacterial strains and culture conditions

L. casei LC2W (China General Microbiological Culture Collection Center, No. 0828) was isolated from traditional dairy products in Inner Mongolia, China, and obtained from Bright Dairy & Food Co., Ltd. (Shanghai, China). *L. casei* 01 was obtained from Chr. Hansens (Hørsholm, Denmark). *L. casei* strains were cultured in MRS agar (Oxoid, Basingstoke, UK) under anaerobic conditions (Bugbox Anaerobic System, Ruskinn, Bridgend, UK) with 95% N_2 and 5% CO_2 at 37 ℃ for 36 h. Single colonies were sub-cultured twice in MRS broth overnight in an MIR-253 incubator (Sanyo, Osaka, Japan) for starter preparation. Both *L. casei* strains were prepared as direct vat-set cultures (2 × 10^{11} CFU/g) in State Key Laboratory of Dairy Biotechnology (Shanghai, China).

Generation of peptides from milk protein

The peptides were obtained through hydrolysis of skim milk according to the method described by Nongonierma and FitzGerald (2013) with slight modifications. Skim milk

105 powder (33.4% protein, Fonterra Ltd., Auckland, New Zealand) was reconstituted in
106 distilled water to produce reconstituted skim milk (RSM; 12%, w/w). Neutral protease
107 NP (Danisco Company, Copenhagen, Denmark) or flavourzyme PB03 (a
108 protease/peptidase complex; Pangbo Biological Engineering Co., Ltd., Nanning, China)
109 were then added to RSM at a concentration of 0.1% (w/w) and dispersed under agitation
110 at 50 ℃ for 60 min using an overhead stirrer (RW20, IKA, Staufenim, Germany). The
111 protease-added RSM was sequentially hydrolyzed at 50 ℃ for 240 min without stirring.
112 Later, the enzyme was inactivated by heating the hydrolysis sample at 90 ℃ for 20 min.
113 The NPMP and FPMP were stored at 4 ℃ until use within 30 d.
114
115 **Peptide supplementation during *L. casei* milk fermentation**
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117 NPMP or FPMP was added to the 12% RSM base, replacing 5, 10, 20, or 40% of the
118 base (w/w). Twelve percent of RSM without peptide supplementation was used as the
119 control. The media was heated to 95 ℃ for 90 min using a GFL1002 water bath (GFL
120 Company, Burgwedel, Germany) and then cooled to 37 ℃. The prepared media was
121 then inoculated with *L. casei* LC2W or *L. casei* 01 (5×10^6 CFU/g) and incubated at
122 37 ℃. The pH values of fermented milk were monitored and measured using a Cinac
123 system (Alliance Instruments, Mery-Sur-Oise, France), with automatic recording every
124 5 min during milk fermentation. The number of viable *L. casei* cells was determined
125 every 24 h for 72 h during fermentation by culturing on MRS agar, and the cell numbers
126 in 72 h-fermented milk samples were further monitored each week during cold storage

(4 ℃) for one month.

The fermented milk samples supplemented with 20% NPMP ($NPMP_{20\%}$) and 20% FPMP ($FPMP_{20\%}$) and FM were incubated at 37 ℃ until the pH value reached 3.70. All samples were then cooled and stored at 4 ℃ for 24 h. Finally, the volatile flavour compounds and FAA compositions were determined.

Change in peptide levels during fermentation

The peptide contents in the three fermented milk samples (FM, $NPMP_{20\%}$, and $FPMP_{20\%}$) at 0, 24, 48, and 72 h were measured according to the method described by Dhakal et al. (2024) with some modifications. The reagent was prepared by mixing 25 mL of 100 mM borax, 2.5 mL of 20% (w/w) sodium dodecyl sulfate, 40 mg of o-phthaldialdehyde solution (dissolved in 1 mL of methanol), and 100 μL of β-mercaptoethanol and adjusting the final volume to 50 mL with deionized water. The samples were filtered using an ultrafiltration tube with a molecular weight cut-off at 10 kDa (Millipore, Billerica, MA), and 50 μL was mixed with 2 mL of reagent. This reaction mixture was combined for 2 min at room temperature (about 25 ℃), and the absorbance at 340 nm was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The peptide concentrations were quantified using casein tryptone (Difco Laboratories, Sparks, MD) as a standard.

Free amino acid analysis

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150 The fermented milk samples were pre-treated to remove the proteins, and the amino
151 acids were extracted following the method described by Ma et al. (2015a). The amino
152 acid content of the samples was analyzed using a high-performance liquid
153 chromatography (HPLC) fitted with a sodium cation exchange amino acid analysis
154 column (4 × 150 mm) and an o-phthalaldehyde post-column derivation system
155 (Pickering, Mountain View, CA). The equipment was coupled with a Waters 510 pump,
156 a 7725i manual injector, and a 363-fluorescence detector (Varian Inc., Walnut Creek,
157 CA). The flow rate was 1.7 mL min^{-1}. The elution was performed by applying a linear
158 gradient of 100% solution A for 1 min, followed by 0 – 100% solution B over the
159 subsequent 48 min (solution A: 0.2 M sodium citrate, pH = 3.0; solution B: 0.2 M
160 sodium borate, pH = 9.8).

161

162 **Analysis of the volatile flavour compounds**

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164 The volatile compounds in the fermented milk were extracted through headspace solid-
165 phase micro-extraction according to the method described by Ma et al. (2015a). The
166 fiber used for manual extraction was DVB/CAR/PDMS (Supelco, Bellefonte, PA). The
167 volatile compounds were analyzed using an Agilent 7890 (II) gas chromatograph
168 (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 5975 series mass
169 selective detector. The SPME fiber was inserted into the injection port that was held at
170 250 °C, and the compounds were thermally desorbed for 4 min under the split (1:10)

conditions. A DB-Wax column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies) was used to separate the volatile compounds. The temperature of the column was maintained at 45 °C for 5 min, ramped at 10 °C min^{-1} to 80 °C, and then further increased to 240 °C at the rate of 5 °C min^{-1}. The carrier gas was helium (1 mL min^{-1}). The mass spectrometer was run in the electron impact mode at 70 eV. The mass scan range was 25 to 400 m/z. The concentrations of volatile flavour compounds in the samples were expressed as the peak area of each compound.

Statistical analysis

All experiments were conducted in triplicate, and the results were presented as average or average ± standard deviation (SD). Statistical differences between treatments were evaluated through ANOVA and Tukey's post hoc test ($p < 0.05$) using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Effect of peptide supplementation on the acidification ability of *L. casei*

After hydrolysis for 5 h, the pH values of the neutral protease and flavourzyme-supplemented RSM samples were changed slightly from 6.65 to 6.41 and 6.37,

192 respectively. A small protein precipitate was observed, indicating that enzyme
193 hydrolysis destabilized the skim milk emulsion. A large protein precipitate was
194 observed in the heat-sterilized media when over 40% NPMP or FPMP was added to the
195 RSM (data not shown). Thus, 5%, 10%, and 20% NPMP or FPMP supplementation
196 were used to evaluate the optimal growth conditions. Compared to the control FM, in
197 the early stage of fermentation (0-24 h), 5-20% of NPMP or FPMP significantly
198 promoted the acidification of *L. casei*. In the middle and late stages of fermentation
199 (24-72 h), FPMP10% and FPMP20% still had a significant stimulating effect on
200 fermentation and acid production of *L. casei* (Table 1). FM reached the pH end-point
201 (3.70) at 90 h, while $FPMP_{20\%}$ and $NPMP_{20\%}$ reached the same pH end-point only at 42
202 h and 67 h, respectively (Fig. 1). These results indicated that FPMP had a positive
203 impact on the acidification ability of *L. casei* strains, suggesting that peptide
204 supplementation shortened the production time, improved production efficiency and
205 reduced the risk of microbial contamination.

206 LAB are microorganisms with fastidious requirements. In this study, FPMP or
207 NPMP supplementation compensated for the weak proteolytic capacity of *L. casei*,
208 notably in the early growth phase to overcome the low concentrations of FAA and
209 peptides in milk. These results were consistent with the previous studies reporting that
210 supplemental peptides could be well utilized by *Lactobacillus rhamnosus* and
211 *Bifidobacterium bifidum* (Cui et al., 2022; Zhang et al., 2021).

212

213 **Effect of peptide supplementation on the growth and propagation of *L. casei***

214

215 The nutrient composition of the medium might affect the viable cell counts in the
216 fermented milk culture. Herein, FPMP supplementation significantly enhanced the
217 propagation of *L. casei.* For instance, the numbers of viable *L. casei* cells at 24, 48, and
218 72 h in the $FPMP_{20\%}$ samples were 8.67, 9.39, and 9.58 log CFU/g, respectively, with
219 a significant increase of 0.44, 0.47, and 0.73 log CFU/g ($p < 0.05$), compared with the
220 control (Fig. 2a). These results indicated that FPMP strongly stimulated both the
221 acidification and propagation of *L. casei*. However, NPMP supplementation did not
222 promote the reproduction of *L. casei* in milk fermentation. There was no significant
223 difference in the number of *L. casei* cells between NPMP20% supplemented sample
224 and the control FM at 24 h, 48 h and 72 h (Fig. 2a). Moreover, cell loss during cold
225 storage in the $NPMP_{20\%}$ samples was the highest among the three samples (FM,
226 $FPMP_{20\%}$, and $NPMP_{20\%}$). The FPMP20% samples maintained the highest number of
227 viable *L. casei* cells throughout the 28 day cold storage. During 14-28 day storage time,
228 the cell counts of the NPMP20% samples were significantly lower ($p < 0.05$) than that
229 of the control FM and FPMP20% samples (Fig. 2b).

230 NPMP promoted acidification (0-24 h), suggesting that these peptides could be
231 utilized by *L. casei*. However, NPMP might impair the propagation during fermentation
232 and survival during the storage of *L. casei*. Therefore, it was speculated that some
233 peptides in NPMP could stimulate the acidification and growth of *L. casei,* while others
234 might inhibit the growth of *L. casei*. The antibacterial activity of peptides derived from
235 milk has been given special attention. Hydrophobic peptides derived from sheep milk

could severely damage the cell membrane integrity (Yang et al., 2024) and the peptides released from yak milk casein using flavourzyme showed strong activity against *Staphylococcus aureus* (Zhang et al., 2023a). Zhang et al. (2010) reported that peptides with different molecular weights had significantly different influences on the growth of yoghurt starter strains (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*), and the peptides with molecular weights below 3 kDa might effectively promote the growth and metabolism of LAB. In another study, different bifidobacteria transported and utilized peptides with different residues due to the specificity and variability of their peptide transport systems (Cui et al., 2022). In this study, the use of different types of enzymes for hydrolysis resulted in different peptide profiles for each hydrolysate. NPMP likely contained protein hydrolysates that inhibited *L. casei*. In contrast, the inhibitory hydrolysates in FPMP were further degraded by flavourzyme consisting of endoprotease and peptidase. As for the comparison of neutral proteases, flavourzyme might obtain more small peptide fragments. Enzymatic hydrolysis is a modest and efficient approach for obtaining soluble peptides from milk protein. Different enzyme types produce hydrolysates with different peptide profiles and have different stimulatory or inhibitory effects. Nevertheless, future studies must focus on screening suitable types of proteases, determining the molecular mass distribution of peptide fractions, analyzing the specificity of peptide transport systems and clarifying the stimulatory role of different peptide fractions in *L. casei*.

Analysis of changes in peptide concentrations

The peptide concentration in RSM was very low (0.036 mg/g, Fig. 3). In the control FM, the peptide concentrations increased as fermentation progressed from 0 – 24 h (Fig. 3), suggesting that the CEP of *L. casei* played a key role in hydrolyzing extracellular caseins to oligopeptides at this stage. As the peptides were transported and utilized intracellularly, the peptide concentrations in the milk gradually decreased from 24 – 72 h. In $NPMP_{20\%}$ and $FPMP_{20\%}$-supplemented samples, the initial peptide concentrations were high at 1.687 mg/g and 1.556 mg/g, respectively. The peptide concentrations in $FPMP_{20\%}$-supplemented milk tended to decrease throughout the fermentation process (Fig. 3), indicating that FPMP could be well utilized by *L. casei*, and the peptide generation rate was lower than the rate of consumption. The peptide concentrations in $NPMP_{20\%}$-supplemented milk decreased from 0 – 24 h but increased slightly from 24 – 48 h. These results further suggested that some NPMP could be preferentially utilized by *L. casei*, while others were not utilized well. The peptide consumption rate in $NPMP_{20\%}$-supplemented milk was lower than the rate of generation, which was consistent with the finding that NPMP could not promote the propagation and survival of *L. casei*.

Free amino acid analysis

FAA analysis is a promising approach to evaluate the protein hydrolysis activity in

fermented dairy products based on the FAA contents (Garavand et al., 2023). After fermentation, there was a notable rise in the FAA concentrations in all three milk samples (Table 2). The increase in the total FAA in the $FPMP_{20\%}$ samples was much greater than in the FM at the end of fermentation (341.06 mg/kg for $FPMP_{20\%}$ and 54.93 mg/kg for FM). It was worth noting that both before and after fermentation, some essential amino acids such as leucine, valine, methionine and isoleucine in FPMP20% samples were much higher than in the FM and $NPMP_{20\%}$ samples (Table 2).

Casein can be degraded to amino acids by LAB only when milk proteins are hydrolyzed to oligopeptides. Herein, FPMP was easily utilized and the FAA concentrations in the $FPMP_{20\%}$ samples were the highest. Flavourzyme degradation obtained the highest FAA contents during the hydrolysis of lentil protein concentrates due to its exopeptidase activity (Vogelsang-O'Dwyer et al., 2023). Although the initial peptide concentrations in the $NPMP_{20\%}$ and $NPMP_{20\%}$ samples were equivalent (1.687 mg/g for $NPMP_{20\%}$ vs. 1.556 mg/g for $FPMP_{20\%}$) with no significant difference ($p > 0.05$), the total FAA levels in the $NPMP_{20\%}$ samples were much lower than in the $FPMP_{20\%}$ samples (409.96 mg/kg for $FPMP_{20\%}$ vs. 226.34 mg/kg for $NPMP_{20\%}$). It was possible that some NPMPs could not be effectively utilized and degraded to FAA, which was consistent with the smaller decrease in peptide concentrations in the $NPMP_{20\%}$ samples.

Flavour compound analysis

Table 3 displays the volatile flavour compounds detected in three fermented milk samples with an end-point pH 3.7. *L. casei* produced heptanone and nonanone as the primary volatile aroma compounds in all three samples, followed by nonanol, pentanone, acetic acid, and undecanone. The amounts of these volatile compounds were the highest in the $FPMP_{20\%}$ samples. The amount of only a few aldehydes and carboxylic acids (for example, butanal, octanal, nonanal, and butanoic acid) were slightly lower in $FPMP_{20\%}$ than in FM or $NPMP_{20\%}$. The flavor is crucial for the food senses. Heptanone was believed to give fermented milk a fruity and cinnamon aroma; nonanone was believed to give fermented milk a green and floral aroma (Shi et al., 2024). The volatile aromatic compounds in fermented products are largely derived from peptides and FAA. These ingredients are transformed into flavour compounds through milk protein degradation and metabolic pathways of branched amino acids such as leucine, isoleucine and valine (Tian et al., 2023; Zhang et al., 2023b). FPMP supplementation significantly enhanced the initial peptide and FAA contents in the fermented milk. Therefore, the characteristic volatile flavour compounds in *L. casei*-fermented milk were enriched, and the enrichment improved when the milk protein was partially hydrolyzed in the initial stage of fermentation. Further, as these hydrolyzed peptides are derived from milk, the "off-flavour" caused by the additions of exogenous ingredients, such as yeast powder, soybean peptone, and tea, can be eliminated. Therefore, pre-treatment of milk using flavourzyme could be considered for large-scale

fermentation, which provides an optimized strategy for improving probiotic fermented dairy products.

Conclusions

In this study, the supplementation of neutral protease or flavourzyme significantly increased the peptide contents in milk. The results showed that the peptides could be efficiently utilized by *L. casei* and the peptide contents in the FPMP-supplemented fermented milk tended to decrease throughout the fermentation process. The peptide contents in the NPMP-supplemented fermented milk decreased in the early stage of fermentation but slightly increased in the middle stage of fermentation, indicating some NPMP were not utilized efficiently by *L. casei*. NPMP promoted the acidification of *L. casei* while impairing the propagation and survival of *L. casei*. FPMP supplementation significantly stimulated the growth and propagation of probiotic *L. casei* during milk fermentation. Moreover, $FPMP_{20\%}$ supplementation significantly increased the FAA levels and the contents of critical flavour components in $FPMP_{20\%}$ fermented milk were significantly higher than in FM and $NPMP_{20\%}$ fermented milk. Furthermore, higher cell numbers, reduced fermentation time, and increased abundance of flavour components were achieved in FPMP-supplemented fermentation with *L. casei*. Overall, this approach could be an economically effective method for large-scale fermentation.

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Author Contributions

Chengjie Ma: Formal analysis; Validation; Writing-original draft; Writing-review & editing; **Miya Su**: Conceptualization; Methodology;Writing-review & editing; **Zhenmin Liu**: Validation; Writing-review & editing; **Kun Wang**: Formal analysis; Writing-review & editing; **Rui Wang**: Investigation; Writing-review & editing.

Conflict of interest

The authors declare no confict of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Tables:

Table 1 Influence of different types and concentrations of peptide supplements on the pH of fermented milk

Table 2 Change of free amino acids after fermentation with and without peptide supplementation

Table 3 Peak area values as determined by gas chromatography mass spectrometer for flavor compounds in fermented milk with and without milk protein peptide

Figure legends:

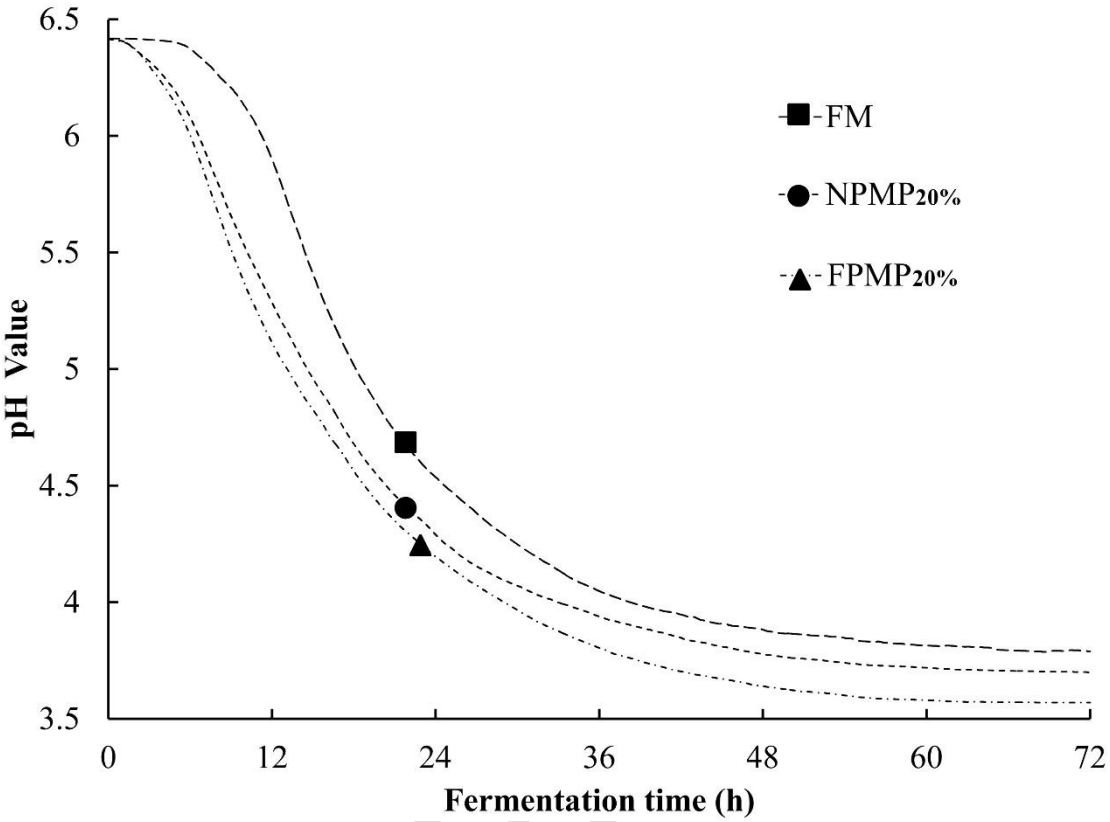


Fig.1 The pH values during fermentation supplemented with and without milk protein peptide by *Lacticaseibacillus casei* LC2W.

FM: fermented milk without supplementation; $NPMP_{20\%}$: fermented milk containing 20% NPMP; $FPMP_{20\%}$: fermented milk containing 20% FPMP.

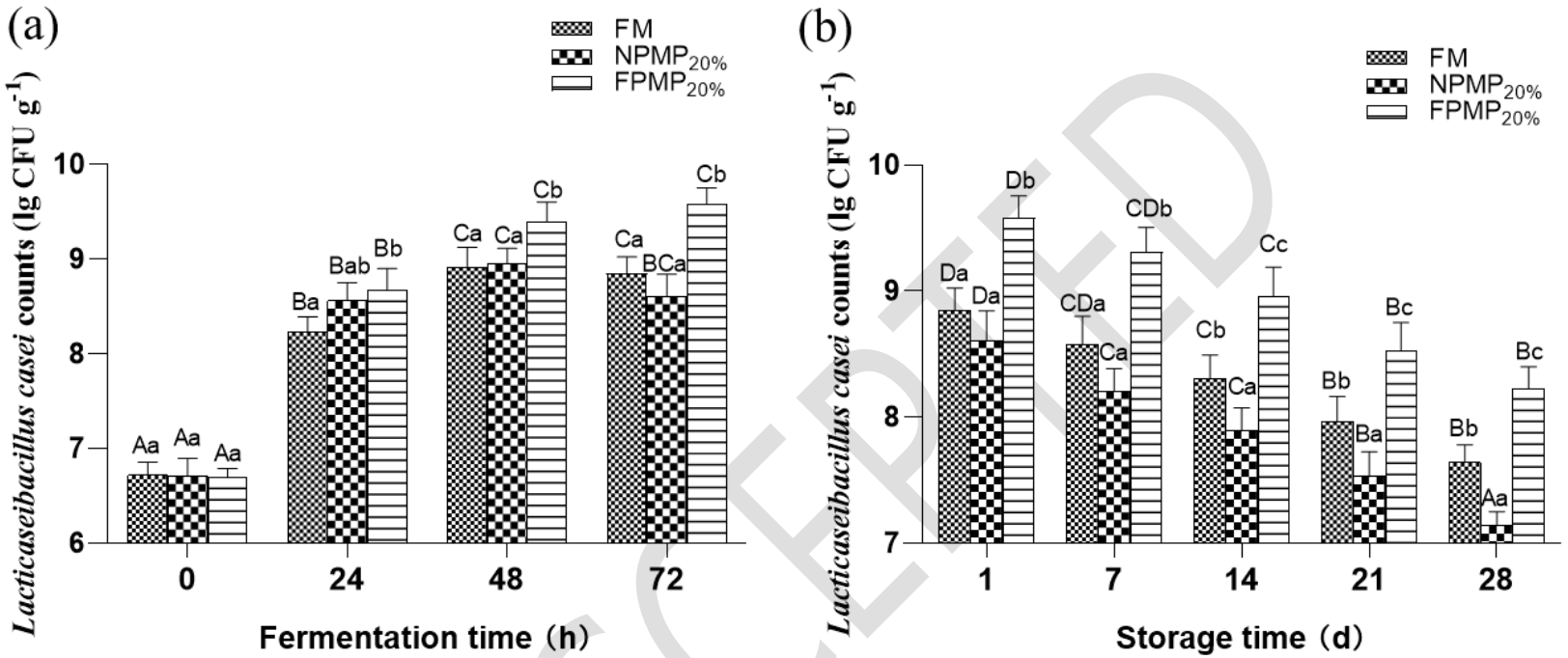
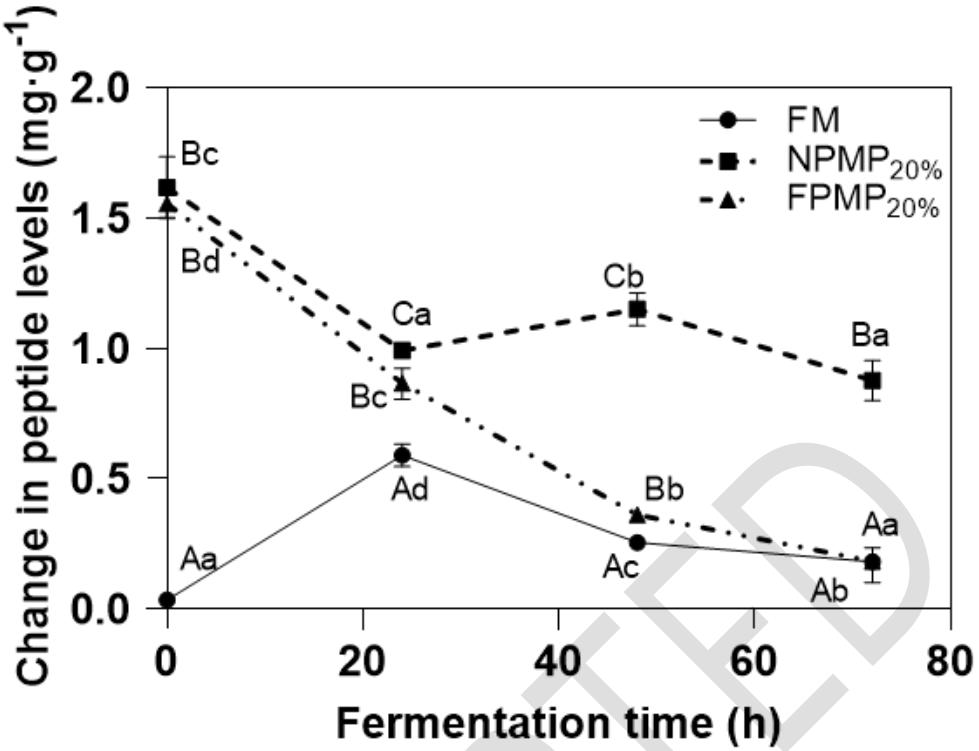


Fig. 2 The viable cell counts of *Lacticaseibacillus casei* during milk fermentation and cold storage with *Lacticaseibacillus casei*. (a) during milk fermentation and (b) during cold storage.

All values are mean ± SD (n=3). The different superscripted lowercase letters indicate significant differences ($p < 0.05$) between different samples at the same time. The different superscripted capital letters indicate significant differences ($p < 0.05$) of the same samples at different fermentation time or storage time.



469

470 **Fig. 3** Change in peptide concentrations of different fermented milk with

471 *Lacticaseibacillus casei* 01.

472 All values are mean ± SD (n=3). The different superscripted lowercase letters indicate

473 significant differences ($p < 0.05$) of the same samples at different fermentation time.

474 The different superscripted capital letters indicate significant differences ($p < 0.05$) of

475 the different samples at the same fermentation time.

Table 1 Influence of different types and concentrations of peptide supplements on the pH of fermented milk

Fermentation time (h)	pH values						
	FM	**$NPMP_{5\%}$**	**$NPMP_{10\%}$**	**$NPMP_{20\%}$**	**$FPMP_{5\%}$**	**$FPMP_{10\%}$**	**$FPMP_{20\%}$**
0	6.42 ± 0.03^{Da}	6.41 ± 0.04^{Ca}	6.40 ± 0.04^{Ca}	6.41 ± 0.03^{Ca}	6.41 ± 0.05^{Ca}	6.39 ± 0.04^{Ca}	6.41 ± 0.03^{Ca}
24	4.51 ± 0.07^{Cc}	4.37 ± 0.08^{Bbc}	4.31 ± 0.07^{Bab}	4.26 ± 0.06^{Bab}	4.35 ± 0.09^{Bb}	4.24 ± 0.07^{Bab}	4.17 ± 0.08^{Ba}
48	3.85 ± 0.06^{Bc}	3.80 ± 0.05^{Abc}	3.77 ± 0.07^{Abc}	3.75 ± 0.05^{Abc}	3.75 ± 0.07^{Abc}	3.69 ± 0.06^{Aab}	3.61 ± 0.07^{Aa}
72	3.76 ± 0.06^{Ac}	3.74 ± 0.05^{Ac}	3.73 ± 0.04^{Ac}	3.68 ± 0.05^{Abc}	3.68 ± 0.04^{Abc}	3.62 ± 0.06^{Aab}	3.57 ± 0.04^{Aa}

The pH values at 0 h were measured after sterilization. FM: fermented milk without supplementation; $NPMP_{5\%}$, $NPMP_{10\%}$, $NPMP_{20\%}$, $FPMP_{5\%}$, $FPMP_{10\%}$ and $FPMP_{20\%}$: fermented milk containing 5% NPMP, 10% NPMP, 20% NPMP, 5% FPMP, 10% FPMP and 20% FPMP. The starter strain was *Lacticaseibacillus casei* 01. All the pH values are mean of three replications ± SD. Samples with different superscripted capital letters in the same column are significantly different ($p < 0.05$); samples with different superscripted lowercase letters in the same row are significantly different ($p < 0.05$).

Table 2 Change of free amino acids after fermentation with and without peptide supplementation

Amino acids	FAA contents before fermentation (mg kg^{-1})			FAA contents after fermentation (mg kg^{-1})		
	FM	$NPMP_{20\%}$	$FPMP_{20\%}$	FM	$NPMP_{20\%}$	$FPMP_{20\%}$
Aspartic	0.03±0.01^{a}	0.03±0.01^{a}	0.70±0.05^{c}	0.35±0.04^{b}	0.74±0.05^{c}	3.15±0.2^{d}
Threonine	0.13±0.02^{a}	0.14±0.02^{a}	1.21±0.05^{c}	0.21±0.03^{b}	1.78±0.13^{d}	3.06±0.12^{e}
Serine	0.12±0.03^{a}	0.12±0.02^{a}	0.71±0.06 ^{b}	1.42±0.12^{c}	1.81±0.11^{d}	2.31±0.15^{e}
Glutamic	0.14±0.03^{a}	0.15±0.03^{a}	0.52±0.03^{b}	1.76±0.11^{c}	13.31±0.25^{d}	15.14±0.3^{e}
Proline	0.28±0.03^{a}	0.27±0.03^{a}	1.13±0.03^{b}	13.98±0.35^{c}	27.34±0.64^{d}	36.22±0.82^{e}
Glycine	0.14±0.02^{a}	0.14±0.03^{a}	0.33±0.02^{b}	0.71±0.04^{c}	1.53±0.05^{d}	2.95±0.08^{e}
Alanine	0.36±0.04^{a}	0.34±0.05^{a}	2.14±0.07^{b}	3.21±0.09^{c}	10.79±0.55^{d}	14.26±0.86^{e}
Cystine	0.45±0.02^{b}	0.46±0.03^{b}	2.61±0.08^{c}	0.35±0.03^{a}	2.51±0.21^{c}	2.57±0.06^{c}
Valine	1.36±0.06^{a}	1.32±0.04^{a}	4.31±0.12^{b}	15.19±0.43^{c}	33.31±0.68 ^{d}	39.84±0.86^{e}
Methionine	0.02±0.01^{a}	0.04±0.01^{b}	11.16±0.32^{d}	0.04±0.02^{b}	2.19±0.24^{c}	11.73±0.42^{e}
Isoleucine	0.64±0.06^{a}	0.63±0.07^{a}	3.18±0.26^{b}	12.61±0.7^{c}	19.02±0.85^{d}	31.62±0.98^{e}
Leucine	0.12±0.02^{a}	0.41±0.02^{b}	22.83±0.82^{d}	6.46±0.48^{c}	50.28±1.13^{e}	131.17±2.4^{f}
Tyrosine	0.62±0.06^{b}	0.59±0.07^{b}	3.41±0.23^{c}	0.49±0.05^{a}	16.62±0.35^{d}	35.87±1.5^{e}
Phenylalanine	0.27±0.01^{b}	0.25±0.02^{b}	2.58±0.21^{c}	0.22±0.01^{a}	15.57±0.57^{d}	32.24±1.25^{e}
Lysine	0.06±0.01^{a}	0.12±0.02^{b}	6.62±0.32^{d}	1.69±0.42^{c}	23.05±0.89^{e}	32.83±2.6^{f}
Histidine	2.03±0.14^{a}	1.98±0.22^{a}	4.42±0.34^{b}	2.16±0.24^{a}	5.03±0.56^{c}	11.02±0.81^{d}
Arginine	0.78±0.07^{a}	0.80±0.08^{a}	1.04±0.12^{b}	1.63±0.15^{c}	1.46±0.13^{c}	3.98±0.32^{d}
Total amino acids	7.55±0.28^{a}	7.79±0.33^{a}	68.90±0.96^{c}	62.48±0.84^{b}	226.34±2.25^{d}	409.96±3.18^{e}

FM: fermented milk without supplementation; $NPMP_{20\%}$: fermented milk containing 20% NPMP; $FPMP_{20\%}$: fermented milk containing 20% FPMP. The starter strain was *Lacticaseibacillus casei* 01. All values are mean ± SD (n=3). Different superscript letters in the same row indicate significant differences between different samples at $p < 0.05$.

Table 3 Peak area values as determined by GC-MS for flavor compounds in fermented milk with and without milk protein peptide supplementation

Compound	Averaged peak area (× 10, 000)		
	FM	$NPMP_{20\%}$	$FPMP_{20\%}$
Acetaldehyde	50.6 ±1.6[a]	89.2±2.9[b]	50.9±2.1[a]
Butanal	35.1±1.2[b]	68.3 ±1.9[c]	20.3±0.8 [a]
Acetone	109.2 ±3.3[a]	208.7±3.1[c]	172.7±4.2[b]
2-Pentanone	496.8±7.1[a]	542.6±9.4[b]	1232.6±14.9[c]
2-Heptanone	1094.8±22.4[a]	2776.4±35.7[b]	6077.1±54.6[c]
Octanal	36.7±1.9[b]	41.9±2.3[c]	23.3±1.2[a]
Propenal	ND	7.1±0.9	ND
2-Heptanol	250.1±5.8[b]	302.1±7.1[c]	213.4±3.9[a]
2-Nonanone	1721.9±31.5[a]	3439.5±56.3[b]	3847.9±82.3[c]
Nonanal	201.3±4.6[b]	319.2±6.3[c]	175.4±3.4[a]
Nonanol	36.4±2.3[a]	78.3±3.5[b]	2298.5±33.4[c]
Acetic acid	59.6±3.6[a]	121.7±5.1[b]	605.2±17.6[c]
Decanal	ND	ND	68.1±3.2
1-Hexanol	25.2±1.3[a]	158.3±4.3[b]	191.7±9.3[c]
1-Octanol	24.7±0.8[a]	37.6±1.4[b]	61.5±1.7[c]
1,6-Octadien-3-ol	ND	7.8	ND
2-Undecanone	226.5±3.3[a]	609.3±7.8[c]	462.2±6.7[b]
Benzoic acid	12.7±0.5[a]	20.8±0.7[b]	21.1±0.6[b]
Butanoic acid	85.3±2.6[c]	61.3±1.5[b]	43.9±1.1[a]
Hexanoic acid	136.2±3.1[c]	21.4±0.8[a]	77.9±2.1[b]
Octanoic Acid	62.5±1.7[c]	18.6±0.5[a]	56.4±1.3[b]

FM: fermented milk without supplementation; $NPMP_{20\%}$: fermented milk containing 20% NPMP; $FPMP_{20\%}$: fermented milk containing 20% FPMP. The starter strain was *Lacticaseibacillus casei* 01. ND: not detected. All values are mean of three replications ± SD. Different superscript letters in the same row indicate significant differences at $p < 0.05$.