The production characteristics of volatile organic compounds and their relation to growth status of *Staphylococcus aureus* in milk environment

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ABSTRACT

In our previous research, 3-methyl-butanol and 3-methyl-butanoic acid were identified as representative and specific volatile organic compounds released by *Staphylococcus aureus* in broth. In this study, we explored the production of the 2 volatiles and their correlation to *Staph. aureus* growth in milk under different conditions. We found significant correlations between the production of 3-methyl-butanoic acid and cell counts of 5 *Staph. aureus* strains in sterile milk, and there were no obvious differences for its production among 5 tested strains. The intensities of the 2 volatiles were similar and positively correlated with bacterial counts in cultures at 25°C and 37°C despite delayed production of volatiles at 25°C; however, neither compound could be detected at 4°C. The production of 3-methyl-butanoic acid was strongly correlated with growth of *Staph. aureus* mixed with *Streptococcus agalactiae*, *Escherichia coli* O157:H7, and *Shigella flexneri*, whereas correlations for 3-methyl-butanol were not statistically significant. Compared with the monoculture of *Staph. aureus*, in mixed cultures, production of 3-methyl-butanol was decreased and that of 3-methyl-butanoic acid was comparatively higher. In pasteurized and raw milks, production of 3-methyl-butanoic acid was correlated with growth of *Staph. aureus*, and 3-methyl-butanoic acid could be detected when *Staph. aureus* populations reached 10^6 to 10^7 cfu/mL in pasteurized milk and 10^5 to 10^6 cfu/mL in raw milk; the correlations for 3-methyl-butanol were not statistically significant. Our results suggest that 3-methyl-butanoic acid is a more suitable marker for high counts of *Staph. aureus* in milk, whereas 3-methyl-butanol is a transient metabolite and easily depressed by environmental factors.

Key words: *Staphylococcus aureus*, milk, bacterial volatile compounds, growth, correlation

INTRODUCTION

Among etiological agents in bovine mastitis, *Staphylococcus aureus* is the most prevalent pathogen. *Staphylococcus aureus* mastitis is a serious problem in the dairy industry, and infected bovines may contaminate individual milk and bulk tank milk. In China, 894 bulk tank milk samples collected from dairy herds were examined for the presence of mastitis pathogens, and *Staph. aureus* was detected in 50.1% of all bulk tank milk samples (Bi et al., 2016). Similarly, the prevalence of *Staph. aureus* in bulk tank milk samples from Ohio dairy herds was estimated to be 48 to 69% (da Costa et al., 2016). In the study of Gücükoğlu et al. (2012), *Staph. aureus* was detected in 45 of 60 (75%) raw milk samples, and the level of *Staph. aureus* contamination was $>10^5$ to 10^6 cfu/mL in 17 of 60 raw milk samples obtained from Samsun province, Turkey. These findings indicate that a high percentage of raw milk samples are contaminated with high cell counts of *Staph. aureus*. Dairy products from raw milk contaminated with *Staph. aureus* constitute a potential risk to public health. Proper pasteurization could inactivate all *Staph. aureus*; however, failure of pasteurization or postprocessing contamination could lead to the presence of viable *Staph. aureus* in milk products after pasteurization. For example, Akindolire et al. (2015) measured pasteurized milk samples from retail outlets of the Northwest province, South Africa, and showed that 10 of 75 pasteurized milk samples were positive for *Staph. aureus*. In the investigation of Vahedi et al. (2013), contamination with *Staph. aureus* was observed in 2 of 100 pasteurized milk samples randomly from supermarkets in Sari, Iran. Mirzaei et al. (2011) examined 200 milk and dairy products in Sarab, Iran, and indicated that 4 pasteurized milk samples were contaminated by coagulase-positive *Staph. aureus*. Thus, milk is a common source of food-borne disease, and detection of *Staph. aureus* in milk is very important.

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In raw milk and milk products, *Staph. aureus* could be detected by using classical microbiological methods and PCR techniques. However, bacteriological culture is time and labor consuming, and it is difficult to perform PCR directly on milk because it is a complex food matrix. Therefore, there is an urgent need to implement rapid and accurate detection technology to control contamination and the spread of *Staph. aureus* in raw milk and milk products.

Microbial volatile organic compounds are interlinked to microbial metabolism and bacterial populations localized on the surfaces of biological samples. Hettinga et al. (2008) suggested a comparable method for mastitis pathogen identification based on volatile metabolites analysis in raw milk, and indicated that *Staph. aureus* produced a different pattern of volatile metabolites from other mastitis pathogens. Their studies reported that *Staph. aureus* produced high amounts of 3-methyl-butanol, especially, and *Staph. aureus* could be differentiated from other mastitis pathogens on the basis of the production of branched fatty acids, such as 2-methyl butyrate. A later study, Hettinga et al. (2009a), showed that 2- or 3-methyl-butanol and 2- or 3-methyl butyrate formed in inoculated milk were similar to the volatile metabolites formed in milk from cows with mastitis. This showed the possibility of detecting *Staph. aureus* in milk based on its volatile metabolites.

Volatile metabolites of *Staph. aureus* in tryptic soy broth were previously analyzed by Filipiak et al. (2012) and showed that *Staph. aureus* released specifically 3-methyl-butanol (at high concentration) and 3-methyl-butanoic acid (at moderate concentration). Bos et al. (2013) found that 3-methyl-butanoic acid and 2-methyl-butanol were biological markers for the presence of *Staph. aureus*. Tait et al. (2014) identified that *Staph. aureus* specifically produced 3-methyl-butanoic acid in 3 media (brain heart infusion, tryptic soy broth, 1% glucose enteric fermentation base broth) and 3-methyl-butanol in 1% glucose enteric fermentation base broth. In our previous study, we also presented that 3-methyl-butanoic acid and 3-methyl-butanol were volatile biomarkers for *Staph. aureus* in tryptic soy broth (Chen et al., 2017).

3-Methyl-butanol and 3-methyl-butanoic acid are regarded as representative and specific volatiles released by *Staph. aureus*; however, the production profile of these volatiles and the relation to *Staph. aureus* growth status have not been reported in milk. In this study, we investigated the production of volatiles and growth of several *Staph. aureus* strains in pure culture and mixtures of *Staph. aureus* with other pathogens at different cultivation temperatures in sterile milk. This exploration

should support the use of representative volatiles as stable markers to detect *Staph. aureus* in milk.

MATERIALS AND METHODS

Strains and Cultures

The strains *Staphylococcus aureus* ATCC 6538, *Streptococcus agalactiae* CICC 10465, *Escherichia coli* O157:H7 CICC 21530, *Salmonella enterica* serovar Enteritidis CICC 21482, and *Shigella flexneri* CGMCC 1.1868 were used. The CICC strains were from the China Center of Industrial Culture Collection (Beijing, China); and CGMCC strains were from China General Microbiological Culture Collection Center (Beijing, China).

Isolate strains from were also acquired from hospital patients with food poisoning in Chongqing, China, designated *Staph. aureus* H3, H4, H5, and H8. For each bacterium, a fresh bacterial culture was prepared in trypticase soy broth (TSB; Qing Dao Hope Bio-Technology Ltd. Corp., QingDao City, China) for 14 h at 37°C with agitation at 150 rpm.

Detection of Growth and Volatile Compounds of *Staph. aureus* in Sterile Milk

Ultra-high-temperature milk was purchased from a local supermarket and used as sterile milk. A fresh bacterial culture of each *Staph. aureus* was inoculated into 1 L of sterile milk in a flask, and the initial inoculation level amounted to 100 to 200 cfu/mL. The incubation conditions were 37°C with agitation at 150 rpm for 24 h. Blank (uninoculated) sterile milks were used as controls. The growth of *Staph. aureus* was determined by enumerating bacterial cells at 2-h intervals. A 1-mL volume of sample culture was collected and serially diluted in saline. Appropriate dilutions were spread on Baird-Parker plates (Qing Dao Hope Bio-Technology Limited Corp.), and 3 replicated samples were obtained at each sampling time. The volatiles were detected by headspace-solid-phase microextraction with GC-MS analysis at 2-h intervals, and the detection method referred to our previous publication (Chen et al., 2017). Briefly, the 5-mL sample was transferred into a 20-mL amber screw-cap vial with addition of 2 g of NaCl. The solid-phase microextraction fiber was 50/30 μ m divinylbenzene/carboxen/polydimethylsiloxane. All steps, including preheating (80°C, 10 min), extraction (80°C, 30 min), desorption (230°C, 2 min), analyzing, and cleaning (250°C, 10 min), were conducted automatically using a Triplus auto-sampler (Thermo Fisher

Scientific, Waltham, MA). The GC-MS analysis was performed using a Thermo Trace GC ultra DSQ II (Thermo Fisher Scientific). Volatile compounds were separated using HP-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m). Three replicate samples were obtained at each sampling time.

Detection of Growth and Volatiles of *Staph. aureus* at Different Temperatures in Sterile Milk

Fresh bacterial culture of *Staph. aureus* 6538 was inoculated into 1 L of sterile milk in a flask at an initial inoculation level of 100 to 200 cfu/mL. The incubation conditions were 37°C with agitation at 150 rpm for 24 h; 25°C with agitation at 150 rpm for 36 h; or 4°C with static cultivation for 22 d. Samples of bacterial culture were collected every 2 or 3 h for 37°C and 25°C cultures and every 2 d for 4°C cultures. The subsequent steps were the same as described above.

Detection of Growth and Volatiles of *Staph. aureus* in Mixed Cultures in Sterile Milk

The combinations of *Staph. aureus* and *Strep. agalactiae*, *Staph. aureus* and *E. coli* O157:H7, *Staph. aureus* and *Salmonella* Enteritidis, and *Staph. aureus* and *S. flexneri* were used as mixed cultures. Fresh bacterial cultures of these target pathogens were inoculated into 1 L of sterile milk in a flask at initial inoculation levels of 100 to 200 cfu/mL. The incubation condition was 37°C with agitation at 150 rpm for 24 h. Blank sterile milks were used as controls. The following steps were the same as described above.

Detection of Growth and Volatiles of *Staph. aureus* in Pasteurized and Raw Milks

Pasteurized milk samples were purchased from a supermarket in the Wuhou district of Chengdu city, and individual raw milk samples were collected from dairy herds in Shuangliu district of Chengdu city, Sichuan province, China. The total bacterial counts were determined to be 1 to 3 $\times 10^6$ cfu/mL for raw milk and 5 to 9 $\times 10^3$ cfu/mL for pasteurized milk. Among the total bacteria, approximately 1 to 2 $\times 10^4$ cfu/mL were recognized to be *Staph. aureus* in raw milk samples, whereas *Staph. aureus* was not found in pasteurized milk samples. At the same time, fresh bacterial culture of *Staph. aureus* 6538 was inoculated into 1 L of pasteurized milk or raw milk in a flask at an initial inoculation level of 100 to 200 cfu/mL. The incubation condition was 37°C with agitation at 150 rpm for 24 h. The growth and volatile production of *Staph. aureus*

were determined at 2-h intervals. Three pasteurized milk samples and 3 raw milk samples were examined. The bacterial counting and the volatile detecting were conducted according to the methods described above.

Data Analysis

We investigated temporal production of volatiles and growth levels of *Staph. aureus* in samples, and statistical analysis was conducted using OriginPro 7.5 (version 7.5776, OriginLab, Northampton, MA). The correlations between measured volatile compound concentrations and bacterial counts were calculated by Spearman correlation coefficient, using SPSS Statistics 17.0 (SPSS Inc./IBM Corp., Chicago, IL).

RESULTS

Production of Volatiles and Relation to Growth of Different *Staph. aureus* Strains

As can be seen in the time-dependent production profile of the volatiles in Figure 1A, the signal intensity of 3-methyl-butanol from *Staph. aureus* 6538 and H4 increased and then decreased slowly, whereas levels of 3-methyl-butanol in *Staph. aureus* H3, H5, and H8 increased up to the maximum followed by a sharp decrease to zero. It is important to note that, in Figure 1, the signal intensity of 3-methyl-butanol from the reference strain ($\times 10^7$ on y-axis) was about 10-fold higher than that from isolate strains ($\times 10^6$ for y-axis). As shown in Table 1, correlations between 3-methyl-butanol intensities and bacterial counts from *Staph. aureus* 6538 ($P < 0.01$) and H4 ($P < 0.05$) were significant, whereas those between 3-methyl-butanol and counts of *Staph. aureus* H3, H5, and H8 were not significant. In addition, signal intensities of 3-methyl-butanolic acid increased from 8 to 10 h and then increased continuously or remained stable; there were no obvious differences in 3-methyl-butanolic acid between the reference strain and isolate strains (Figure 1A). As shown in Table 1, correlations between 3-methyl-butanolic acid intensities and bacterial counts from 5 target *Staph. aureus* were all significant ($P < 0.01$).

Production of Volatiles and Growth of *Staph. aureus* at Different Temperatures

Neither 3-methyl-butanol nor 3-methyl-butanolic acid was detectable until 18 to 20 h at 25°C or 6 to 8 h at 37°C (Figure 1B and 1C), indicating that production of volatiles from *Staph. aureus* was delayed at 25°C because of the slower growth of *Staph. aureus* compared

Figure 1. Time-dependent production of 3-methyl-butanol and 3-methyl-butanoic acid for *Staphylococcus aureus* isolates (hospital strains, H3, H4, H5, and H8) in sterile milk (A), for *Staph. aureus* ATCC 6538 at 37°C in sterile milk (B), for *Staph. aureus* ATCC 6538 at 25°C in sterile milk, and (D) for *Staph. aureus* ATCC 6538 mixed with other pathogens (*Escherichia coli*, *Streptococcus agalactiae*, *Salmonella* Enteritidis, or *Shigella flexneri*) in sterile milk. Note that the y-axis scale for production of 3-methyl-butanol in pure cultures of *Staph. aureus* ATCC 6538 is $\times 10^7$ counts/s, whereas that for other strains and mixed cultures is $\times 10^6$ counts/s. Error bars are SD.

Figure 2. Growth curves of different strains of *Staphylococcus aureus*, mixed cultures (with *Escherichia coli*, *Streptococcus agalactiae*, *Salmonella* Enteritidis, or *Shigella flexneri*), and of *Staph. aureus* ATCC 6538 at 25 and 37°C in sterile milk. Error bars are SD.

with that at 37°C (Figure 2). We did not find dramatic differences in levels of the 2 volatiles produced between 25°C and 37°C. Furthermore, at 37°C and 25°C, correlations between signal intensities of the 2 volatiles and bacterial counts of *Staph. aureus* were significant ($P < 0.01$; Table 2). However, at 4°C, 3-methyl-butanol and 3-methyl-butanolic acid could not be detected during the 22-d cultivation. The bacterial count of *Staph. aureus* increased from 100 to 200 cfu/mL to 2×10^3 to 3

$\times 10^3$ cfu/mL after 22 d, and the metabolism of *Staph. aureus* was strongly inhibited at 4°C.

Production of Volatiles and Growth of *Staph. aureus* in Different Mixed Cultures

The signal of 3-methyl-butanol from mixed cultures increased to a maximum and then decreased quickly to zero (Figure 1D). Correspondingly, in mixed cultures of

Table 1. Correlation coefficients between bacterial counts and signal intensities of the volatile compounds 3-methyl-butanol and 3-methyl-butanolic acid produced from 5 *Staphylococcus aureus* cultures

Strain	3-Methyl-butanol	3-Methyl-butanolic acid
<i>Staph. aureus</i> 6538	0.799**	0.893**
<i>Staph. aureus</i> H3	−0.154	0.914**
<i>Staph. aureus</i> H4	0.708**	0.852**
<i>Staph. aureus</i> H5	0.084	0.871**
<i>Staph. aureus</i> H8	−0.154	0.933**

** $P < 0.01$.

Table 2. Correlation coefficients between bacterial counts and signal intensities of the volatile compounds 3-methyl-butanol and 3-methyl-butanolic acid produced from *Staphylococcus aureus* cultures under different temperatures

Temperature (°C)	3-Methyl-butanol	3-Methyl-butanolic acid
37	0.779**	0.893**
25	0.813**	0.910**
4	— ¹	—

¹Not detected.

** $P < 0.01$.

Table 3. Correlation coefficients between bacterial counts and signal intensities of the volatile compounds 3-methyl-butanol and 3-methyl-butanolic acid produced from mixed cultures

Mixed culture	3-Methyl-butanol	3-Methyl-butanolic acid
<i>Staphylococcus aureus</i> and <i>Escherichia coli</i> O157:H7	−0.502	0.972**
<i>Staph. aureus</i> and <i>Streptococcus agalactiae</i>	−0.221	0.952**
<i>Staph. aureus</i> and <i>Salmonella</i> Enteritidis	0.038	0.152
<i>Staph. aureus</i> and <i>Shigella flexneri</i>	−0.215	0.927**

** $P < 0.01$.

Staph. aureus with *Strep. agalactiae*, *Salmonella* Enteritidis, *E. coli* O157:H7, or *S. flexneri*, correlations between 3-methyl-butanol intensities and bacterial counts of *Staph. aureus* were not significant (Table 3). On the other hand, production of 3-methyl-butanolic acid in mixed cultures increased continuously throughout the period, except that it was heavily suppressed in the mixed culture with *Salmonella* Enteritidis. In mixed cultures, correlations between 3-methyl-butanolic acid intensities and bacterial counts of *Staph. aureus* were significant ($P < 0.01$) except for that of *Staph. aureus* and *Salmonella* Enteritidis. The signal intensities of 3-methyl-butanol from mixed cultures ($\times 10^6$ for y-axis) were reduced to only 10% of that from *Staph. aureus* pure culture ($\times 10^7$ for y-axis; Figure 1). The signal intensity of 3-methyl-butanolic acid from the other mixed cultures (with *E. coli* O157:H7, *Strep. agalactiae*, and *S. flexneri*) was higher than that of pure *Staph. aureus* culture, except for the mixed culture of *Staph. aureus* and *Salmonella* Enteritidis.

Detectable Growth Level of *Staph. aureus* in Pasteurized and Raw Milks

The correlations between 3-methyl-butanol and bacterial counts of *Staph. aureus* were not significant (Table 4), but those between 3-methyl-butanolic acid and *Staph. aureus* counts were significant ($P < 0.01$). Further, 3-methyl-butanol was detected in 2 blank pasteurized milk samples and 3 blank raw milk samples;

therefore, the increase in 3-methyl-butanol signal was suitable for judging growth of *Staph. aureus*. The elevated 3-methyl-butanol signal in pasteurized milk was observed as *Staph. aureus* reached about 10^6 cfu/mL, whereas we did not detect any increase 3-methyl-butanol signal during the cultivation process in 3 raw milk samples (Table 5). Interestingly, 3-methyl-butanolic acid was not detected in 3 blank pasteurized milk or 3 blank raw milk samples. At the same time, 3-methyl-butanolic acid could be detected in pasteurized milk as *Staph. aureus* reached 10^6 to 10^7 cfu/mL, and in raw milk as *Staph. aureus* reached 10^5 to 10^6 cfu/mL (Table 6).

DISCUSSION

3-Methyl-butanol, as a branched-chain volatile aldehyde, has been reported to be a key flavor compound, and can be produced from *Staphylococcus xylosus* (de Vos Petersen et al., 2004), *Proteus vulgaris*, *Psychrobacter* sp. (Deetae et al., 2007), and lactic acid bacteria (Afzal et al., 2017). It is well known that 3-methyl-butanol is a transitory compound, which is usually biosynthesized from leucine catabolism using α -ketoacid decarboxylase enzyme or α -ketoacid dehydrogenase enzyme complex, and immediately oxidized into 3-methyl-butanolic acid (Beck et al., 2002; Afzal et al., 2013). In this study of *Staph. aureus* in milk, the 3-methyl-butanol signal increased beginning in the early exponential growth phase, reached a maximum value, and then decreased in the exponential and stationary growth phases; 3-methyl-butanolic acid could

Table 4. Correlation coefficients between bacterial counts and signal intensities of the volatile compounds 3-methyl-butanol and 3-methyl-butanolic acid produced from *Staphylococcus aureus* cultures in pasteurized milks and raw milks

Milk sample	3-Methyl-butanol	3-Methyl-butanolic acid
Pasteurized milk 1	0.367	0.920**
Pasteurized milk 2	0.266	0.928**
Pasteurized milk 3	0.382	0.887**
Raw milk 1	— ¹	0.810**
Raw milk 2	—	0.886**
Raw milk 3	—	0.671*

¹No increase in 3-methyl-butanol detected during culture.* $P < 0.05$; ** $P < 0.01$.**Table 5.** Growth of *Staphylococcus aureus* corresponding to the increase in 3-methyl-butanol signal

Sample	Growth (cfu/mL)	Increase in 3-methyl-butanol (counts $\times$ s)
Pasteurized milk 1	1.38×10^6	2.44×10^5
Pasteurized milk 2	3.75×10^6	1.52×10^6
Pasteurized milk 3	2.59×10^6	1.87×10^6
Raw milk 1	— ¹	—
Raw milk 2	—	—
Raw milk 3	—	—

¹No increase in 3-methyl-butanol was detected during culture of 3 raw milk samples.

Table 6. Growth of *Staphylococcus aureus* corresponding to detectable signal intensity of 3-methyl-butanoic acid

Sample	Growth (cfu/mL)	Detectable 3-methyl-butanoic acid (count $\times$ s)
Pasteurized milk 1	3.97×10^6	7.93×10^7
Pasteurized milk 2	4.65×10^7	3.12×10^7
Pasteurized milk 3	3.09×10^7	3.36×10^7
Raw milk 1	2.30×10^5	9.73×10^6
Raw milk 2	1.54×10^6	1.32×10^7
Raw milk 3	6.78×10^5	1.04×10^7

be detected in the later exponential growth phase and increased steadily in the stationary growth phase. The low amounts of 3-methyl-butanol and the high amounts of 3-methyl-butanoic acid during the later growth period of *Staph. aureus* in milk were observed clearly in the current study, demonstrating that 3-methyl-butanol is a transient metabolite that would rapidly disappear from culture medium after production, leading to nonsignificant correlations between 3-methyl-butanol intensities and bacterial counts of *Staph. aureus* in most cases. However, 3-methyl-butanoic acid is a stable product in the metabolism of *Staph. aureus*, resulting in significant correlations between 3-methyl-butanoic acid intensities and bacterial counts of *Staph. aureus*.

Multiple bacteria occur simultaneously in real milk samples, so mixed cultures of *Staph. aureus* and other pathogens more closely mimic the actual situation. In the present study, we made binary mixed cultures of *Staph. aureus* and other pathogens. Production of 3-methyl-butanol generally decreased in all mixed cultures compared with *Staph. aureus* pure culture; however, the level of 3-methyl-butanoic acid was higher in mixed cultures than in *Staph. aureus* pure culture except for the coculture with *Salmonella* Enteritidis. Extracellular oxidation-reduction potential is an environmental factor that affects bacterial metabolic fluxes (Deetae et al., 2011; Matsuoka and Kurata, 2017). Kieronczyk et al. (2006) reported that oxidative conditions favor the production of aldehydes by amino acid catabolism, such as conversion of leucine to 3-methyl-butanol, and reducing conditions stimulate the production of carboxylic acids (e.g., 3-methyl-butanoic acid). Therefore, the decreased production of 3-methyl-butanol and increased production of 3-methyl-butanoic acid in the current study might be caused by the weaker oxidation state in mixed cultures than in pure culture. In this study, the release of 3-methyl-butanoic acid was suppressed for the combination of *Staph. aureus* with *Salmonella* Enteritidis. Growth inhibition of *Staph. aureus* by *Salmonella* Enteritidis was not observed during the logarithmic growth phase (Figure 2); rather, the maximum cell counts in the mixed culture of *Staph. aureus* and *Salmonella* Enteritidis were lower than in the pure

culture of *Staph. aureus* during the stationary growth phase. Therefore, the decrease of 3-methyl-butanoic acid in the mixed culture of *Staph. aureus* and *Salmonella* Enteritidis might be caused by decomposition of *Salmonella* Enteritidis. Mpatswenumugabo et al. (2017) and Mekonnen et al. (2017) demonstrated that *Staph. aureus* and CNS were the frequently isolated mastitis pathogens from raw milk. A mixed culture of *Staph. aureus* with other CNS was not used in this study; however, the specificity of 3-methyl-butanoic acid for *Staph. aureus* might be supported by other research. For example, Hettinga et al. (2008, 2009b) noted that 3-methyl-butanol was produced by *Staph. aureus* and CNS, respectively, whereas 3-methyl-butyrate was only detectable in *Staph. aureus*. In the future, we will focus on the effects of CNS on volatiles of *Staph. aureus* through the mixed culture experiment and explore the different influencing factors.

In the present study, total bacterial counts were 1×10^6 to 3×10^6 cfu/mL for raw milk from dairy herds and 5×10^3 to 9×10^3 cfu/mL for pasteurized milk from supermarkets. Under this complex microbial flora, we could observe the growth of *Staph. aureus* and its production of volatiles. 3-Methyl-butanoic acid was detected in both pasteurized milk with 10^6 to 10^7 cfu/mL of *Staph. aureus* and raw milk with 10^5 to 10^6 cfu/mL of *Staph. aureus*. Similarly, Hettinga et al. (2009b) revealed that important volatile metabolites were released and accumulated to a detectable level after 8 h of incubation when the number of *Staph. aureus* in milk reached approximately 10^7 cfu/mL. In TSB culture of *Staph. aureus*, 3-methyl-butanoic acid was significantly released at a cell count of 5×10^7 cfu/mL (Filipiak et al., 2012). The studies confirmed that the concentration of volatile compounds increased and could be detected when the count of *Staph. aureus* reached a threshold value, similar to reports for other bacteria (Mayr et al., 2003; Ragaert et al., 2006; Silcock et al., 2014). On the other hand, milk and its products have been associated with staphylococcal food poisoning (Johler et al., 2015; Vitale et al., 2015; Jans et al., 2017), which is caused by consumption of food contaminated with enterotoxins produced by *Staph. aureus*. Generally, staphylococcal food poisoning is confirmed either by the recovery of at least 10^5 *Staph. aureus* per gram or milliliter of food remnants or by the detection of staphylococcal enterotoxins (Hennekinne et al., 2012). The dose of enterotoxins reaches a certain level when the *Staph. aureus* population in contaminated food exceeds 10^5 cfu/g or cfu/mL, and this toxin level could produce symptoms of staphylococcal intoxication (Necidová et al., 2012). In the present study, we detected 3-methyl-butanoic acid when *Staph. aureus* counts in milk reached 10^5 to 10^7 cfu/mL, which suggested that the detectable level

of 3-methyl-butanoic acid could be a marker for milk contaminated with *Staph. aureus* being hazardous to consumers. To our knowledge, no studies have focused on the relationship between the release of volatile metabolites and production of enterotoxins from *Staph. aureus* to date. Our future studies will illustrate the relationship between these 2 aspects.

CONCLUSIONS

3-Methyl-butanoic acid was a stable end product and its production correlated well with the growth of *Staph. aureus* at different temperatures and in different mixtures with other pathogens (except *Salmonella* Enteritidis) in sterile milk. 3-Methyl-butanol was a transient metabolite and its production was not significantly correlated with growth of *Staph. aureus* in most cases. In pasteurized and raw milks, production of 3-methyl-butanoic acid was correlated significantly with the growth of *Staph. aureus*, and the volatile compound could be detected with *Staph. aureus* populations of 10^6 to 10^7 cfu/mL (pasteurized milk) and 10^5 to 10^6 cfu/mL (raw milk). The corresponding correlations for 3-methyl-butanol were not significant. The production characteristics of volatiles and their correlation with growth of *Staph. aureus* in milk points to a method for rapid and nondestructive detection by odor analysis for the milk industry.

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