Inoculum size of co-fermentative culture affects the sensory quality and volatile metabolome of fermented milk over storage

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ABSTRACT

*Lactocaseibacillus paracasei* PC-01 is a probiotic candidate isolated from naturally fermented yak milk in Lhasa, Tibet, and it has been shown to possess excellent milk fermentation properties. This study used *Lactocaseibacillus paracasei* PC-01 as a co-fermentation strain to investigate the effect of inoculum size with a commercial starter in milk fermentation on the product flavor and profile of volatile metabolites over 28 d of cold storage. *Lactocaseibacillus paracasei* PC-01 was allowed to ferment in pasteurized milk with or without the commercial starter (YF-L904) at 42°C until the pH decreased to 4.5. The finished fermented milks were stored at 10°C for 28 d. Milk samples were taken at hour 0 (before fermentation) and then at d 1, 14, and 28 of cold storage. Different inoculum sizes of *Lactocaseibacillus paracasei* PC-01 had no significant effect on pH or titratable acidity during storage of fermented milk. Viable counts of strain PC-01 continued to increase during cold storage of the fermented milk. Generally, as storage of fermented milk proceeded, the overall sensory quality score decreased in all groups. However, the overall sensory scores of PC-01-M were generally higher than those of other groups, suggesting that a medium dose of *Lactocaseibacillus paracasei* PC-01 had the most obvious effect of slowing the decline in sensory quality of fermented milk during storage. Changes in sensory scores and consumer preferences were accompanied by increases in both the quantity and variety of key volatile metabolites in fermented milk during fermentation, post-ripening (d 1), and storage. Major differentially abundant metabolites, including acetaldehyde, methyl ketones, medium-chain and short-chain fatty acids, 2,3-butanedione, and acetoin, were enriched in fermented milks rated highly in the sensory evaluation. Our data confirmed that the inoculum size of co-fermentative culture affected the sensory quality and volatile metabolome of fermented milk over storage, and an optimal range of co-fermentative culture was titrated in this work.

Key words: *Lactocaseibacillus paracasei* PC-01, inoculum size, fermented milk, gas chromatography-mass spectrometry, volatile metabolites

INTRODUCTION

Yogurt is a dairy product that has a long history of consumption around the world. It is produced by milk fermentation, which was fermented by *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Codex Alimentarius, 2003). As the public is more health conscious (Shiby and Mishra, 2013), nutritive foods that add desirable effects are preferred. One good example is probiotic yogurt, which contains beneficial microbes that are known to promote health.

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO/FAO, 2002). Fermented milk is one of the best probiotic carriers, and commonly used probiotics in yogurt are mostly lactobacilli and bifidobacteria, which are generally considered safe for food use (Lourens-Hattingh and Viljoen, 2001; Floch et al., 2016). Because probiotics have to survive through the gastrointestinal tract, where they confer beneficial effects to the host, high viability during fermentation and after fermentation is necessary. It is recommended that the quantity of viable cells be maintained at a level >10⁶ cfu/mL during the shelf life of the product to ensure their ability to exert beneficial effects (Koh et al., 2019; Sakandar and Zhang, 2021). However, these bacteria also play a role in shaping the sensory quality of the fermented milk during production and storage.
Flavor directly influences consumer preferences for products (Ott et al., 2000). Many studies have demonstrated that adding probiotics confers positive effects on the sensory quality of fermented milk because co-fermentation of probiotics and starter cultures increases the production of desirable flavors. The influence of probiotics on the flavor compounds of fermented milk is strain-specific. Peng et al. (2022) found that adding *Lacticaseibacillus casei* Zhang and *Bifidobacterium lactis* V9 together with starter cultures in co-fermentation increased the content of acetaldehyde in fermented milk. Similarly, Tian et al. (2017) reported that application of *Lacticaseibacillus casei* LC2W increased the levels of butyric acid and methyl ketone in fermented milk. Dimitrellou et al. (2019) found that the addition of *Lactobacillus casei* ATCC 393 significantly affected the concentrations of key volatile compounds, such as acetic acid, acetoin, and 2-butanone in yogurt. These studies demonstrated that applying probiotic bacteria in combination with a conventional starter culture would improve the sensory quality, especially flavor, of fermented milk products.

Sensory evaluation is the most intuitive and important way to evaluate the flavor of a product, but the results are greatly affected by the state of tasters when the evaluation is performed. Metabolomics objectively profiles the small molecules present in samples, and it is increasingly used to detect chemical contents in food samples. Solid-phase microextraction (SPME) is a straightforward and sensitive method for extracting volatile metabolites from fermented milk (Merkle et al., 2015), and it is compatible with downstream analysis by GC-MS for determining the composition and concentration of chemicals in samples (Sfakianakis and Tzia, 2017).

Although some previous works have analyzed the changes in volatile metabolites and related metabolic pathways during probiotic fermented milk production, the specific role of probiotics as active players in shaping product sensory quality seems to have been neglected. Particularly, few studies have addressed the effect of inoculum size of probiotics as a co-fermentative microbe in fermented milk production. It would be of interest to titrate a suitable inoculum size of probiotics for co-fermentation, so that they could be added in an adequate amount to serve their beneficial functions in the host and impart desirable flavor to the fermented milk, while not adversely affecting the sensory quality of the fermented product, especially during or after product storage.

*Lacticaseibacillus paracasei* PC-01 was isolated from naturally fermented yak milk in Lhasa, Tibet. A previous study showed that this strain had high tolerance to simulated gastrointestinal fluids and bile salts, suggesting high probiotic potential (Lv et al., 2021), and the strain has been shown to possess excellent milk fermentation properties. Thus, the objective of this study was to use *Lacticaseibacillus paracasei* PC-01 as a co-fermentation strain to investigate the inoculum size effect of its application together with a commercial starter in milk fermentation on the product flavor and profile of volatile metabolites over a 28-d cold storage period. A sensory evaluation in conjunction with metabolomics analysis was performed in this study. Our findings also provide guidelines for an optimal dose range when using closely related strains as co-fermentation cultures in fermented milk production.

**MATERIALS AND METHODS**

**Starter Cultures and Probiotic Strain**

*Lacticaseibacillus paracasei* PC-01 was provided by the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. The strain has been preserved at the China General Microbiological Culture Collection Center (preservation number CGMCC No. 17537). The commercial yogurt starter culture (YF-L904 in direct-vat-set form, purchased from Chr. Hansen Co. Ltd.) comprised *Lactobacillus delbrueckii* ss. *bulgaricus* and *Streptococcus thermophilus*.

**Preparation of Fermented Milk**

Stirred fermented milk was made from Holstein cow milk (3.0 g/100 g of CP, Kjeldahl factor = 6.38, 3.7 g/100 g of fat). A high-pressure homogenizer was used to dissolve and homogenize sucrose (6%) into the cow milk at 20 MPa (Bai et al., 2020). Then, the milk mixture was pasteurized at 90°C for 10 min, followed by cooling to 37°C. After cooling, the milk was divided into 4 batches, and each was inoculated with different combinations of starter cultures: YF-L904 (0.03 g/L) alone or YF-L904 (0.03 g/L) plus differing concentrations of *Lacticaseibacillus paracasei* PC-01 (2 × 10^6 cfu/g, 5 × 10^6 cfu/g, or 1 × 10^7 cfu/g). The 4 groups of fermented milk were designated control (YF-L904 only), PC-01-L, PC-01-M, and PC-01-H (inoculated with YF-L904 plus low, medium, and high concentrations of *Lacticaseibacillus paracasei* PC-01, respectively). After inoculation, the milk samples were fermented at 42°C until the pH decreased to 4.5 (5.5 h). The finished fermented milks were poured into sanitized screw-cap bottles and ripened at 4°C for 1 d. Then the fermented milk samples were stored at 10°C for 28 d (Salvador and Fiszman, 2004). Fermented milk samples were taken every 2 wk during storage. The count of viable bacteria, determi-
nation of acidity, and evaluation of sensory properties were carried out immediately after sampling. For GC-MS, the samples were stored at −40°C until analysis.

**Measurement of pH and Titratable Acidity**

The pH was measured at 20°C using a FE28 pH meter. The titratable acidity (TA) was determined by titration with 0.1 mol/L NaOH using phenolphthalein as color indicator (Guo et al., 2021).

**Plate Counts**

Fifteen grams of fermented milk was mixed with 135 g of sterilized 0.9% (wt/vol) saline before serial dilution. Vancomycin-added de Man, Rogosa, and Sharpe (MRS) agar was used as a selective medium for growing *Lacticaseibacillus paracasei* PC-01. Appropriate amounts of diluted fermented milks were inoculated on the agar plates, followed by incubation at 37°C for 72 h before colony counting (Aryana and McGrew, 2007).

**Sensory Evaluation**

Ten trained panelists (4 men and 6 women) were selected to participate in the sensory evaluation. The sensory evaluation adopted a 100-point system to score odor, flavor, texture, and taste (Table 1). Participants were guided to taste the fermented milks and to drink water between samples to cleanse the palate according to specific scoring rules (Pan, 2020).

**Determination of Volatile Components by SPME-GC-MS**

An SPME system was used to extract volatile metabolites in the fermented milk samples. Before sampling, an SPME fiber was inserted into the injection port of a 7890B GC (Agilent Technologies Inc.) and allowed to age at 250°C for 5 min. For each analysis, 5 mL of fermented milk and 50 μL of internal standard (1,2-dichlorobenzene) were added into a 15-mL flask with the SPME fiber (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane; Supelco). The internal standard was diluted with ultrapure water, and the final concentration of internal standard solution in each sample was 10 μg/L. The sample was extracted for 1 h at 50°C with low-speed stirring (400 r/min) with a magnetic stirrer bar. After the extraction step, the SPME fiber was immediately inserted into the injection port of a 7890B GC for 3 min at 270°C to desorb the volatile compounds into the GC. Volatile compounds determined by GC-MS were identified by matching their mass spectra with those deposited in the National Institute of Standards Technology (NIST) Mass Spectral Database (https://chemdata.nist.gov/). The internal standard method was used for quantitative analysis of volatile compounds. The relative abundances of identified volatile compounds were semi-quantified as peak areas in total ion chromatography, and the concentrations of the volatile components were calculated by dividing volatile peak areas by internal standard peak areas from the same sample (Dan et al., 2018).

**Statistical Analysis**

All measurements were repeated at least 3 times. The results of average plate counts were converted to the base-10 logarithm of colony-forming units per gram of fermented milk (log10 cfu/g). MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used for statistical analysis of GC-MS data. Before multivariate statistical analysis, all data were normalized by Pareto scaling and log transformation (base 10). Multiple statistical methods, including principal component analysis (PCA), heatmap visualization, t-test, fold-change analysis, and

<table>
<thead>
<tr>
<th>Sensory item (possible points)</th>
<th>Standard</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smell (30)</td>
<td>The sample smells good, with rich aroma of fermented milk.</td>
<td>25–30</td>
</tr>
<tr>
<td></td>
<td>The sample has special aroma of fermented milk.</td>
<td>20–24</td>
</tr>
<tr>
<td></td>
<td>The sample has no special aroma of fermented milk or has slightly peculiar smell.</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Flavor (30)</td>
<td>The sample tastes delicious, with slight sweetness and sour and mellow yogurt smell.</td>
<td>25–30</td>
</tr>
<tr>
<td></td>
<td>The sample tastes sweet or sour without off-flavor.</td>
<td>20–24</td>
</tr>
<tr>
<td></td>
<td>The sample tastes too sweet or sour with a small amount of off-flavor.</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Texture (20)</td>
<td>No bubbles, uniform coagulation, smooth appearance, no whey precipitation, milky white.</td>
<td>15–20</td>
</tr>
<tr>
<td></td>
<td>No bubbles, uniform coagulation, slightly rough appearance, a small amount of whey is precipitated, slightly yellow.</td>
<td>10–14</td>
</tr>
<tr>
<td></td>
<td>Bubbles, uneven coagulation, rough appearance, whey precipitation, slightly gray or yellow.</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Taste (20)</td>
<td>The sample tastes smooth and creamy.</td>
<td>15–20</td>
</tr>
<tr>
<td></td>
<td>The sample tastes not smooth enough.</td>
<td>10–14</td>
</tr>
<tr>
<td></td>
<td>The sample tastes rough.</td>
<td>&lt;10</td>
</tr>
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partial least squares-discriminant analysis (PLS-DA) with a variable importance on projection (VIP), were used to analyze differences between treatment groups. Volatile metabolites with \( P < 0.05 \), fold-change >2 or <0.5, and VIP >1 were selected as significantly different metabolites (Peng et al., 2022).

RESULTS

pH and Titratable Acidity

The pH and TA are key indicators for assessing fermented milk. They have an effect not only on the sensory quality of the product but also on the viable probiotic count during storage. All 4 fermented milks showed similar trends in pH and TA during fermentation and product storage (Figure 1). After 5.5 h of fermentation, the pH generally decreased below 4.5 and reached the fermentation end point (data not shown).

The pH in all treatment groups generally declined from 4.3 to around 4.0 during postfermentation storage, with no significant difference between groups \( (P > 0.05) \). When the fermented milk reached the fermentation end point, the average TA was moderate \( (82.5–83.5°T) \), with no significant differences between groups. After the 28-d storage, the TA of fermented milk gradually increased to around 120°T, with no significant difference between groups \( (P > 0.05) \).

Viable Counts of Lacticaseibacillus paracasei PC-01

Viable counts of *Lacticaseibacillus paracasei* PC-01 increased throughout the fermentation and storage period (Figure 2). However, the bacterial level was highest for PC-01-H throughout, because of the large inoculum size applied initially compared with the other 2 fermented milks, whereas PC-01-L had the lowest viable counts throughout. At d 28, the viable counts of PC-01-L, PC-01-M, and PC-01-H were 7.84 ± 0.01, 8.13 ± 0.01, and 8.22 ± 0.00 log cfu/mL, respectively (Figure 2).

![Figure 1](https://example.com/figure1.png)

*Figure 1.* Changes in pH and titratable acidity (TA) of fermented milks during milk fermentation, post-ripening (0 h to 1 d) and storage (1 to 28 d). Control = inoculated with commercial starter (YF-L904) only; PC-01-L, PC-01-M, and PC-01-H = inoculated with both YF-L904 and various inoculum sizes of *Lacticaseibacillus paracasei* PC-01 \( (2 \times 10^6 \text{ cfu/g, } 5 \times 10^6 \text{ cfu/g, and } 1 \times 10^7 \text{ cfu/g, respectively}) \). Error bars represent SD \( (n = 3) \).
Sensory Evaluation of Fermented Milk

The sensory evaluation of fermented milk was scored by smell, flavor, texture, and taste. The PC-01-M group outscored the other 3 groups in terms of smell, taste, and total score \((P < 0.05)\) on the first day of storage (Figure 3). Additionally, the smell scores of all PC-01 groups were significantly higher than those of the control treatment \((P < 0.05)\). The sensory scores of fermented milks generally decreased with storage time, probably because of the accumulation of lactic acid and other metabolites. After 14 and 28 d of storage, the sensory scores of all groups continued to decline (Figure 3). However, the total sensory score of the PC-01-M group was always significantly higher than that of other groups \((P < 0.05)\).

Differences in Volatile Metabolome Between Control and Probiotic Fermented Milks on the First Day of Storage

A total of 57 volatile metabolites were detected across all groups after postfermentation ripening (1 d) by GC-MS. Symbols representing PC-01 groups (with low, medium, and high doses of probiotic inoculum) were closely clustered and were distinct from those representing the control group, suggesting clear differences between the volatile metabolomes between PC-01 and control groups (Figure 4a). Consistently, the heatmap of the profiles of volatile metabolomes showed that the 3 PC-01 groups shared higher similarity compared with the control group (Figure 4b).

A total of 43 different volatile metabolites were identified in control and PC-01-L (Figure 5a). The 2 groups shared 18 common metabolites, whereas 8 and 17 volatile metabolites were unique to control and PC-
Two discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: decanoic acid and octanoic acid (Figure 5a). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

In total, 39 different volatile metabolites were identified in control and PC-01-M (Figure 5b). The 2 groups shared 21 common metabolites, whereas 5 and 13 volatile metabolites were unique to control and PC-01-M, respectively. Seven discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: 2,3-butanedione, decanoic acid, acetaldehyde, octanoic acid, 2-undecanone, 2-nonanone, and acetoin (Figure 5b). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

A total of 46 different volatile metabolites were identified in control and PC-01-H (Figure 5c). The 2 groups shared 19 common metabolites, whereas 7 and 20 volatile metabolites were unique to control and PC-01-H, respectively. Eight discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change, i.e., acetone, octanoic acid, 3,4-dimethyl-2-hexanol, decanoic acid, 2-undecanone, 2-nonanone, acetaldehyde, and 2-heptanone (Figure 5c). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

Adding Lactobacillus paracasei PC-01 seemed to affect both the content and concentration of some volatile metabolites in the fermented milk. Compared with that of the control, concentrations of octanoic acid and decanoic acid in PC-01 groups increased significantly. The concentrations of some common fermented milk flavor compounds, including acetaldehyde, 2-undecanone, and 2-nonanone, were significantly higher in the PC-01-M and PC-01-H groups than in the control. Furthermore, the typical fermented milk flavor compounds, 2,3-butanedione and acetoin, were greatly elevated in PC-01-M.

**Differences in Volatile Metabolome Between Control and Probiotic Fermented Milks After 14 Days of Storage**

A total of 80 volatile metabolites were detected across all groups after 14 d of storage. Similar to the PCA analysis of fermented milk volatile metabolomes at d 1, the symbols representing the 3 PC-01 groups were...
closely clustered and distinct from those representing the control group; however, a more obvious trend of probiotic dose-based clustering patterns was also observed among the 3 PC-01 subgroups, suggesting that there were probiotic dose-dependent variations in the overall fermented milk metabolome after 14 d of storage (Figure 6a). Consistent results were seen in the cluster heatmap of fermented milk volatile metabolomes, with a greater difference existing between control and 3 PC-01 subgroups than among the 3 PC-01 subgroups. Moreover, the volatile metabolomes of PC-01-M and PC-01-H, but not PC-01-L, formed a close cluster, suggesting that the volatile metabolomes of PC-01-M and PC-01-H were more similar (Figure 6b).

A total of 63 different volatile metabolites were identified in control and PC-01-L (Figure 7a). The 2 groups shared 28 common metabolites, whereas 14 and 21 volatile metabolites were unique to control and PC-01-L, respectively. Two discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: pyruvic acid and 3-hydroxybutanal (Figure 7a). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

A total of 60 different volatile metabolites were identified in control and PC-01-M (Figure 7b). The 2 groups shared 25 common metabolites, whereas 16 and 19 volatile metabolites were unique to control and PC-01-M, respectively. Ten discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: pyruvic acid, 5-hydroxymethylfurfural, butanoic acid, hydroxyacetone, acetoin, 4,8-dimethyl-1-nonanol, 7-oxooctanoic acid, acetaldehyde, tetradecanoic acid, and dodecanoic acid (Figure 7b). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

A total of 55 different volatile metabolites were identified in control and PC-01-H (Figure 7c). The 2 groups shared 15 common metabolites, whereas 25 and 15 volatile metabolites were unique to control and PC-01-H, respectively. Nine discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: pyruvic acid, 5-hydroxymethylfurfural, butanoic acid, tetradecanoic acid, dodecanoic acid, heptanal, hydroxyacetone, acetaldehyde, and acetoin (Figure 7c). Their identified and quantified information is shown in supplemental data.
Some obvious changes occurred in the contents of some metabolites after 14 d of storage. The concentrations of pyruvic acid were dramatically reduced in all PC-01 groups compared with control. Similar trends of significant declines ($P < 0.05$) were seen in some other metabolites, including 5-hydroxymethylfurfural, hydroxyacetone, tetradecanoic acid, dodecanoic acid, and acetaldehyde, in PC-01-M and PC-01-H compared with control, whereas the concentrations of acetoin and butanoic acid increased significantly.

### Differences in Volatile Metabolome Between Control and Probiotic Fermented Milks After 28 Days of Storage

A total of 69 volatile metabolites were detected across all groups. Volatile fermented milk metabolome-based PCA revealed distinct clustering patterns of symbols representing the 4 groups (control, PC-01-L, PC-01-M, and PC-01-H) on the score plot, suggesting obvious differences between groups (Figure 8a). However, it is interesting to note that control and PC-01-L groups were located closer to each other (both in the left quadrants) than to cluster PC-01-M (at lower right quadrant) or cluster PC-01-H (at upper right quadrant), respectively (Figure 8a). The cluster heatmap of volatile metabolomes (Figure 8b) revealed clustering of the control and PC-01-L pair, and of the PC-01-M and PC-01-H pair, confirming that the overall volatile metabolite profiles were more similar between control and PC-01-L, and between PC-01-M and PC-01-H, implicating that using larger inoculum sizes (medium or high doses) of \textit{Lacticaseibacillus paracasei} PC-01 had a greater effect on the overall volatile metabolome when the fermented milks were stored for a prolonged period (e.g., over 14 d). In contrast, applying a low dose of \textit{Lacticaseibacillus paracasei} PC-01 did not result in drastic changes in the overall volatile metabolome, even at a late time point of 28 d, comparing with control without adding the probiotic strain.

A total of 59 different volatile metabolites were identified in control and PC-01-L (Figure 9a). The 2 groups shared 28 common metabolites, whereas 16 and 15 volatile metabolites were unique to control and PC-01-L, respectively. Venn diagrams show the number of common and differential metabolites between treatment pairs. Variable importance on projection (VIP) plots show the differential volatile metabolites identified by partial least squares-discriminant analysis (PLS-DA), $t$-test, and fold-change analysis between treatment pairs.

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**Figure 5.** Differential abundant metabolites identified between control and (a) PC-01-L, (b) PC-01-M, and (c) PC-01-H on the first day of storage. Control = inoculated with commercial starter (YF-L904) only; PC-01-L, PC-01-M, and PC-01-H = inoculated with both YF-L904 and various inoculum sizes of \textit{Lacticaseibacillus paracasei} PC-01 ($2 \times 10^6$ cfu/g, $5 \times 10^6$ cfu/g, and $1 \times 10^7$ cfu/g, respectively). Venn diagrams show the number of common and differential metabolites between treatment pairs. Variable importance on projection (VIP) plots show the differential volatile metabolites identified by partial least squares-discriminant analysis (PLS-DA), $t$-test, and fold-change analysis between treatment pairs.
A total of 46 different volatile metabolites were identified in control and PC-01-M (Figure 9b). The 2 groups shared 20 common metabolites, whereas 16 and 10 volatile metabolites were unique to control and PC-01-M, respectively. Seven discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: butanoic acid, acetaldehyde, 2,3-pentanediol, 2-methyl-3-pentanone, 1,2-cyclopentanedione, 3-methyl-1-hexanone, and acetoin (Figure 9b). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

A total of 52 different volatile metabolites were identified in control and PC-01-H (Figure 9c). The 2 groups shared 21 common metabolites, whereas 16 and 15 volatile metabolites were unique to control and PC-01-H, respectively. Eleven discriminatory volatiles were identified between the 2 groups by PLS-DA with VIP, t-test, and fold-change: 2-furanmethanol, 4,8-dimethyl-1-nonanol, acetaldehyde, 2,3-pentanediol, 1,2-cyclopentanediol, 2-methyl-3-pentanone, 2-undecenoic acid, octadecanoic acid ethene ester, 2,3-butanediol, 2-propyl-1-heptanone, and 2,2-dimethyloctanol (Figure 9c). Their identified and quantified information is shown in supplemental data (https://data.mendeley.com/datasets/73xw89p9ps/1).

After 28 d of storage, the concentrations of acetaldehyde and 2,3-pentanediol were considerably lower in all PC-01 groups than in the control. Compared with the control, the concentrations of acetoin and butanoic acid were significantly higher in PC-01-M and the concentration of 2,3-butanediol was higher in PC-01-H.

DISCUSSION

Probiotics are live bacteria that confer health-promoting effects to the host when consumed in an adequate amount. Probiotic activity depends largely on the viability and activity of these bacteria. Fermented milk is thought to be a good probiotic carrier for maintaining the viability of these beneficial bacteria. However, it is likely that, because of their activity, these viable bacteria could cause postfermentation changes when used in fermented milk. Thus, it would indeed be of interest to titrate a suitable inoculum size of probiotics to avoid drastic changes in the sensory quality of fermented milk during postfermentation storage. To our knowledge, only a few studies have compared the
effects of inoculum size of probiotic co-fermentation bacteria (3 different concentrations of *Lacticaseibacillus paracasei* PC-01 in this study) on the sensory quality and changes in fermented milk metabolome during storage.

No significant difference was observed in the viable counts, pH, TA, or time to reach the fermentation end point between groups during milk fermentation and storage, even after 28 d of cold storage, regardless of the presence of *Lacticaseibacillus paracasei* PC-01, suggesting that they were not the major bacteria responsible for milk acidification. Notably, viable counts of *Lacticaseibacillus paracasei* PC-01 continued to increase after fermentation even at a low pH, confirming that they were viable and highly active throughout the product’s shelf life.

Consumer preference is directly determined by the sensory characteristics of fermented milk. Sensory evaluation is a comprehensive index closely linked to physical and chemical indicators of the fermented milk, such as acidity and composition of the milk metabolome. Generally, the sensory score of fermented milk of all groups decreased with storage time, probably because of the accumulation of acids and other metabolites after prolonged storage. However, PC-01-M group outscored the other 3 groups in terms of smell, taste, and total score ($P < 0.05$) at the start of the cold storage (1 d). Moreover, during cold storage, the total sensory scores of PC-01-M and PC-01-H were substantially higher than that of the control; the smell, flavor, and total sensory scores of PC-01-M were consistently highest among all groups. The results of PCA of the fermented milk volatile metabolomes were in line with that of the sensory scores. A probiotic dose-dependent trend of variation was observed in the overall fermented milk volatile metabolome, with a milder effect observed when using the smallest inoculum size of *Lacticaseibacillus paracasei* PC-01. Compared with the control, the application of higher doses of *Lacticaseibacillus paracasei* PC-01 (PC-01-M and PC-01-H) caused more obvious changes in fermented milk volatile metabolomes after 14 or 28 d of storage. As the storage of fermented milks was extended to 28 d, the overall sensory score of PC-01-H was lower than that of PC-01-M, accompanied by a significantly higher level of 2,3-butanedione but significantly lower amounts of acetoin and butanoic acid in PC-01-H compared with PC-01-M. Such undesirable metabolite changes in PC-01-H likely compromised its sensory quality. These results suggested that the addition of *Lacticaseibacillus paracasei* PC-01 was generally desirable. However, different inoculum sizes of *Lacticaseibacillus paracasei* PC-01 affected the sensory quality of the final products in a dose-dependent manner, and a medium dose of *Lacticaseibacillus paracasei* PC-01...
would be optimal from the perspectives of sensory quality and stability of fermented milk.

The basic metabolites of fermented milk are produced by the action of co-fermentation of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus (Beshkova et al., 1998). Many studies have found that adding probiotics to fermented milk affected the composition and concentration of volatile metabolites during fermentation and storage (Tian et al., 2017; Farag et al., 2021), changing product flavor and quality. This work profiled the volatile metabolites using GC-MS to assess chemical changes in fermented milks at post-ripening (1 d) and every 2 wk during storage (14 d and 28 d). Because the sensory scores of the probiotic fermented milk were consistently higher than those of the control fermented milk, differences in volatile metabolites between the 2 groups were investigated. Several discriminating volatile metabolites were identified, including acetaldehyde, 2,3-butanedione, acetoin, 2-undecene, 2-nonanone, 2-heptanone, butyric acid, decanoic acid, octanoic acid, tetradecanoic acid, and dodecanoic acid, most of which are indeed common metabolites in fermented milk that contribute to the organoleptic characteristics of fermented dairy products. In fact, these metabolites are known to be generated by lactic acid bacteria, and their related metabolic pathways are summarized in Figure 10 (Cheng, 2010; Martin et al., 2011; Fincheira et al., 2017).

Acetaldehyde is a typical flavor component in fermented milk that imparts fresh and green apple flavor (Cheng, 2010; Chen et al., 2017). Lactic acid bacteria can synthesize acetaldehyde through a variety of metabolic pathways (Liu, 2014); for example, by converting threonine to acetaldehyde by threonine aldolase or by dehydrogenation of ethanol. Acetaldehyde can also be produced from pyruvic acid (Settachaimongkon et al., 2014), either directly by decarboxylation of pyruvate or indirectly through the production of the intermediate acetyl-CoA first. Compared with control, the concentrations of acetaldehyde in PC-01-M and PC-01-H groups were significantly higher at d 1, probably because of highly active pyruvate metabolism during fermentation and post-ripening in the presence of a relatively large amount of Lacticaseibacillus paracasei PC-01. The expression of pyruvic acid metabolic genes has been shown to be upregulated in Lacticaseibacillus paracasei PC-01 cells during the lag phase (Feng, 2021). After 14 and 28 d of cold storage, the acetaldehyde...
levels in PC-01 groups were clearly lower than those of
the control. A previous report noted that *Lactobacillus*
could improve acetaldehyde metabolism (Nosova et al.,
2000).
2,3-Butanedione and acetoin are key volatile metab-
olites that contribute to the distinctive aroma of
fermented milk (Geng et al., 2018; Zheng et al., 2020).
2,3-Butanedione, also known as diacetyl, imparts
fermented milk cream and vanilla flavors (Cheng,
2010; Chen et al., 2017). Pyruvic acid can be con-
verted to 2,3-butanedione through the intermediate
α-acetolactate (Cheng, 2010). At d 1, the concentra-
tion of 2,3-butanedione in PC-01-M was considerably
higher than that in the control. After 28 d of storage,
the content of 2,3-butanedione in PC-01-H was signifi-
cantly higher than that in the control. Acetoin can be
converted from 2,3-butanedione by diacetyl reductase
(Comasio et al., 2019), which contributes to the sweet
and buttery flavor of fermented milk. The acetoin fla-
vor softens the harshness of 2,3-butanedione. The com-
bination of acetoin and 2,3-butanedione enriches the
mild, pleasant, and buttery flavor of fermented milk
(Cheng, 2010). Acetoin concentrations in PC-01-M
were consistently and significantly higher than those
in control at post-ripening (d 1) and throughout stor-
age. Our data suggested that adding a medium dose
of *Lacticaseibacillus paracasei* PC-01 as co-fermentative
bacteria significantly enhanced the flavor of fermented
milk, which was accompanied by an increase in a vari-
ety of desirable flavor molecules.
Saturated fatty acids in fermented milk can be con-
verted into methyl ketones through β-oxidation (Hu et
al., 2020). Our results showed that on d 1, the con-
centrations of 2-nonanone and 2-undecone in PC-01-M,
as well as the concentrations of 2-nonanone, 2-unde-
cone, and 2-heptanone in PC-01-H, were considerably
greater than those in control. 2-Nonanone and 2-hep-
tanone have a fruity flavor, whereas 2-undecone has a
floral and herbal flavor (Cheng, 2010). The results
suggested that the addition of a medium or high dose
of *Lacticaseibacillus paracasei* PC-01 together with the
starter bacteria accelerated β-oxidation of SFA during
fermentation and post-ripening, increasing the contents
of methyl ketones. In contrast, Sun et al. (2021) found
that the effect of lactic acid bacteria on methyl ketones
varied between strains.
Milk acidification in fermented milk production pro-
cess was a result of the action of lactic acid bacteria in
metabolizing carbohydrates, lipids, and proteins into
various kinds of acids. Probiotics can hydrolyze lipids
to release free fatty acids, leading to an increase in
short-chain and medium-chain fatty acids (C2 to C12)
in fermented milk (Hu et al., 2020). A previous study
found that supplementing the probiotic strain *Lacti-

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**Figure 9.** Differential abundant metabolites identified between control and (a) PC-01-L, (b) PC-01-M, and (c) PC-01-H after 28 d of storage. Control = inoculated with commercial starter (YF-L904) only; PC-01-L, PC-01-M, and PC-01-H = inoculated with both YF-L904 and various inoculum sizes of *Lacticaseibacillus paracasei* PC-01 (2 × 10⁶ cfu/g, 5 × 10⁶ cfu/g, and 1 × 10⁷ cfu/g, respectively). Venn diagrams show the number of common and differential metabolites between treatment pairs. Variable importance on projection (VIP) plots show the differential volatile metabolites identified by partial least squares-discriminant analysis (PLS-DA), t-test, and fold-change analysis between treatment pairs.
caseibacillus casei Zhang could increase the levels of decanoic acid and octanoic acid in fermented milk (Sun et al., 2021), whereas applying both Lacticaseibacillus casei LC2W and Lacticaseibacillus paracasei IMC502 significantly increased the content of butyric acid (Tian et al., 2017; Gu et al., 2020). Our data showed that, at d 1, contents of octanoic acid and decanoic acid in fermented milks inoculated with Lacticaseibacillus paracasei PC-01 were significantly higher than those of the control group, indicating stronger and accelerated actions of the added probiotics in proteolysis and fat oxidation during fermentation and post-ripening. Both octanoic acid and decanoic acid impart desirable flavor quality to the resultant fermented milks. Octanoic acid provides a soapy, goat, or fruity flavor, whereas decanoic acid brings a soap flavor (Cheng, 2010). Adding a medium dose of Lacticaseibacillus paracasei PC-01 also resulted in a significantly higher level of butyrate in fermented milk stored for 14 and 28 d compared with control. Similarly, the butyric acid content in the PC-01-H group increased significantly after 14 d of storage. Higher levels of short-chain fatty acids not only improve the flavor of fermented milk but also likely confer desirable health benefits, such as regulating immunity, maintaining digestive health, and lowering gastrointestinal inflammation (Campos-Perez and Martinez-
Lopez, 2021). Saturated fatty acids with more than 12 carbon atoms have a high perception threshold, so they have only a minor effect on flavor (Hu et al., 2020). Our results showed that, after 14 d of storage, the contents of tetradecanoic acid and dodecanoic acid in PC-01-M and PC-01-H were significantly lower than those in control, suggesting that the addition of a medium or high dose of Lactcaseibacillus paracasei PC-01 enhanced oxidation of SFA into medium- and short-chain fatty acids during cold storage, coinciding with the relatively active bacterial growth and increase in butyric acid in these groups at these time points.

The flavor of fermented milk is the result of the overall combination of volatile metabolites. It is obvious that an increased amount of key volatile flavor compounds improves the sensory quality of fermented milk. The application of a medium or high dose of Lactcaseibacillus paracasei PC-01 significantly slowed the decline in sensory quality of fermented milk over cold storage, which was accompanied by increases in both the quantity and variety of key volatile metabolites in fermented milk. Major differentially abundant metabolites, including acetaldehyde, methyl ketones, medium- and short-chain fatty acids, 2,3-butanedione, and acetoin, were enriched in fermented milks rated highly in the sensory evaluation, suggesting that these metabolites exerted positive effects on the fermented milk flavor. As storage continued, the growth of Lactcaseibacillus paracasei PC-01 continued, leading to further increase in TA, a decrease in pH, and changes in key volatile metabolites, which subsequently contributed to further sensory decline.

CONCLUSIONS

This study used Lactcaseibacillus paracasei PC-01 as a model co-fermentation strain to investigate the effect of inoculum size together with a commercial starter in milk fermentation on product flavor and the profile of volatile metabolites during a 28-d cold storage period. Our results suggested that the amount of probiotic co-fermentation strain had significant effect on the sensory quality of the fermented milk over storage, and that a medium dose of Lactcaseibacillus paracasei PC-01 (5 × 10⁶ cfu/g) is optimal for the sensory quality of the fermented milk. Alterations in the sensory quality of fermented milk were accompanied by obvious changes in the contents of some milk metabolites. Major differentially abundant metabolites, including acetaldehyde, methyl ketones, medium- and short-chain fatty acids, 2,3-butanedione, and acetoin, were enriched in fermented milks rated highly in the sensory evaluation, suggesting that these metabolites affected the fermented milk flavor positively.

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