The potential risks of paclobutrazol residue on yogurt fermentation from the level of chiral enantiomers

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ABSTRACT

In recent years, pesticide residues in food have increasingly become the focus of public attention. However, the standard system of pesticide maximum residue limits in fermented food is imperfect, which can lead to potential safety risks to consumers. In this context, the aim of the study was to assess the potential effects of paclobutrazol residue on the yogurt fermentation process. We examined the stereoselective behaviors of the 2 paclobutrazol enantiomers from the perspective of chirality during the yogurt fermentation process. The results indicated that no significant degradation occurred for either of the 2 enantiomers (2R, 3R-paclobutrazol, 2S, 3S-paclobutrazol), and no visible enantiomer conversion behavior was observed. In addition, the reason paclobutrazol did not significantly degrade was explained from the perspective of the microbial function. Results from 16S rRNA sequencing indicated that paclobutrazol significantly affected the microbial composition and inhibited metabolic function of microorganisms to exogenous substances, which impeded the degradation of residual pesticide in yogurt. Furthermore, the stable residue of exogenous substance may cause potential food safety problems. Microbial α -diversity analysis indicated that fermentation time played a more important role on diversity than did paclobutrazol concentration. Moreover, *Staphylococcus* was found in yogurt after treatment with paclobutrazol; *Staphylococcus aureus* causes dangerous infectious diseases in humans. We devised a method to investigate the presence of pesticide residues during food fermentation and provided a theoretical basis for food safety assessment.

Key words: paclobutrazol, yogurt, food fermentation process, 16S rRNA sequencing

INTRODUCTION

Fermented foods have a long history and are popular among consumers. One traditional fermented dairy product, yogurt, is mostly made by milk and starter with the action of microorganisms (Srisuvar et al., 2013). Yogurt has been proven to have excellent nutritional value on account of its protein, calcium, vitamins, and beneficial microorganisms (Belli et al., 2013; Danneskiold-Samsøe et al., 2019). Therefore, long-term consumption of yogurt is good for human health.

Pesticides play an important role in agricultural production due to their high effectiveness for controlling pests and protecting crops. However, with the extensive use of pesticides, more pesticides are retained and enriched in the environment (Pimentel, 1995; Muñoz-Leoz et al., 2011; Zhi and Ji, 2012; Tejada et al., 2017), and pesticide residues affect food safety (Gao et al., 2019). Many reports illustrate that pesticides are directly toxic to microorganisms and other organisms (Russell et al., 2011; Chen et al., 2019). Recently, Zhang et al. (2019) proved that exogenous substances may change the composition of a microbial community, thus affecting the fermentation process. Yogurt produces plentiful microorganisms during fermentation, which play an important role in the formation of nutritional value and flavor in fermented foods (McMurtrie et al., 2019). It is worrisome that pesticide residues in raw materials may have an effect on the composition of the microbial community, potentially compromising the quality and safety of yogurt (Leite et al., 2012). Nonetheless, few studies have investigated the effects of pesticides on microorganisms produced during yogurt fermentation and the standard system of pesticide maximum residue limit in fermented foods. Therefore, the safety of fermented food is a problem worthy of attention, and the composition, function, and diversity of microorganisms during yogurt fermentation deserves further study.

Paclobutrazol [(2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol] is one of the most commonly used plant growth regulators in the world (Gilley and Fletcher, 1998). Paclobutrazol consists of 2 isomers that have different bioactivities. The form

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of (2S,3S)-paclobutrazol (S-paclobutrazol) is active as a plant growth regulator, and (2R,3R)-paclobutrazol (R-paclobutrazol) has high fungicidal activity (Sugavanam, 1984). Although the products and applications of paclobutrazol are in racemic forms, and chiral pesticides may exhibit stereoselective behaviors when they enter into the environment (Yang and Ji, 2015; Zhi et al., 2015), only a few reports have examined the differences between the 2 enantiomers. Paclobutrazol has fungitoxicity by inhibiting the synthesis of ergosterol in fungi (Yun et al., 2014), and has been demonstrated to negatively affect microbial communities and nontarget organisms (Chen et al., 2019; Kuo et al., 2019; Liu et al., 2019). Furthermore, the main target organisms of paclobutrazol are plants. Some researchers have demonstrated that contamination of feed, with aflatoxins or pesticides, for example, can cause cows to produce contaminated milk (Xiong et al., 2018). Therefore, residual paclobutrazol in treated plants may enter a cow's body as she feeds, eventually contaminating the milk and resulting in food safety risks.

Recently, high-throughput sequencing technology has proven to be a valuable tool for the analysis of sample microbial information. By using this technology, some researchers studied stress responses, potential functions, and metabolic capacities of microbial isolates from fermented foods such as kefir and cheese (Zhi et al., 2014; Liu et al., 2015). In addition, some studies focused on investigating the microbial community composition of fermented foods (Wolfe et al., 2014; Bourrie et al., 2016). The 16S rRNA sequencing is one type of high-throughput sequencing technology, and has been widely used to study the composition, diversity, and dynamics of microbial communities that can influence the quality and character of fermented dairy foods (Marsh et al., 2013; Kergourlay et al., 2015).

In this study, we applied high-throughput sequencing technology to pesticide residue analysis to study the stereoselective behaviors and influence of paclobutrazol in 2 yogurt fermentation systems. Information from this study may aid in predicting potential risks to fermented food and human health after the application of chiral pesticides, as well as provide a method for the safety assessment of pesticide residues in food.

MATERIALS AND METHODS

Chemicals and Reagents

Paclobutrazol (standards) were obtained from the Institute for Control of Agrichemicals at the China Ministry of Agriculture (Beijing, China). Standards of the 2 optically pure isomers were prepared from Green Herbs

Co. Ltd. (Beijing, China). The stock standard solution (1 mg/mL) was dissolved by acetone and stored at 4°C in darkness. Analytical-grade methanol, acetonitrile, anhydrous sodium sulfate, and sodium chloride were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The purification materials primary secondary amine (PSA) and C₁₈E were purchased from Welch Materials Inc. (Shanghai, China). The ultrapure water was made by a Milli-Q system (Bedford, MA). The HPLC-grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ). Milk was purchased from China Mengniu Dairy Company (Hohhot, Inner Mongolia, China) and commercial yogurt was purchased from Inner Mongolia Yili Industrial Group Limited (Hohhot, Inner Mongolia, China) by Share Ltd. Baking powder was purchased from Angel Yeast Co. Ltd. (Yichang, Hubei, China).

Fermentation Pretreatment

To compare the differences in composition, diversity, and function of fermented microbes under different conditions, 2 fermentation systems with 2 different starters (commercial yogurt and commercial baking powder) were conducted. The fermentation process followed a previous method by Benozzi et al., (2015). We weighed 250.0 g of milk and 12.5 g of commercial yogurt into the fermentation container, and the entire fermentation process lasted 24 h. All the above operations were conducted in the dark at 42°C. When baking powder was the starter, we used 0.25 g of commercial baking powder (instead of 12.5 g of commercial yogurt); all other conditions were the same. The microbes of commercial yogurt were *Lactobacillus bulgaricus*, *Bifidobacterium animalis* ssp. *lactis*, and *Streptococcus thermophilus*; the microbes of commercial baking powder were *L. bulgaricus*, *B. animalis* ssp. *lactis*, *S. thermophilus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, and *Lactobacillus reuteri*.

Sample Contamination and Sampling

To evaluate the potential influence of xenobiotic substances on fermented microbes, 0.5 or 1.0 mL of paclobutrazol stock standard solution (1 mg/mL) was added into each yogurt container, yielding final analyte concentrations of 2 and 4 mg/kg of paclobutrazol, respectively. Our different samples consisted of commercial yogurt, no contamination (**CK**), commercial yogurt (2 mg/kg of paclobutrazol; **L**), commercial yogurt (4 mg/kg of paclobutrazol; **H**), commercial baking powder, no contamination (**TC**), commercial

baking powder (2 mg/kg of paclobutrazol; **TL**) and commercial baking powder (4 mg/kg of paclobutrazol; **TH**), respectively. The samples for quantitative analysis were collected according to the appropriate time points (0, 4, 8, 12, and 24 h) and stored at -20°C , and samples collected for DNA isolation were stored at -80°C . We compared the following treatment/timepoint combinations: yogurt fermented with starter culture without paclobutrazol at the beginning of fermentation (**CK0h**), yogurt fermented with yogurt as starter culture without paclobutrazol at the end of fermentation (**CK24h**), yogurt fermented with yogurt as starter culture with 4 mg/kg of paclobutrazol at the end of fermentation (**H24h**), yogurt fermented with yogurt as starter culture with 2 mg/kg of paclobutrazol at the end of fermentation (**L24h**), yogurt fermented with baking powder without paclobutrazol at the beginning of fermentation (**TC0h**), yogurt fermented with baking powder without paclobutrazol at the end of fermentation (**TC24h**), yogurt fermented with baking powder with 2 mg/kg of paclobutrazol at the end of fermentation (**TL24h**), and yogurt fermented with baking powder with 4 mg/kg of paclobutrazol at the end of fermentation (**TH24h**).

Sample Pretreatment

The extraction method was based on a modified QuEChERS method (Di et al., 2019). We weighed 10.0 g of sample and added it into a 50-mL polypropylene centrifuge tube. Then, 20 mL of acetonitrile and 2.0 g of sodium chloride were added into the tube and immediately covered. The mixture was stirred for 5 min on a vortex mixer, followed by ultrasonic extraction (Kunshan Ultrasonic Instrument Co. Ltd., Jiangsu, China) for 10 min. After extraction, the sample was centrifuged at 3,500 rpm for 5 min. We repeated the above steps 3 times to ensure high extraction efficiencies. The combined supernatant was passed through a

funnel with 5 g of anhydrous sodium sulfate into the pear-shaped flask. After, the extraction solvent was dried by vacuum rotary evaporation at 40°C . Next, 1 mL of methanol was used to dissolve the dried extraction solvent again and move it to a 2-mL centrifuge tube containing 100 mg of C_{18}E and 100 mg of PSA. The mixture was stirred for 3 min on a vortex mixer for purification and centrifuged at 8,000 rpm for 5 min. The supernatant was passed through a $0.22\text{-}\mu\text{m}$ filter membrane into a 2-mL sample vial. Finally, a $20\text{-}\mu\text{L}$ aliquot of the extract sample was injected into the HPLC.

HPLC Analysis

Paclobutrazol enantiomers were separated and determined on an Agilent 1260 HPLC (Santa Clara, CA) equipped with a G1311A pump, G1322A degasser, G1329A ALS, and G1314B VWD. The injection volume was $20\text{ }\mu\text{L}$, and UV detection wavelength was 230 nm. The signal was received and processed by using Agilent ChemStation software. The mobile phase was mixture of methanol and water (70/30, vol/vol) with a flow rate of 0.8 mL/min at 30°C . The chiral column was CDMPC-CSP (cellulose-tri [3, 5-dimethylphenylcarbamate], $\text{W}250 \times 4.6\text{ mm id}$) purchased from Welch Materials, Inc. (Shanghai, China) and the column temperature was controlled by an AT-930 heater and cooler column attemperator (Tianjin Automatic Science Instrument Co. Ltd., Tianjin, China).

For quality control in the determination of the target analytes, a recovery study of R-paclobutrazol and S-paclobutrazol at different levels (1 and 5 mg/kg of paclobutrazol) was conducted to ensure the accuracy and precision of the method. In the blank yogurt sample, different concentrations of paclobutrazol solution were added (1 and 5 mg/kg of paclobutrazol). Extraction and HPLC analysis were performed using the above methods. The recovery of the 2 enantiomers was analyzed and is displayed in Table 1.

Table 1. Recoveries and relative standard deviations (RSD) of samples spiked with paclobutrazol ($n = 3$)

Sample	Chemical	Paclobutrazol (mg/kg)	Recovery (%)				RSD (%)
			Sample 1	Sample 2	Sample 3	Average (%)	
Yogurt	R-paclobutrazol	1	94.20	92.92	84.83	90.65	4.58
	S-paclobutrazol	1	93.28	97.39	90.55	93.74	3.00
	R-paclobutrazol	5	85.86	92.92	86.50	88.43	3.61
	S-paclobutrazol	5	86.43	97.39	89.60	91.14	5.05
Milk	R-paclobutrazol	1	95.20	99.16	93.99	96.12	4.32
	S-paclobutrazol	1	90.18	92.75	99.67	94.20	2.98
	R-paclobutrazol	5	102.58	101.29	93.59	99.15	3.22
	S-paclobutrazol	5	102.13	99.81	96.89	99.61	4.62

DNA Extraction and Sequencing Analysis

The total genetic DNA of the yogurt was extracted and purified by the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA). For the amplification of the V3-V4 region of the 16S rRNA gene, 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used as forward and reverse primers, respectively. The PCR reaction conditions were as follows: 2 min at 98°C followed by 30 cycles at 98°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and finally at 72°C for 5 min. The main microbial communities of the tested samples were detected based on Illumina MiSeq 2500 platform (Illumina Inc., San Diego, CA). The sequenced bacteria genomes deposited in GenBank were downloaded from National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>) for downstream analyses. If the similarity between sequences was higher than 97%, it was defined as an operational taxonomic unit (OTU), and each OTU corresponds to a representative sequence. An E-value <0.01 means the results were credible.

RESULTS AND DISCUSSION

Method Validation

To evaluate the accuracy of sample pretreatment method, we investigated linearity, correlation coefficient, the recovery of paclobutrazol in the samples, relative standard deviations, and limit of detection (LOD). A dilution series of paclobutrazol spiked samples was prepared and analyzed to checking the method with linear calibration. Good linearity was achieved in all cases with correlation coefficients better than 0.9982. The complexity of the sample matrix may have affected the experiment results; therefore, all samples were spiked with paclobutrazol at 1 and 5 mg/kg and the results are summarized in Table 1. The recoveries of the 2 paclobutrazol enantiomers ranged from 88.43 to 99.61%, with relative standard deviations ranging from 2.98 to 5.05%. The LOD for target analytes were calculated at a signal to noise ratio of 3, and the LOD values of S-paclobutrazol and R-paclobutrazol were 0.42 and 0.39 mg/L, respectively. These results indicated that the sample pretreatment method developed in this work performed well in quantitating the target analytes.

The relative HPLC chromatogram is shown in Figure 1 and demonstrates that the 2 paclobutrazol enantiomers could completely separate with a resolution of 5.76 under the established chromatographic conditions. According to the studies, the first peak was (2R,3R)-

paclobutrazol and the second peak was (2S,3S)-paclobutrazol (Wang et al., 2014; Wu et al., 2015).

Stereoselective Behaviors of Paclobutrazol During Yogurt Fermentation

To investigate the stereoselective behaviors of paclobutrazol under different conditions, 2 fermentation systems (commercial yogurt as starter and commercial baking powder as starter) with 2 paclobutrazol concentration levels were conducted. During 24 h of fermentation, samples were collected and detected at 5 time points (0, 4, 8, 12, and 24 h). The change trend of paclobutrazol enantiomers in the fermentation systems when yogurt was treated with 4 mg/kg of paclobutrazol racemate during fermentation is shown in Figure 2A and 2B. The concentrations of the 2 paclobutrazol isomers did not change and no significant degradation was observed. The same results were obtained when the spiked concentration was 2 mg/kg of paclobutrazol. The reasons for this might be that the yogurt fermentation time was short and no microorganisms that could degrade paclobutrazol were produced during the fermentation process.

In this study, the enantiomer fraction (EF) was used to evaluate the enantioselectivity and it was calculated as follows:

$$EF = \frac{[R]}{[S] + [R]},$$

where [R] refers to the concentration of R(-)-enantiomer, and [S] refers to the concentration of S-(+)-enantiomer. Consequently, an EF >0.5 indicates faster degradation of the S-(+)-enantiomer, an EF <0.5 indicates faster degradation of the R(-)-enantiomer, and a value of 0.5 indicates that the degradation process is nonenantioselective. The changing trend of EF values are shown in Figure 2C and 2D when the spiked concentration was 4 mg/kg paclobutrazol. Results demonstrate that there were no significant stereoselective behaviors in any of the samples. The same results were obtained when the spiked concentration was 2 mg/kg of paclobutrazol.

Sequencing Data Quality

High-throughput sequencing was conducted on 8 group samples: CK0h, CK24h, L24h, H24h, TC0h, TC24h, TL24h, TH24h (0 and 24 h indicate the beginning and end of fermentation). A total of 1,686,669 paired-end reads were observed. Clean tags were ob-

Figure 1. The relative HPLC chromatogram of paclobutrazol.

tained from raw tags after cleaning, and 1,391,040 tags were available, with average length from 427 to 430 bp. There were 1,359,268 effective tags, and the average effective percent was 80.53%. The detected OTU in different samples are shown in Table 2, and a total of 97 OTU were detected with clustering at 97% similarity.

Bacterial Composition of Yogurt Samples

To evaluate the potential influence of paclobutrazol on fermented microbes, we analyzed 16S rRNA sequencing. Results from Figure 3 indicate that the dominant bacteria were *Lactobacillus* and *Streptococcus* in all samples. However, the composition and relative abundances of fermented bacteria were different depending on different fermentation systems and sampling times.

Figure 3A shows the relative abundances of fermented bacteria with commercial yogurt as starters. In the initial fermentation process, *Streptococcus* and *Lactobacillus* were dominant in CK0h (solvent control), and the relative abundances were 81.59 and 6.09%, respectively. After 24 h of fermentation, the relative abundances of *Streptococcus* were decreased to 50.05, 50.46,

and 54.52% in CK24h, L24h, and H24h, respectively. However, the relative abundances of *Lactobacillus* were 49.82, 49.21, and 45.24% in CK24h, L24h, and H24h, respectively. There was a significant increase in the relative abundance of *Lactobacillus* compared with that at the beginning of fermentation ($P < 0.01$, $Q < 0.01$). The Q -value was calculated from the P -value, and it means that the relative abundance of *Lactobacillus* in CK0h was significantly different from CK24h, L24h, and H24h. This implies that *Lactobacillus* became the major flora at the end of fermentation.

From Figure 3B we show that *Streptococcus* (59.32%), *Lactobacillus* (29.68%), *Bifidobacterium* (5.98%), and *Weissella* (3.33%) were the dominant bacteria in TC0h (solvent control), with commercial baking powder as starters. After 24 h of fermentation, the relative abundances of *Streptococcus* were decreased and *Lactobacillus* were increased, which was consistent with the commercial yogurt starters. It was noteworthy that the relative abundances of *Staphylococcus* significantly increased in TL24h compared with TC24h. Previous studies indicate that *Staphylococcus* is a widely distributed gram-positive bacterium (Wertheim et al., 2005),

and can be detected in air, water, and a variety of foods (Pastells et al., 2015). Notably, *Staphylococcus* as a pathogen can cause many dangerous infectious diseases, such as pneumonia and septicemia (Borsa et al., 2016; Meng et al., 2017), and *Staphylococcus* can induce skin abscesses in mice (Lei et al., 2018). Other studies demonstrated *Staphylococcus* can produce enterotoxins in food processing, and consequently, a risk of food poisoning (Schelin et al., 2017; Guidi et al., 2018; Grispoli et al., 2019). The residue of paclobutrazol could affect the microbial system during yogurt

fermentation processing, which will affect the quality of the final yogurt. In addition, the microorganism which could degrade paclobutrazol was not found, and further explained why the concentration of paclobutrazol did not change.

Alpha-Diversity of Bacterial Community

In this work, the alpha-diversities of different treatment groups were analyzed and compared. The diversity indexes, which contained OTU, ACE, Chao

Figure 2. The changes in paclobutrazol concentration in isomers and enantiomer fraction (EF) value during yogurt fermentation process (A = yogurt fermented with yogurt starter, 4 mg/kg of paclobutrazol; B = yogurt fermented with baking powder, 4 mg/kg of paclobutrazol; C = yogurt fermented with yogurt starter, 4 mg/kg of paclobutrazol; D = yogurt fermented with baking powder, 4 mg/kg of paclobutrazol). An EF value of 0.5 indicates that the degradation process was nonenantioselective. An EF near EF = 0.5 means the degradation of paclobutrazol was nonenantioselective.

Table 2. Operational taxonomic unit (OTU) and α -diversity of microbial communities at initial time (0 h) and after 24 h of fermentation

Sample ¹	Diversity index				
	OTU ₉₇	ACE	Chao1	Simpson	Shannon
CK0h	84.67 ^a	85.17 ^a	85.67 ^a	0.65 ^a	1.16 ^{ab}
CK24h	28.33 ^c	56.64 ^a	40.84 ^b	0.50 ^{bcd}	0.70 ^c
H24h	37.00 ^{bc}	72.94 ^a	53.79 ^b	0.50 ^{bcd}	0.71 ^c
L24h	30.33 ^{bc}	52.27 ^a	40.82 ^b	0.50 ^{cd}	0.72 ^c
TC0h	44.33 ^{bc}	51.72 ^a	52.73 ^b	0.40 ^d	1.35 ^a
TC24h	75.00 ^a	82.14 ^a	80.28 ^{ab}	0.55 ^{abc}	0.90 ^{bc}
TH24h	39.67 ^{bc}	53.16 ^a	48.78 ^b	0.65 ^a	0.67 ^c
TL24h	46.33 ^b	73.95 ^a	68.57 ^{ab}	0.64 ^{ab}	0.67 ^c

^{a-d}Different superscripts indicate significance in different samples.

¹CK = yogurt fermented with yogurt starter, no contamination; H = yogurt fermented with yogurt starter, 4 mg/kg of paclobutrazol; L = yogurt fermented with yogurt starter, 2 mg/kg of paclobutrazol; TC = yogurt fermented with baking powder, no contamination; TH = yogurt fermented with baking powder, 4 mg/kg of paclobutrazol; TL = yogurt fermented with baking powder, 2 mg/kg of paclobutrazol.

1, Simpson, and Shannon, are shown in Table 2. An increase in the Simpson value indicated a low diversity of bacteria. Conversely, an increase in the Chao1, ACE, and Shannon values indicated an increase in the diversity of bacteria. High microbial diversity could be found in CK0h and TC0h, and as fermentation progressed, bacterial diversity decreased. This phenomenon could be attributed to the proliferation of dominant bacteria during fermentation. Moreover, the diversity of bacteria in TH24h was lower than that in TC24h; this suggested that different concentrations of paclobutrazol could affect alpha-diversity, and a relatively high concentration of paclobutrazol reduced diversity.

Beta-Diversity of Bacteria Community

Beta diversity, which reflects the degree of similarity between pairs of communities, was estimated by conducting principal component analysis and nonmetric multidimensional scaling between samples, and the results are displayed in Figure 4. In the 2 fermentation systems (commercial yogurt and baking powder as starters), CK24h, L24h, and H24h were close to each other and far from CK0h; TC24h, TL24h, and TH24h were close to each other and far from TC0h. The results of principal component analysis and nonmetric multidimensional scaling were consistent, and indicated

Figure 3. Bacterial composition of yogurt samples at genus level with different starters and concentrations of paclobutrazol (A = yogurt fermented with yogurt starter; B = yogurt fermented with baking powder). CK = yogurt fermented with yogurt starter, no contamination; H = yogurt fermented with yogurt starter, 4 mg/kg of paclobutrazol; L = yogurt fermented with yogurt starter, 2 mg/kg of paclobutrazol; TC = yogurt fermented with baking powder, no contamination; TH = yogurt fermented with baking powder, 4 mg/kg of paclobutrazol; TL = yogurt fermented with baking powder, 2 mg/kg of paclobutrazol. 0h indicates initial time of fermentation and 24h indicates the end of the fermentation process.

Figure 4. Principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS) of yogurt samples (A = PCA in CK0h, CK24h, H24h, L24h; B = PCA in TC0h, TC24h, TH24h, TL24h; C = NMDS in CK0h, CK24h, H24h, L24h; D = NMDS in TC0h, TC24h, TH24h, TL24h). CK = commercial yogurt, no contamination; H = commercial yogurt, 4 mg/kg of paclobutrazol; L = commercial yogurt, 2 mg/kg of paclobutrazol; TC = commercial baking powder, no contamination; TH = commercial baking powder, 4 mg/kg of paclobutrazol; TL = commercial baking powder, 2 mg/kg of paclobutrazol. 0h indicates initial time of fermentation and 24h indicates the end of the fermentation process. PC1 = principal component 1; PC2 = principal component 2.

that fermentation time played a more important role in bacterial diversity than did paclobutrazol.

Functional Analysis of Cluster of Orthologous Groups and Kyoto Encyclopedia of Genes and Genomes

To infer the functional genome composition and compare the differences between samples, PICRUST software was used (Shen et al., 2017). In addition,

the sequencing results were compared with Cluster of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to predict the functions of sample bacteria. The pathway was analyzed on KOBAS version 2.0 web server (Xie et al., 2011).

From the COG analysis (Figure 5A), we concluded that the significant differences between CK24h and CK0h were mainly concentrated in cellular processes and signaling, metabolism, and information storage

Figure 5. The different functions of Cluster of Orthologous Groups (COG) between CK0h and CK24h (A); TC24h and TC0h (B); TH24h and TC24h (C); TL24h and TC24h (D). CK = commercial yogurt, no contamination; TC = commercial baking powder, no contamination; TH = commercial baking powder, 4 mg/kg of paclobutrazol; TL = commercial baking powder, 2 mg/kg of paclobutrazol. 0h indicates initial time of fermentation and 24h indicates the end of the fermentation process.

Figure 6. The different functions of Kyoto Encyclopedia of Genes and Genomes (KEGG): between CK0h and CK24h (A); TC24h and TC0h (B); TC24h and TH24h (C); TL24h and TC24h (D). CK = commercial yogurt, no contamination; TC = commercial baking powder, no contamination; TH = commercial baking powder, 4 mg/kg of paclobutrazol; TL = commercial baking powder, 2 mg/kg of paclobutrazol. 0h indicates initial time of fermentation and 24h indicates the end of the fermentation process.

and processing (3 aspects of 13 functions). Compared with CK0h, CK24h was significantly enriched in carbohydrate transport and metabolism, replication, recombination and repair, cell cycle control, cell division, and chromosome partitioning. Moreover, compared with the H24h treatment, L24h and CK24h had no significant influence on COG functions. We conjectured that the degradation of paclobutrazol was not significant during fermentation and might be related to these results.

For the samples that used baking powder, compared with TC0h, TC24h was significantly enriched in carbohydrate transport and metabolism, indicating that carbohydrate transport and metabolism were crucial functions as fermentation progressed (Figure 5B). Compared with TC24h, both samples spiked with paclobutrazol (TH24h and TL24h) were significantly enriched the functions of cell cycle control, cell division, and chromosome partitioning (Figure 5C), demonstrating that addition of paclobutrazol could promote these functions. In addition, 10 functions differed between TC24h and TL24h (Figure 5D), including significant enrichment of translation, ribosomal structure and biogenesis, nucleotide transport and metabolism, cell cycle control, cell division, and chromosome partitioning in TL24h.

The results of the KEGG analysis are shown in Figure 6. Compared with CK0h, CK24h was significantly enriched in metabolism, human diseases, organismal systems, cellular processes, environmental information processing, genetic information process, environmental information processing, and genetic information processing (Figure 6A). The main metabolic pathway functions of CK24h were enriched in carbohydrate metabolism, replication and repair, and translation. For CK0h, the main enrichments were amino acid metabolism, global and overview maps, and metabolism of cofactors and vitamins. For KEGG function prediction, carbohydrate metabolism was significantly enriched in CK24h, which was the same as COG function prediction. In addition, compared with H24h, L24h and CK24h had no significant influence on KEGG function, which was the same as COG. From analysis of the KEGG metabolic pathway, we confirmed that the lack of change in paclobutrazol concentration during yogurt fermentation process was related to the relative absence of microbial degradation.

The main differences of KEGG functions between TC24h and TC0h are shown in Figure 6B. The function significantly enriched in TC24h was carbohydrate metabolism, while the function more enriched in TC0h was AA transport and metabolism. The differences between TC24h and TH24h were the same as those for TC24h and TL24h (Figure 6C). Compared with TC24h and TL24h (Figure 6D), the functions of TC24h

were significantly enriched in xenobiotics biodegradation and metabolism. The above results show that the addition of paclobutrazol could inhibit xenobiotic biodegradation and metabolism, which further explained why paclobutrazol did not degrade during yogurt fermentation. The related mechanisms need to be further investigated.

CONCLUSIONS

In this study, we explored the effect of paclobutrazol on yogurt fermentation. The results illustrated that the concentration of the 2 isomers showed no visible degradation, and there were no significant stereoselective behaviors occurring in any of the samples. We explained the lack of degradation of paclobutrazol in the context of microbial composition and function, and found that stable residues of paclobutrazol can cause potential food safety risks. Paclobutrazol did affect microbial community structure during yogurt fermentation. However, microbial α -diversity analysis indicated that fermentation time played a more important role in diversity than paclobutrazol concentration. Relative abundances of *Staphylococcus* were significantly increased in paclobutrazol-added samples when commercial baking powder was used as a starter, bringing potential risk to the quality of yogurt. Moreover, the addition of paclobutrazol inhibited some functions of microorganisms, including xenobiotic biodegradation and metabolism, which might mean that some pollutants, including paclobutrazol, could not degrade during fermentation. Based on the above results, we infer that presence of paclobutrazol residues could weaken microbial degradation and metabolism in yogurt, and the presence of *Staphylococcus* may pose a risk to food safety.

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