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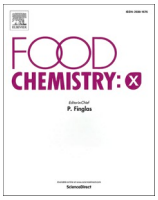

The effective of bacterial community dynamics driven by different starter cultures on the flavor development of Chinese fermented sausages

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ABSTRACT

This study aimed to understand the community successions driven by different starters and their effects on the flavor development of Chinese fermented sausages. The results showed that the bacterial genus (67.6%) and pH (32.4%) were the key factors influencing the volatile profile. Inoculated the starters composed of *Pediococcus* and staphylococci maintained the stable community succession patterns dominated by staphylococci (samples T and S). Although the highly acidic environment (pH < 5.2) caused the community to exhibit a fluctuation in succession pattern, the inoculation of *Latilactobacillus paracasei* (sample Y) maintained microbial diversity and was conducive to the accumulation of aldehydes and esters. In sample P, inoculated the starter with *Latilactobacillus* and *Staphylococcus* also maintained microbial diversity, the moderately acidic environment (pH > 5.4) resulted in a stable succession pattern of the microbial community, and it was not conducive to the accumulation of aldehydes, alcohols and esters.

1. Introduction

Traditional Chinese spontaneous fermented sausages were a mixture of lean pork, pork fat, salt, sugar, nitrite, spices, and/or Chinese liquor stuffed into casings, then left them to ferment and dry continuously in an open environment (Hu et al., 2020; Wang et al., 2022). In this process, the microorganisms originating from raw materials and the environment play a crucial role in the formation of flavor profiles (Hu et al., 2020; Wang et al., 2022). However, the effects of spontaneous fermentation relying on empirical methods on sausage flavor formation are complex and uncertain (Franciosa, Alessandria, Dolci, Rantsiou, & Cocolin, 2018; Xiao, Liu, Chen, Xie, & Li, 2020). At present, the fermented sausage industry is relying on starter cultures to provide a standardized flavor (Hu et al., 2020; Wang et al., 2022; Xiao et al., 2020). Most of the starter cultures consist of lactic acid bacteria (LAB), which is responsible for environmental acidification, and *Staphylococcus*, which contributes to the development of color and flavor (Hu et al., 2020; Sánchez Mainar, Stavropoulou, & Leroy, 2017; Xiao et al., 2020). Moreover, there is growing interest in the use of probiotic starter cultures in fermented meat products, as they can offer potential health benefits and sensory properties (Franciosa et al., 2018; Sánchez Mainar et al., 2017).

Recently, many studies have indicated that the inoculation of starter culture can improve the volatile organic compound (VOC) profile of Chinese fermented sausage (Chen, Kong, Han, Xia, & Xu, 2017; Wang et al., 2022; Yang et al., 2022). For example, the inoculation of *Pediococcus pentosus*, *Latilactobacillus sakei* and *Staphylococcus xylosus* could

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increase the content of ester compounds (Chen et al., 2017). While the inoculation of *Latilactobacillus plantarum* and *S. xylosus* could increase the content of aldehydes and ketones (Xiao et al., 2020). To understand the effect of starter culture on the volatile flavor profile of fermented sausage, previous studies focused on a series of biochemical reactions, driven by starter cultures, related to the formation of volatile flavor compounds, such as proteolysis, fatty hydrolysis, carbohydrate metabolism, amino degradation and esterase reaction (Chen et al., 2017; Ferrocino et al., 2018; Toldrá, Astiasaran, Sebranek, Talon, & Hui, 2015; Wang et al., 2022; Xiao et al., 2020). However, those studies ignored the role of microbial community structure in the formation of flavor profiles, frequently. It is difficult to understand the effect of substance metabolism which is driven by starter cultures on flavor profile formation, especially in fermented sausage with a complex microbial community.

To understand the relationship between microbial communities and volatile compounds in Chinese fermented sausages better, some studies have been performed to investigate and analyze the correlation between microbial communities and volatile flavor compound composition of fermented sausages at a particular time in the entire fermentation period, usually after complete fermentation (Hu et al., 2019., Hu et al., 2020; Yang et al., 2022). For example, in Harbin dry sausages, a Chinese traditional fermented sausage, *L. sakei*, and *L. plantarum* were correlated with the production of carboxylic acids and alcohols, while the *Lactococcus lactis* and *Latilactobacillus alimentarius* were positively related to the production of ketones, aldehydes and esters (Hu et al., 2020). However, sausage fermentation, as a complex biochemical progress involving a variety of species (Ferrocino et al., 2018), is difficult to elucidate the community assembly mechanisms and this effect on the development of flavor profiles by focusing on the biological information of the endpoint fermentation. At present, although several studies have investigated the microbial and VOCs dynamics during sausage fermentation, briefly analyzed changes in the community and volatile composition, which makes it difficult to improve the understanding of the mechanism of community evolution in fermented sausage and its impact on the development of volatile flavor profiles (Ferrocino et al., 2018; Hu, Wang, Kong, Wang, & Chen, 2021; Xiao et al., 2020). In other fermented foods, such as cheese, it has been shown that moisture content and the interaction between fungi and bacteria through the regulation of pH influence the assembly dynamics of microorganisms in cheese rind (Wolfe, Button, Santarelli, & Dutton, 2014). However, in the study of fermented sausage, the correlation between microbial dynamics and volatile metabolites, as well as which factors affect the community assembly process remains unclear.

To understand the mechanism of bacterial community formation in fermented sausage and its effect on the development of volatile flavor profiles, we selected three commercial starter cultures (namely THM-17, SBM-52 and PROMIX-5) (Yang et al., 2022) and a probiotic starter culture (*Latilactobacillus paracasei*) to prepare fermented sausage. 16S rDNA sequencing and gas chromatography/mass spectrometry were performed to investigate changes in community dynamics and volatile flavor profiles driven by different starter cultures. Besides, the dynamic changes of the physicochemical indexes were also investigated, and then, the sensory attributes of different samples were evaluated by panelists. This study may help to understand the mechanism of bacterial community assembly and its effect on the development of volatile flavor profiles in Chinese fermented sausage.

2. Materials and methods

2.1. Manufacturing fermented sausages

Chinese fermented sausages were prepared with lean pork (80%) and pork fat (20%) which were purchased from a local supermarket (Jinzhou, Liaoning, China), other ingredients used were: glucose (3 g/kg), salt (25 g/kg),), nutmeg (1 g/kg), clove (1 g/kg), cinnamon (1 g/kg) were also purchased from the local supermarket, and sodium ascorbate (0.5 g/kg), sodium caseinate (1 g/kg), sodium nitrite (0.15 g/kg) were purchased from Sichuan Jinshan Pharmaceutical Co, ltd (Emei, Sichuan, China). Three commercial starter cultures included THM-17 composed of *P. pentosaceus* and *S. xylosus* (9×10^{10} CFU/g); SBM-52 composed of *P. pentosaceus*, *P. acidilactici*, *S. xylosus*, and *S. carnosus* (1×10^{11} CFU/g); and PROMIX-5 composed of *S. xylosus*, *L. sakei*, and *L. plantarum* (2×10^{11} CFU/g) produced in Clerici Sacco, Cadorago, Italy and purchased from an import retail shops in Shanghai, China. And the *L. paracasei* (1×10^{10} CFU/g) was isolated from yogurt (Putranto, Mustopa, Kusumawati, & Prastyowati, 2020).

After mincing and mixing, the meat mixture was divided into five batches: *L. paracasei* with 10 log CFU/g was added to the meat mixture and named Y; According to the using instruction, three commercial starter cultures, THM-17, SBM-52 and PROMIX-5, were added to the meat mixture with the final concentration 0.02% (w/w) and named T, S and P, respectively. The last batch was named CK without inoculating any starter culture. Each batch of the mixture was stuffed into an artificial collagen casing (Mackessen (Shanghai) Food Co., ltd (Shanghai, China)) of 20–22 mm in diameter and approximately 12 cm in length, the initial weight of each fresh sausage was approximately 100 g. For each group, about 90 sausages were yielded. Fermentation and ripening were carried out in a KBF 240 humidity chamber (BINDER, Germany). The sausages were fermented at 18 °C–20 °C and 90%–95% relative humidity for 3 days, and then ripened at 10 °C–15 °C and 70%–85% relative humidity for 18 days. These fermented sausages at 0 (meat mixtures), 3, 7, 12, and 21 days of fermentation/ripening were taken and stored in a refrigerator (Haier FCD-195SE, China) at −20 °C until further analysis for subsequent analysis (Yang et al., 2022).

2.2. Sensory evaluation

The sensory attributes were evaluated by 19 selected panelists and the method was described by (Yang et al., 2022). According to ISO 8586:1993, each panelist had completed 100 h of generalized training and an average of 1 year of experience in descriptive sensory testing, including fermented sausages. And all sensory descriptors in Table S1 have been selected from the descriptions of fermented sausages given by panelists.

2.3. pH, water activity, color analysis, and moisture content

During the fermentation and ripening, the pH, water activity (a_w), color, and moisture content of different samples were recorded. The pH was detected by using a pH-meter PH200 (CLEAN, Shanghai, China). The a_w was detected by using a water activity meter (HD-6, Wuxi Huake Instrument Co., Ltd., Wuxi, China). The color parameters lightness (L^*), redness (a^*) and yellowness (b^*) were detected by using a Minolta CR-400 color meter (Konica Minolta, USA). And the moisture content was measured by dehydration at 100 °C to a constant weight (Yang et al., 2022).

2.4. Microbiological analysis

Each fermented sausage was finely sliced. 25 g minced sausage was added to 225 ml sterile saline solution and homogenized with a homogenizer (IKA, Germany) for 2 min at 6000 rpm. The homogenates were used to prepare decimal dilutions and 0.1 ml of each dilution was spread on the appropriate agar media. Lactic acid bacteria (LAB) counts were done on Man Rogosa Sharpe Agar (MRS) (Grbio, Shanghai, China) at 30 °C for 48 h. Staphylococci counts were done on Mannitol Salt Agar (MSA) (Grbio, Shanghai, China) incubated at 30 °C for 48 h.

2.5. Texture profiles

Each fermented sausage during fermentation and ripening was used to detect the texture. The texture profile was detected by using a TA-XT

Plus (Stable MicroSystems, UK). The samples (cylinders) approximately 1.5 cm thick and 1.5 cm in diameter, after discarding the artificial casing of the sausages, were equilibrated to room temperature and compressed twice to 50% of their original thickness at a constant speed of 1 mm/min. The following parameters were detected: hardness, cohesiveness, gumminess, chewiness and springiness (Lorenzo, Gómez, & Fonseca, 2014).

2.6. Volatile profiles

Each fermented sausage was minced and weighed 2.0 g into headspace vials. Samples were equilibrated at 40 °C for 15 min and the extraction of headspace volatile compounds was done using a 50/30 μm CAR/DVB/PDMS fiber (Agilent Technologies, USA) at 40 °C for 60 min. 2-Methylheptan-3-one (Aladdin, Shanghai, China) was used as an internal standard (Yang et al., 2022). The compounds of each sample adsorbed by the fiber were desorbed in a 7890B gas chromatograph coupled with a 5977B mass spectrometer (Agilent Technologies, USA) for 5 min at 250 °C. The compounds were separated on a DB-WAX capillary column (Agilent Technologies, USA, 30 m, 250 μm i.d., film thickness 0.25 μm). Helium was used as carrier gas with a flow rate of 1 ml/min. The GC oven temperature program began at 40 °C, held for 5 min, ramped to 110 °C at 3 °C/min and held at 110 °C for 5 min, then to 150 °C at 2 °C/min and held at 150 °C for 5 min, and finally to 250 °C at 10 °C/min, held at 250 °C for 5 min. Mass spectrometry was obtained by electron impact at 70 eV ionization energy. The scan range was 20–500 *m/z*, and the transfer tube temperature was 250 °C. The compounds were identified by comparison with mass spectra from the NIST11 library database (Agilent). Quantitative data (μg/g) was obtained by calculating the relative peak area of each compound compared with that of the internal standard (Ferrocino et al., 2018).

2.7. DNA extraction and sequencing analysis

Samples were sent to the Chinese Academy of Inspection and Quarantine for genomic DNA extraction and Illumina MiSeq high-throughput sequencing. Each sample (5.0 g) was extracted genomic DNA by QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Germany) according to instructions. After genomic DNA extraction was completed, 1% agarose gel electrophoresis was used to detect the extracted genomic DNA. The primers used to amplify the V3-V4 region of the 16S rRNA bacterial gene were 343F (5′-CTCCTACGGRRSGCA GCAG-3′) (Liu, Lozupone, Hamady, Bushman, & Knight, 2007) and 806R (5′-GGACTACNVGGGTW TCTAAT-3′) (Caporaso et al., 2011). The sequencing platform was MiseqPE300 and the databases of Silva 128 were used for sequence alignment.

2.8. Statistical analysis

ANOVA, principal component analysis was performed to visualize the discrimination among different samples using the R program (v.4.0.2). Variance decomposition was performed to understand the contribution of different factors to the formation of volatile compounds using the R program (v.4.0.2). The correlations between selected genus and volatile compounds were calculated using Pearson's correlation coefficients and visualized using R program (v.3.6.3). All samples were analyzed in triplicates in this work.

3. Results and discussion

3.1. Sensory characteristics of different chinses fermented sausages

To understand the effects of different starter cultures on the sensory properties, we selected four starter cultures and a probiotic, or used no starter culture, to produce fermented sausages and assess their differences in sensory quality. Surprisingly, sample Y, inoculated with *L. paracasei*, received the highest scores for overall acceptability (7.06), followed by samples S (6.67) and T (6.22), and then sample P (5.53) (Fig. 1a, b). As shown in Table S1, samples S and T had similar characteristics in odor, with moderately floral, fruity, cheesy, meaty, slightly sour and rancid aromas. Sample Y was more floral, fruity, cheesy and meaty, whereas less floral and fruity and more metallic, sour and rancid aromas were perceived in sample P. Although the basic physical and chemical indexes were different among those samples, we did not observe significant differences in their appearance and texture ($P > 0.05$), and the differences in a_w (0.75–0.78), moisture (22.0%–25.0%), color and texture of the final products could not be distinguished by panelists (Fig. 1c–e). Thus, we focused on the effects of the different starter cultures in terms of odor, which was an important factor influencing the overall acceptability of fermented sausages (Toldrá et al., 2015).

3.2. Development of volatile profiles in different samples

A total of 58 VOCs were identified in different samples. The VOCs identified here were: eight aldehydes, nine ketones, 16 esters, eight alcohols, 11 acids and six other compounds (Fig. 2a). Different samples usually clustered together in the upper part at 0–3 days, and clustered in the bottom at the end of ripening, except sample CK (21 days). Then, we focused on the dynamic changes of VOCs in samples P, T, S and Y. There was a similar trend in the variation of VOCs in different samples, with a slow increase within the first 3 days, followed by a massive increase during the ripening period (Fig. 2b). In the early stage of fermentation, microbial metabolize substances more for their own growth, so the volatile profile did not change significantly, while in the middle and last stages of fermentation, the microbial community tended to show a stronger functionality, that is, more VOCs would be produced (Toldrá et al., 2015). It was obvious that samples S and Y had a large percentage of esters (21.96% and 28.45%, respectively) at the end of ripening (Fig. 2b), which may contribute to fruity and floral characteristics (Corral et al., 2015; Olivares, Navarro, & Flores, 2009). Samples P and T were characterized by a high content of ketones (32.02% and 27.31%, respectively), which may contribute to a lactic odor and a mushroom aroma (Toldrá et al., 2015). To understand the effect of microbial growth on the changes in VOCs level better, we selected several key VOCs (see Table S2 in the supplemental material) which might be associated with the typical flavor of fermented sausage for further analysis and discussion.

Benzeneacetaldehyde, as an important compound was related to the roses, floral and fresh aromas (Toldrá et al., 2015). As shown in Fig. 3, it was absent at day 0, and increased during the fermentation process, with its level being significantly increased in sample Y (Fig. 3a). Octanal is an important compound in fermented sausage which contributes to a floral note (Olivares et al., 2009; Toldrá et al., 2015). And it increased slowly from day 0 to day 12, but stabilized thereafter, with its levels being higher in samples Y and T compared with samples S and P (Fig. 3a). 3-Methyl-butanal, another important aldehyde, is a low-threshold flavor compound that contributes to fruity, cheese and rancid aromas (Olivares et al., 2009; Toldrá et al., 2015). It increased slowly in samples P and S, and stayed at a low concentration at the late stage of drying (Fig. 3a).

Acetic acid, which contributes to the vinegar note, was the most abundant acid in each sample, with its level increasing rapidly after day 12 in samples P and S (Fig. 3b). The origin of acetic acid is mainly the microbial metabolism of glucose, and we were interested in the detection of the lowest level of this compound in sample Y. It might imply that non-volatile organic acids might result in the low pH of sample Y; 3-methyl-butanoic acid was detected in samples S, T P and Y, but 2-methyl-butanoic acid alone was detected in sample Y, at a low level (Fig. 3b). These results were consistent with other studies, in which the predominant *Staphylococcus* in fermented sausages catabolizes isoleucine and leucine to form these acids; in turn, they have an important effect on aroma development because of their cheesy, sweet or rancid odors (Toldrá et al., 2015). In addition, we found some unpleasant acids

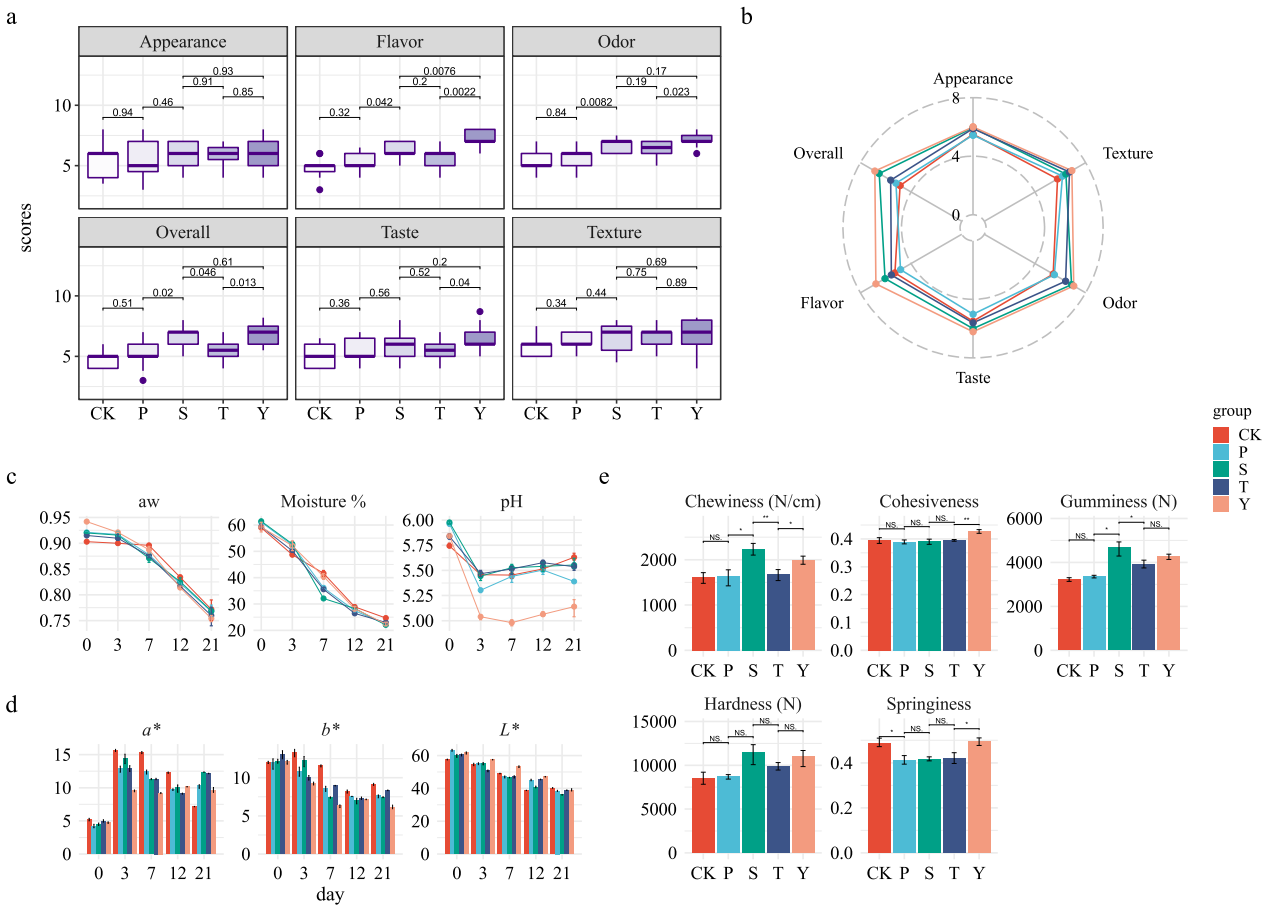

Fig. 1. Sensory and quality of different fermented sausages. (a) The distribution of sensory score. (b) Radar graph displaying the odor, taste, texture, appearance, Flavor, and overall acceptability scores expressed by panelists. (c) The dynamic changes of water activity (a_w), moisture, and pH of fermented sausages. (d) Color formation of different samples. (e) Texture profiles of the end-products. *$P < 0.05$; **$P < 0.01$; No significant difference: NS.

in each sample, such as octanoic acid, hexanoic acid and heptanoic acid, especially in samples S, T and P (Fig. 3). This might be one of the reasons for the lower overall acceptability of these samples compared to the sample Y.

Regarding ketones, acetoin was the VOC with the highest concentration in all samples, especially in sample P (Fig. 3c). Acetoin is a by-product of carbohydrate metabolism in LAB (Ferrocino et al., 2018). The carbohydrate metabolism of *Latilactobacillus* is stronger than that of *Pediococcus* at temperatures below 20 °C (Toldrá et al., 2015). This might lead to a higher level of acetoin in sample P compared to samples S and T (Fig. 3c). Moreover, previous studies reported that a higher level of acetoin was detected in fermented sausages inoculated with *L. sakei* than in those inoculated with *P. pentosaceus* (Montanari, Bargossi, Gardini, Lanciotti, & Tabanelli, 2015). However, the excessive content of acetoin in fermented sausages might contribute to a sour note (Ferrocino et al., 2018; Pilevar & Hosseini, 2017). Interestingly, the inoculation of *L. paracasei* in sample Y reduced the production of acetoin compared with other samples. Generally, *L. paracasei* is used as a starter culture for yogurt or cheese. These products were always at relatively high temperatures (above 35 °C), and more acetoin could be produced. In contrast, we prepared fermented sausages at a lower temperature (10 °C–15 °C), which might decrease the accumulation of acetoin in sample Y.

As for alcohols, 1-octen-3-ol, which is closely associated with the characteristic mushroom odor, is often described as an important volatile component of fermented sausages because of its low odor threshold values (Olivares et al., 2009). This compound was not detected in the raw pieces and increased slowly during the fermentation process (Fig. 3d). 2,3-Butanediol was another abundant compound in each sample (Fig. 3d). The low content of this compound detected in sample P suggests that it might be converted to acetoin by butanediol dehydrogenase (Ferrocino et al., 2018), which increased the acetoin level in sample P (Fig. 3c, d).

Esters were the most important compounds for the fermented sausage aroma because of their low odor threshold values (Olivares et al., 2009). The levels of these compounds increased slowly during the early fermentation stage, then rapidly throughout the maturation period; such as ethyl hexanoate and ethyl 3-methylbutyrate (Fig. 3e). The fermentation could be roughly divided into three stages based on the change trends of esters level. In short, we observed a slow esters level growth rate in stage I (0–3 days); a fast esters level growth rate in stage II (3–12 days); and a flat esters level growth rate in stage III (12–21 days) (Fig. 3e). Notably, there was a correlation between the accumulation of esters level and the evolution pattern of LAB and staphylococci in each sample. A rapid increase in the esters level started at a colony count of LAB and staphylococci > 7.4 log CFU/g and then slowed down when the colony count reached about 8.5 log CFU/g (Fig. 3e, f). On the other hand, we noticed that the rapid (sample T) or jumpy (sample P) growth of LAB and staphylococci in stages I and II was not conducive to the accumulation of esters. In contrast, the stable growth of LAB and staphylococci in sample S during the fermentation process yielded high esters levels (Fig. 3e, f). It is possible that, in samples T and P, the rapid and jump growth of LAB and staphylococci in stages I and II were not conducive to the accumulation of ester precursors, resulting in lower ester levels at stage III. Although the bacterial growth pattern in sample Y was similar to that of sample T, the esters level in sample Y continued

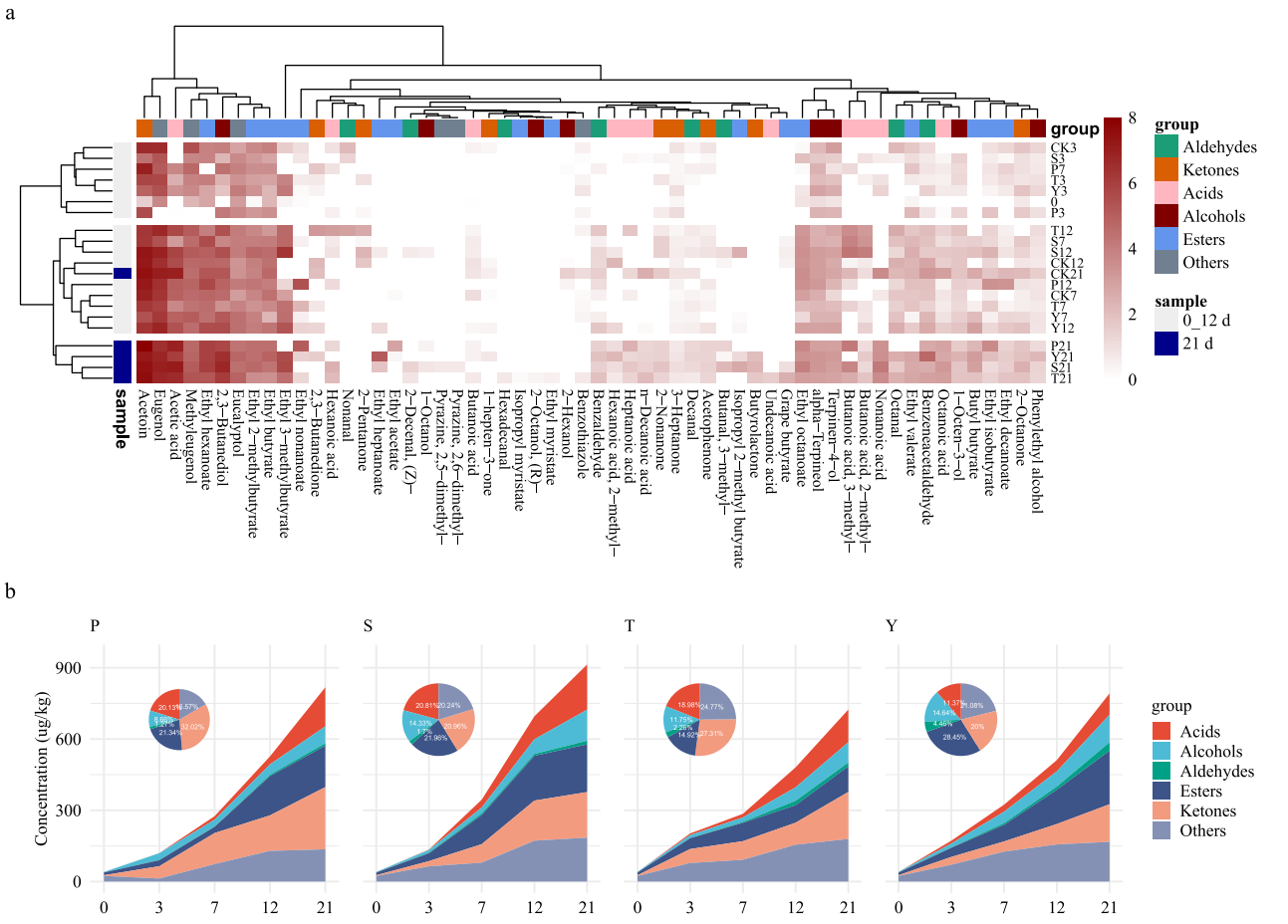

Fig. 2. The dynamic changes of volatile profiles during fermentation. (a) Heatmap of volatile compound levels of the samples at different times. (b) The levels of different types of volatile organic compounds during fermentation. The numbers in the legend indicated the logarithmic value of the volatile compound levels (c), i.e., the value = $\log_2^{(c+1)}$.

to increase during fermentation and was relatively high at the end of ripening. indicating that *L. paracasei* as a starter culture achieved similar results to those of the commercial starters (in particular SBM-52).

3.3. Dynamic succession of microbial communities in different samples

One of the main objectives of fermented food microbial community research is to understand the dynamic of species abundance and their influence factors during fermentation (Wolfe et al., 2014). Here, we described the variability of microbial community succession processes in different samples (Fig. 4). Since the same raw materials, process and fermentation conditions are used. Therefore, we mainly suggested that the differences in microbial community assembly patterns among the samples were driven by different starter cultures. In the early fermentation stage, the indigenous bacteria (*Brochothrix*, *Photobacterium, Weissella* and *Acinetobacter*) which stemmed from raw meat, air, water and other environmental factors (Toldrá et al., 2015; Van Reckem et al., 2021) have spread and colonized in sausages (Fig. 4). Although the initial microbial composition of each sample had some similarities at the genus level, as fermentation progressed, it had a dramatic change (Fig. 4a). For example, according to the cluster analysis, there is a similar microbial composition between samples P and T at day 0 (Fig. 4a). Then the community succession showed a different dynamic pattern, that is, *Latilactobacillus* and *Staphylococcus* were the dominant genera in sample P, while *Staphylococcus* was the dominant genera in sample T (Fig. 4a, c, d). Furthermore, concerning microbial diversity, a previous investigation on microbial community succession of Chinese Chi-flavor type Baijiu had indicated a pattern of initial decrease and then increase in microbial diversity (Zhao et al., 2022). Our study mirrors this finding. Specifically, as shown in Fig. S1, the microbial diversity of samples CK, T, P and Y showed a similar trajectory of initial decrease followed by an increase in microbial diversity, while the microbial diversity of sample S exhibited a continuous decrease and stabilizing during the middle and late stages of fermentation (12–21 d). Notably, samples CK, Y, and P experienced the lowest microbial diversity on day 3 of fermentation, while sample T reached its lowest diversity on day 12.

To understand the effects of the different starter cultures on community succession in fermented sausage more clearly, we used area plots to display the dynamic assembly process of microbial communities. (Fig. 4b–f). The dynamics of LAB showed a similar trend in different samples: they grew rapidly first (0–3 days), followed by a decline (3–12 days), and finally grew slowly (12–21 days), whereas staphylococci grew rapidly from day 0 to day 7, and exhibited slight growth from day 7 to day 21 (Fig. 4b–f). The growth of these two types of bacteria affected the dynamics of the entire microbial communities, which ultimately led to three types of microbial structures (Fig. 4g): samples T and S were largely dominated by *Staphylococcus*, and it was consistent with the results of our previous study (Yang et al., 2022); whereas sample P was dominated by *Latilactobacillus* and *Staphylococcus*; and sample Y was dominated by *Latilactobacillus*. These findings were consistent with the results of the cluster analysis (Fig. 4a).

At the first stage of fermentation (0–3 days), compared with CK, the presence of *Latilactobacillus* in samples Y (*L. paracasei*), *Pediococcus* in samples T and S showed a strong competitive advantage in that they

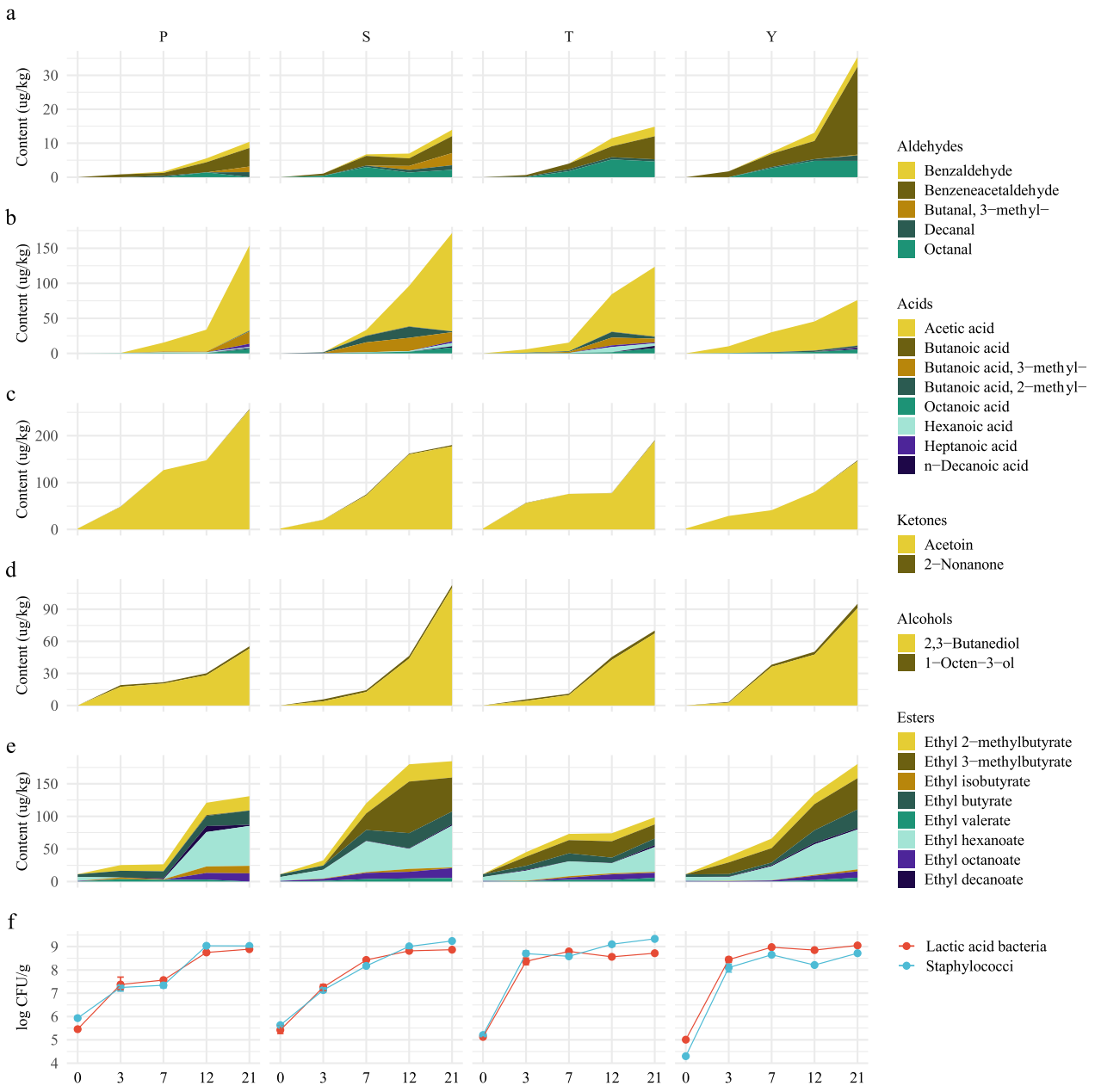

Fig. 3. The dynamic changes of key flavor compounds and microbiological counts. Dynamics of aldehydes (a), acids (b), ketones (c), alcohols (d) and esters (e) levels during fermentation. (f) Microbiological counts ($\log_{10}^{(CFU/g)}$) in different samples throughout the manufacturing process.

could grow rapidly and inhibit other indigenous bacteria, such as, *Photobacterium*, *Acinetobacter*, *Weissella* and rare genera with a relative abundance (RA) < 0.1% (Fig. 4b–f). Moreover, we found that the initial ratio of *Lactococcus*, *Latilactobacillus* and *Pediococcus* largely affected the type of dominant LAB (Fig. 4b–f), probably because the bacteriocin produced by one LAB could inhibit another LAB, and a genus was more numerous when it had an advantage in such an antagonism (Gao, Cao, Cai, & Sørensen, 2021; Talon & Leroy, 2014). The relationship between LAB and *Staphylococcus* was quantity dependent. When the proportion of *Latilactobacillus* or *Lactococcus* was higher than that of *Staphylococcus* at day 0, the relationship between them seemed to represent commensalism (Fig. 4a, d, f), and when the proportion of *Staphylococcus* was higher at day 0, the relationship between *Staphylococcus* and *Latilactobacillus* (or *Lactococcus*) seemed to be amensalism that was not conducive to the growth of LAB (Fig. 4b, d). In contrast, the relationship between *Pediococcus* and *Staphylococcus* resembled a mutualism. These findings indicated that the initial proportion of LAB and *Staphylococcus* and their interaction greatly affected the formation of the microbial community structure of fermented sausage. Moreover, this effect is mainly reflected in the early fermentation stage (0-3d). From day 3 to day 7, the RA of *Latilactobacillus*, *Pediococcus* or *Lactococcus* decreased, which was accompanied by the proliferation of staphylococci. It seemed that the rapid growth of *Staphylococcus* was detrimental to LAB (Fig. 4b–f), however, this relationship warranted further verification.

After day 7 (7–21 d), the microbial communities in different samples showed different drift patterns. The communities of samples S, T and P showed stable succession patterns, and the microbial communities of samples S and T were dominated by *Staphylococcus* but without diversity, while the microbial community of sample P was more diverse and dominated by *Latilactobacillus* and *Staphylococcus*; the microbial community of sample Y showed a fluctuation assembly process pattern, it was also diversity and dominated by *Latilactobacillus* (Fig. 4b–f, h). The relationship between community species diversity has been the focus of microecological research. There is a study indicated that the

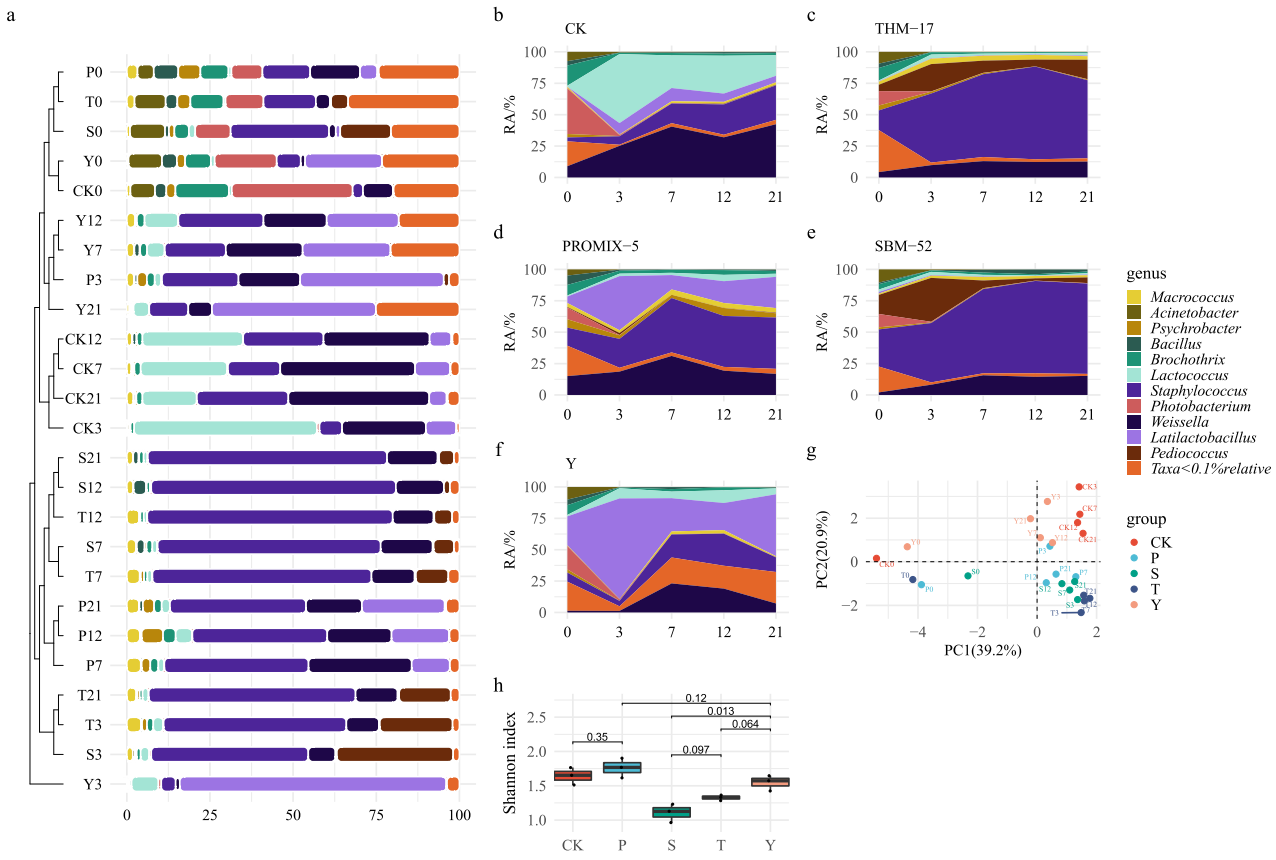

Fig. 4. The dynamic changes of microbiota in different samples during fermentation. (a) Cluster analysis of microbiota at different fermentation stages. The dynamic changes of microbiota in CK (b), T (c), P (d), S (e), Y(f). (g) PCA plot of the samples at different fermentation stages. (h) Alpha diversity plot of Shannon index for different samples at the end of fermentation.

stability of a microbial community was related to its diversity (Hu, Amor, Barbier, Bunin, & Gore, 2022). When the microbial community is more species diversity, the community succession tends to be more fluctuations. Interestingly, although the microbial diversity of sample P was higher than that of sample Y (Fig. 4h), the fluctuation effect was not as dramatic as sample Y (Fig. 4d, f). It was found that environmental changes in the process of community succession, such as the decrease of pH, would increase the intensity of species interaction and thus reduce the stability of the communities (Hu et al., 2022; Ratzke, Barrere, & Gore, 2019). Thus, the lower pH in sample Y cussed its microbial structure to exhibit a more dramatic fluctuation pattern. By contrast, the microbial communities without species diversity, and the mild pH environment in samples T and S led to stable community succession patterns.

Moreover, we found that *Latilactobacillus*, but not *Pediococcus*, could increase the microbial diversity of samples Y and P at the end of ripening (Fig. 4h), probably because *Pediococcus* produced pediocins, which have a large spectrum of inhibition (Talon & Leroy, 2014). Although the use of *Pediococcus* reduced microbial diversity (Fig. 4h), it facilitated the growth of *Staphylococcus*, which might be beneficial to the development of the aroma of samples S and T; in contrast, increasing the diversity of starters (e.g., SBM-52: *P. pentosaceus*, *P. acidilactici*, *S. xylosus* and *S. carnosus*) resulted in a better odor in sample S compared with sample T. In general, staphylococci are poor competitors in fermented sausages with a slow growth ability during fermentation (Ravyts et al., 2010). Therefore, increasing the RA of staphylococci in the initial bacterial community by using starter cultures is conducive to the quick colonization of staphylococci, ensuring its quantity–dependent competitiveness in the community evolution. And it might be beneficial to the flavor development of fermented sausage. Meanwhile, it was obvious that reducing the initial RA of staphylococci was detrimental to the development of odor and aroma, such as sample P. (Fig. 1a and Fig. 3a–e). Moreover, fermented sausages inoculated with PROMIX-5 or a probiotic bacterium (*L. paracasei*) did not reduce microbial diversity, and the RA of staphylococci was extremely low in sample Y; however, probiotic bacteria became the dominant genus and led to the development of a better flavor (Fig. 1a). This implies that the pattern of microbial diversity in sample Y was better in terms of aroma formation than was that in sample P.

3.4. Correlation between bacteria and volatile flavor compounds

In this study, we used the same raw materials and fermentation conditions for each sample; thus, the composition of the microbial communities and environmental factors had an important effect on the sausage flavor (Toldrá et al., 2015). Thus, we used a variance partitioning analysis to explore the contribution of abiotic and biotic factors to the formation of flavor compounds. As shown in Fig. 5a, the bacterial genus contributed to 67.6% of the formation of flavor, with *Staphylococcus*, *Lactococcus*, *Latilactobacillus* and *Weissella* being the prominent genera ($P < 0.01$), whereas abiotic factors (pH) contributed to 32.4% of the formation of flavor.

With respect to the influence of bacteria on volatile flavor compounds, analogous research conducted on other fermented foods such as Chinese semi-dry Hakka rice wine and traditional sweet rice wine revealed that bacterial genera exhibiting high RA played a role in the generation of diverse VOCs (Qian et al., 2023). Thus, a correlation analysis was performed to investigate the connection between the bacterial genera with high RA and VOCs, employing a clustering heatmap for visualization. As shown in Fig. 5b, there were significant positive

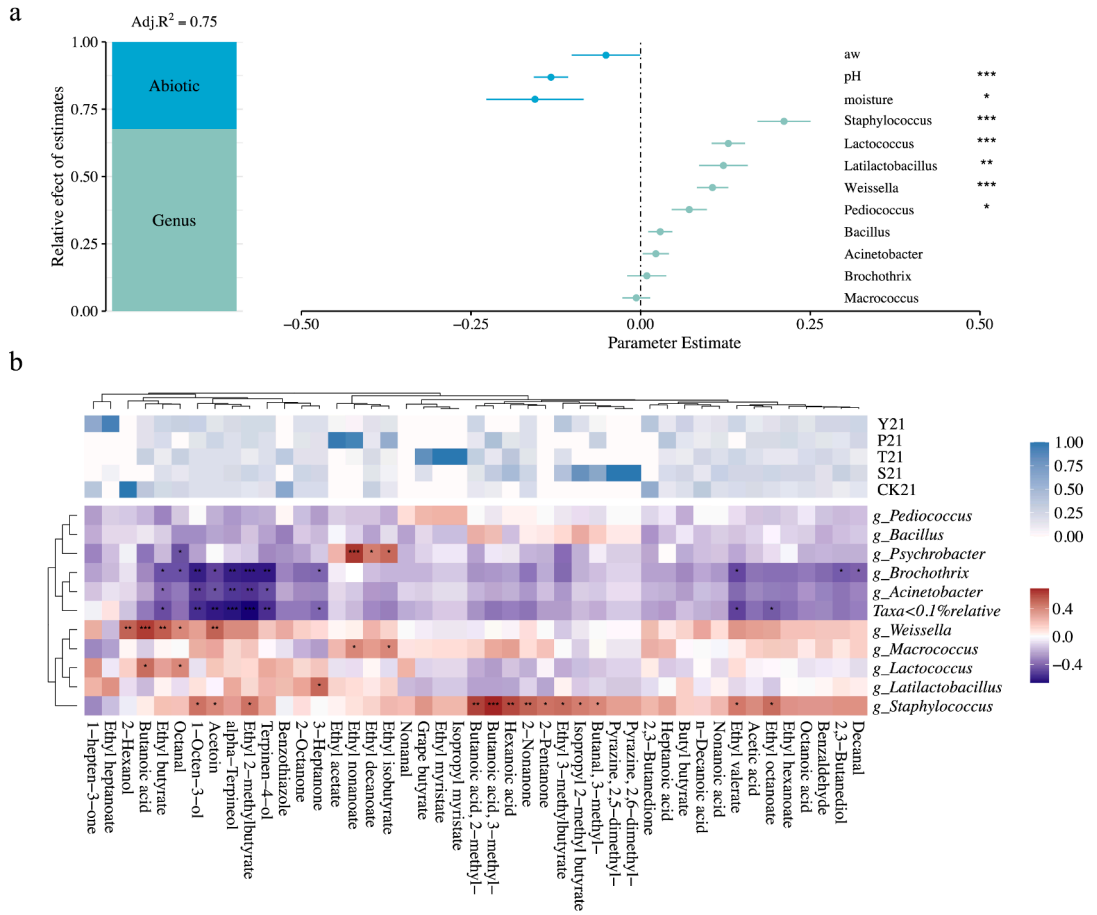

Fig. 5. Effect of abiotic and biotic factors on the flavor compounds development. (a) Average parameter estimates of model predictors, associated 95% confidence intervals and relative importance of each factor, expressed as the percentage of explained variance. (b) Heatmap of correlation analysis between microbial genus and volatile organic compounds based on Pearson's correlation coefficients. The adjusted (adj.) R^2 of the averaged model was 0.75. The numbers from 0.00 to 1.00 indicated the relative abundance of flavor compounds. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

correlations between *Staphylococcus* and most of the flavor compounds during fermentation, in particular 3-methyl-butanal, 2-nonanone, 2-methyl-butanoic acid, 3-methyl-butanoic acid, 1-octen-3-ol, ethyl valerate, ethyl 2-methylbutyrate, ethyl 3-methylbutyrate and ethyl octanoate ($P < 0.05$); moreover, it was clear that these compounds accumulated in large quantities after 3 days of fermentation and were present at high levels in samples S or T, in which *Staphylococcus* were overwhelmingly predominant from day 7 to day 21. Conversely, we found several associations between LAB and several flavor compounds during fermentation. The presence of *Weissella* was positively correlated with ethyl butyrate, ethyl decanoate, ethyl 2-methylbutyrate, acetoin, acetic acid, butanoic acid and heptanoic acid during fermentation. In turn, *Pediococcus* was correlated with isopropyl myristate, ethyl myristate, grape butyrate and nonanal; *Lactococcus* was correlated with octanal, acetoin, butanoic acid and ethyl butyrate; and *Latilactobacillus* was correlated with 2-octanone, ethyl acetate, ethyl heptanoate, butyl butyrate, etc. (Fig. 5b). Moreover, *Psychrobacter* and *Macrococcus*, which exhibited a high RA in sample P (Fig. 4d), were positively associated with acetoin, acetic acid, 1-octen-3-ol, ethyl isobutyrate, ethyl nonanoate and ethyl isobutyrate ($P < 0.05$). Although the species level was not distinguished in this study, due to the high inoculation level, the *Latilactobacillus* and *Staphylococcus* in the different fermentation progress were largely derived from the addition of the starter cultures (Stavropoulou et al., 2018). Those findings indicated that the dominant *Staphylococcus* or a diverse microbial community with a predominant presence of *Latilactobacillus* (*L. paracasei*) might both contribute to sausage aroma development.

Commonly, the starter cultures of fermented sausages are composed of LAB and *Staphylococcus* (Pilevar & Hosseini, 2017). In general, the endo- and exopeptidases, released by LAB (such as *Pediococcus*), contribute to increasing the concentration of free amino acids which can be utilized by *Staphylococcus* because of its low proteolytic activity (Tremonte et al., 2010). Thus, the *Pediococcus* in THM-17 could inhibit more background microorganisms and provide a suitable environment for the growth of *Staphylococcus* to produce flavor compounds (Fig. 4c, 6b). Moreover, by increasing the microbial diversity of both *Pediococcus* and *Staphylococcus* (SBM-52: *P. pentosaceus*, *P. acidilactici*, *S. xylosus* and *S. carnosus*), the aroma profile of fermented sausages (in particular ethyl esters) could be improved, probably because diverse staphylococcal populations increased ecosystem functioning compared with monocultures, and different staphylococci will be predominant at different stages of fermentation, as the pH dynamic changes, thus taking full advantage of their functional properties (Giri, Shitut, & Kost, 2020; Søndergaard & Stahnke, 2002; Stavropoulou, De Maere, et al., 2018). The species combination pattern of PROMIX-5 was designed to increase the microbial diversity of fermented sausages, to promote aroma development (Fig. 4h). However, such a combination pattern seemed to be unsuitable for producing fermented sausages in China, probably because of the high RA of *Weissella* and its associated unpleasant compounds (e.g., acetic acid, butanoic acid and heptanoic acid) in sample P. Bacterial starters are often made up of a balance between LAB and staphylococci, but can also be composed of LAB exclusively (Talon & Leroy, 2014). We found that the addition of probiotics (*L. paracasei*) to sample Y was correlated with a decrease in the RA of *Weissella* and that

such a microbial structure, dominated by probiotics, was beneficial to the aroma of fermented sausages, as many panelists preferred sample Y (Fig. 1a, b). Some studies have shown that probiotic bacteria improve the flavor of fermented sausages because of their proteolytic, glycolytic and lipolytic activities (Bis-Souza et al., 2019). For example, exopeptidases from *Latilactobacillus* are responsible for the generation of free amino acids from the muscle proteins, whereas the aldehydes, alcohols and acids derived from the degradation of leucine, valine, phenylalanine and methionine have very low-threshold values (Leroy, Verluyten, & De Vuyst, 2006). However, most probiotics belong to the category of *Latilactobacillus*, and it has been reported that *Latilactobacillus* has a limited ability to convert amino acids to form branched-chain flavor compounds (Gutsche, Tran, & Vogel, 2012); therefore, the formation of flavor is the result of a combination of microbial species (Franciosa, Ferrocino, Giordano, Mounier, & Cocolin, 2021).

Regarding the effect of pH on the volatile flavor compounds (Fig. 5a). In fact, pH contributed to flavor profiles by influencing the growth of species, especially *Staphylococcus*, which had a significant positive correlation with most of the flavor compounds. Here we mainly discussed the effect of pH changes driven by LAB on the growth of *Staphylococcus*. Firstly, we found that the dynamic changes of the microbial community depended on the abiotic factors (with principal coordinate one (PC1) being significantly associated with the pH measured across samples) (Fig. 6a) and microbial interaction (Fig. 6b). To better understand the potential microbial interactions in the fermentation process, correlation analysis was conducted on the information of bacteria genus at different samples and their fermentation time nodes (Fig. S1). The results showed that *Staphylococcus* and *Pediococcus* remained a significant positive correlation throughout the fermentation process in different samples (Fig. S1a–e); inversely, a significant negative correlation between *Latilactobacillus* and *Staphylococcus* at days 3, 7, 12 and 21 (Fig. S1b–e). Therefore, we suggested that *Latilactobacillus* and *Pediococcus* may affect the growth of *Staphylococcus* by changing the environmental pH. In detail, during the early fermentation stage (0–3d), sample Y (*L. paracasei*) and sample P (*L. sakei*, *L. plantarum* and *S. xylosus*) were inoculated with *Latilactobacillus*, with higher acid production efficiency (Leroy et al., 2006; Stavropoulou, Filippou, et al., 2018), these *Latilactobacillus* used the carbohydrates to produce acid rapidly, causing the pH of samples Y and P to drop to 5.04 and 5.30 (Fig. 1c), respectively. The low pH environment was not conducive to the growth of staphylococci (Van Reckem et al., 2021), so the RA of staphylococci in samples Y and P did not grow as rapidly as in samples T and S during the early fermentation period (Fig. 4a, c–e). In the middle and late stages of fermentation (3–21 d), the pH rises due to the transamination reaction of amino acids and the esterification reaction of organic acids, which consume the acids in the environment. In this stage, although they remained a negative correlation between *Latilactobacillus* and *Staphylococcus* in statistical analysis, the inhibitory effect of acidic conditions on staphylococci was alleviated, and the RA of staphylococci in samples P and Y gradually increased. In samples T (*P. pentosaceus* and *S. xylosus*) and S (*P. pentosaceus*, *P. acidilactici*, *S. xylosus* and *S. carnosus*), *Pediococcus* with slower acid production efficiency were inoculated (Leroy et al., 2006; Stavropoulou, Filippou, et al., 2018). Their pH was maintained above 5.44 throughout the whole fermentation (Fig. 1c), and the mild acid environment allowed *Staphylococcus* to grow rapidly, which dominated the overall microbial community structure by day 7 of fermentation (Fig. 4c, e). These results indicated that LAB affects the growth of *Staphylococcus* which was related to most VOCs by changing environmental acidity, thus might shaping the flavor profile differences among different samples.

Why did the addition of probiotics (*L. paracasei*) promote the development of aroma? Leroy and Cocolin et al. highlighted the importance of microbial diversity in the development of fermented sausage flavor (Franciosa et al., 2018; Franciosa et al., 2021; Ravyts et al., 2010). However, inoculated starter cultures exhibit a loss of the peculiar organoleptic characteristics in traditional fermented sausages (Franciosa et al., 2018; Yang et al., 2022), because it might reduce the complexity of microbial systems. The results of our correlation analysis implied the existence of a strong interaction between the bacteria such as *Latilactobacillus* and Staphylococcus, which played an important role in sausage flavor formation (Fig. 5 and Fig. 6b). When glucose is used up, other compounds, such as lactate, gluconate, glucose-6-phosphate, pyruvate, acetate, amino acids, fatty acids, nucleotides and urea, are metabolized by almost all bacteria in the microbial community of fermented sausages (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). Therefore, the rapidly metabolize glucose ability of *Latilactobacillus* might improve the community diversity in sample Y may be explained by the fact that different genotypes or species are more likely to use different substrates (Giri et al., 2020). In brief, except *Latilactobacillus*, the RA of all other genera was low in sample Y (Fig. 4h); such a microbial structure might be able to fully utilize various substrates in fermented sausages, resulting in a good flavor profile similar to that of sausages produced using starter cultures, such as SBM-52,

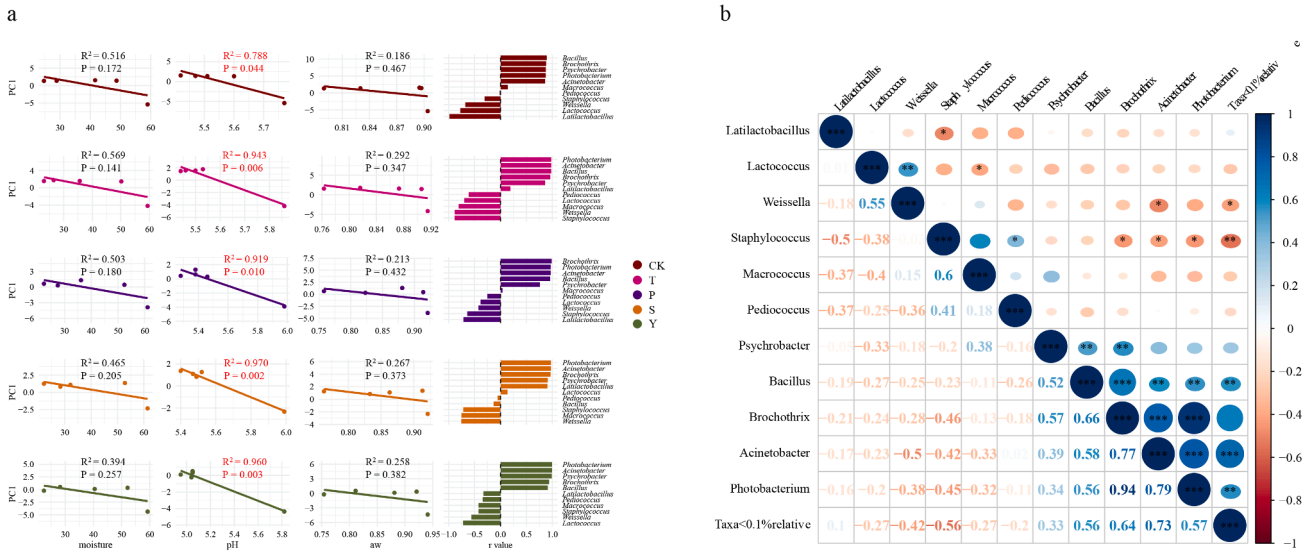

Fig. 6. Abiotic and biotic drivers of microbiota composition. (a) Plots of PC1 versus three environmental variables. (b) Pearson correlations between bacterial genus. The bar plot in (a) shows the correlations between pH and bacterial genus in each sample. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

because the previous study reported that indigenous bacteria could catabolize various substrates into ketones, aldehydes, alcohols and esters, yielding cheesy, fresh, fruity, sweet and floral notes at low levels (Casaburi et al., 2015). Moreover, *L. paracasei* provided an acidic environment in fermented sausages (Fig. 1c), reduction in pH could inhibit the hydrolysis activity of the enzymes, which are involved in the reaction of an acid and alcohol compounds and released by staphylococci, and thus reduced ester hydrolysis (Casaburi, Villani, Toldrá, & Sanz, 2006; Toldrá et al., 2015), these might explain the high ester content detected in sample Y, despite the low percentage of *Staphylococcus*.

4. Conclusion

During fermentation, pH (32.4%) and bacterial genus (67.6%) are the key abiotic factor and biological factors that affect the flavor profile. About the correlation between microbial community succession pattern and VOCs, in samples S and T, *Staphylococcus* was the overwhelmingly dominant genus in the microbial communities, and the stable and continuous growth of *Pediococcus* and *Staphylococcus* in sample S was more favorable to aroma development, in particular the ethyl esters, such as ethyl 2-methylbutyrate and ethyl 3-methylbutyrate; In sample P, the inoculation of a starter culture consisting of *Latilactobacillus* and *Staphylococcus* maintained the microbial diversity, and the moderately acidic environment (pH $>$ 5.4) resulted in a stable succession pattern of the microbial community which was not conducive to the accumulation of aldehydes, alcohols and esters; In sample Y, the inoculation of *L. paracasei* maintained the microbial diversity and the higher acid production efficiency of *L. paracasei* made the microbial community showed a fluctuation assembly process pattern. Moreover, the high RA of *Latilactobacillus* (*L. paracasei*) and the diversity of microbial communities (especially some low abundance species), which was also beneficial to the formation of esters such as ethyl 2-methylbutyrate, ethyl 3-methylbutyrate, ethyl butyrate and ethyl hexanoate.

CRediT authorship contribution statement

Di Zhang: Conceptualization, Software, Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Peng Yang:** Conceptualization, Data curation, Validation, Formal analysis, Methodology, Writing – original draft. **Kaihao Liu:** Formal analysis, Methodology, Writing – original draft. **Liu Wu:** Formal analysis, Methodology, Writing – original draft. **Guoliang Li:** Funding acquisition, Supervision, Writing – review & editing. **Huan Zhang:** Funding acquisition, Supervision, Writing – review & editing. **Xiaozhong Ma:** Supervision, Writing – review & editing. **Liangyan Rong:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Ruren Li:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100838.

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