FEEDS

Profiling of the viable bacterial and fungal microbiota in fermented feeds using single-molecule real-time sequencing

Jie Yu,^{†,‡} Qiangchuan Hou,[‡] Weicheng Li,[‡] Weiqiang Huang,[‡] Lanxin Mo,[‡] Caiqing Yao,[‡] Xiaona An,[‡] Zhihong Sun,[‡] and Hong Wei^{†,1}

[†]Department of Laboratory Animal Science, College of Basic Medical Sciences, Third Military Medical University, Chongqing, China, [‡]Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Key Laboratory of Dairy Products Processing, Ministry of Agriculture, Inner Mongolia Agricultural University, Huhhot 010018, China

¹Corresponding author: weihong63528@163.com

Abstract

Fermented concentrated feed has been widely recognized as an ideal feed in the animal industry. In this study, we used a powerful method, coupling propidium monoazide (PMA) pretreatment with single-molecule real-time (SMRT) sequencing technology to compare the bacterial and fungal composition of feeds before and after fermentation with four added lactic acid bacteria (LAB) inoculants (one *Lactobacillus casei* strain and three *L. plantarum* strains). Five feed samples consisting of corn, soybean meal, and wheat bran were fermented with LAB additives for 3 d. Following anaerobic fermentation, the pH rapidly decreased, and the mean numbers of LAB increased from 10^6 to 10^9 colony-forming units (cfu)/g fresh matter. SMRT sequencing results showed that the abundance and diversity of bacteria and fungi in the feed were significantly higher before fermentation than after fermentation. Fifteen bacterial species and eight fungal genera were significantly altered following fermentation, and *L. plantarum* was the dominant species (relative abundance 88.94%) in the post-fermentation group. PMA treatment revealed that the bacteria *Bacillus cereus*, *B. circulans*, *Alkaliphilus oremlandii*, *Cronobacter sakazakii*, *Paenibacillus barcinonensis*, and *P. amylolyticus* (relative abundance >1%) were viable in the raw feed. After fermentation, their relative abundances decreased sharply to <0.2%; however, viable *L. plantarum* was still the dominant species post fermentation. We inferred that our LAB additives grew rapidly and inhibited harmful microorganisms and further improved feed quality. In addition, coupling PMA treatment with the Pacific Biosciences SMRT sequencing technology was a powerful tool for providing accurate live microbiota profiling data in this study.

Key words: bacterial and fungal community, fermented feeds, lactic acid bacteria, propidium monoazide treatment, single-molecule real-time sequencing

Introduction

Given the rapid increase in the intensification of livestock and poultry farming in China, livestock and poultry farming methods have changed from traditional free-range to farm feedlot. Therefore, standard feeds did not meet the nutritional

needs of the animals. Silage, concentrated feed, and total mixed ration have been used as primary feeds in the modern breeding industry. However, the use of plant-derived materials such as legume seeds, leaf meals, leaf protein concentrates, and root tuber meals as feed ingredients is limited by the presence

Abbreviations

CP	crude protein
DM	dry matter
ESI	electrospray ionization
FM	fresh matter
LAB	lactic acid bacteria
LDA	linear discriminant analysis
LEfSe	linear discriminant analysis effect size
MS	mass spectrometry
OTU	operational taxonomic unit
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PMA	propidium monoazide
QTOF	quadrupole time-of-flight
SMRT	single-molecule real-time
UPLC	ultra performance liquid chromatography

of a wide variety of harmful substances (Francis et al., 2001). Important among these are glucosinolates, sinapic acid, and gossypol, which seriously harm both animal and human health (Liener et al., 1990). In addition, some raw materials contain pathogenic microorganisms (Duniere et al., 2017) and emit an odor that reduces feed palatability. Therefore, the research and development of rich nutrition, good flavor, and excellent quality feed products are of great significance for animal husbandry.

Fermented concentrated feed is prepared by spontaneous fermentation or by the use of lactic acid bacteria (LAB) culture as microbial inoculum under anaerobic conditions. LAB can convert water-soluble carbohydrates into organic acids, consisting primarily of lactic acid. As a result of this acidification, the composition of the microbial community in the feed is altered, with a decrease in pathogenic microorganisms (Amado et al., 2012), and biodegradation of toxins in the raw materials (Ahlberg et al., 2015). In addition, some LAB inoculants may also serve as probiotics, as they improve animal performance by increasing milk yield and by enhancing immunity and feed efficiency (Dhama et al., 2011; Vibhute et al., 2011). Therefore, feed-associated microorganisms could significantly impact the quality of fermented feed and animal health. Feed and silage contain a large number of microorganisms including LAB, yeast, mold, clostridia, and acetic acid bacteria (Muck, 2010). Although the effects of LAB additives are well known, it is unknown how the microbial composition changes during fermentation. Therefore, a better understanding of the live microbial communities involved in fermentation with LAB inoculants could provide additional insight into approaches to improve feed quality and animal health.

A number of studies have investigated the live microbial communities in fermented feed and silage and primarily used culture-dependent methods (Mceniry et al., 2010; Yang et al., 2010). However, live microbial communities described solely on the basis of culturing are often incomplete, as many species are unculturable or poorly represented by the culturing process (Duniere et al., 2017). Metagenomic sequencing of microbial DNA is a culture-independent method that could provide a comprehensive microbiota profile independent of phenotypic traits and cultivability of individual microbes. In particular, an advanced high-throughput sequencing tool, the third generation Pacific Biosciences (PacBio, Menlo Park, CA, USA) single-molecule real-time (SMRT) sequencing technology, generates long reads and allows high taxonomic resolution at the species level when coupled with full length 16S ribosomal RNA (rRNA)

gene sequencing (Mosher et al., 2014). Moreover, the PacBio SMRT technology can largely shorten the time required for detecting and analyzing the overall microbial profile of a sample to within 24 h. It has been successfully used in many studies to describe microbial communities in complex environments, including silage (Bao et al., 2016), fermented dairy products (Li et al., 2017; Yao et al., 2017), and infant formula (Zheng et al., 2016). High-throughput sequencing technology is based on polymerase chain reaction (PCR) amplification of environmental DNA and DNA-derived signals, which originate from both living and dead cells. This method is unable to differentiate between viable and nonviable cells, leading to interpretations that overestimate the viable microbial population present (Vaishampayan et al., 2013). Thus, it is necessary to develop an accurate and comprehensive approach to detect viable microbial communities in the fermented feed that reflect the history of dead microorganisms and the changes in live microorganisms during the fermentation process.

Studies have suggested the treatment of samples with propidium monoazide (PMA) before DNA extraction as a method that allows selective detection of only live cells (Nocker et al., 2006). The principle is based on the integrity of cell membranes, as PMA can only penetrate cells with damaged membranes and intercalate with their DNA (Nocker et al., 2007). Thus, PMA strongly inhibits the PCR amplification of dead cells, and molecular methods can be used to detect the live cells. Recently, PMA treatment has been used in combination with real-time or quantitative PCR, denaturing gradient gel electrophoresis, microarray technology, and next-generation sequencing to detect *Bifidobacterium breve* strain Yakult in human feces (Fujimoto et al., 2011); analyze the bacterial communities of water samples (Nocker et al., 2010); and quantify viable microbial cells during Gouda cheese ripening (Erkus et al., 2016). These studies have demonstrated the applicability of PMA treatment in detecting live microorganisms under a wide variety of conditions. However, the microbial profiles described by previous studies are restricted to the genus level of precision due to the relatively low taxonomical resolution of traditional DNA sequencing, which can only determine a partial sequence of the 16S rRNA gene.

Therefore, PMA pretreatment combined with SMRT sequencing technology was used to assess the viable bacterial and fungal populations present in fermented feed samples produced by the addition of four different LAB additives. We focused on comparing the viable bacterial and fungal composition of feed before and after fermentation. In addition, we analyzed the quality of the fermented feed using conventional indicators such as pH, chemical composition, microbial counts, and mycotoxin formation.

Materials and Methods

Fermented concentrated feed preparation

Three ingredients were used to prepare the mixture: corn (dry matter [DM], 86%), soybean meal (DM, 89%), and wheat bran (DM, 87%). The materials were smashed by a feed hammer mill (2,800 g, 2.0 screens) and mixed 1:1:1 based on fresh weight. The compound LAB inoculant consisted of four strains, namely *Lactobacillus casei* F-08, *L. plantarum* P-8, *L. plantarum* KT-PLA1, and *L. plantarum* Ps-9, which were obtained from the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. LAB inoculant containing 4×10^{10} colony-forming units (cfu)/mL was suspended in 10-fold the weight of sterile water and evenly

mixed with the feed at the final concentration of 10^5 cfu/g, after which sterile water was added to the mixed feed until the water content reached 35%. For small-scale feed preparation, 30 kg of mixed feed (total 150 kg) was placed in a plastic bucket specialized for fermentation and stored at 30 °C for 72 h. Five samples were collected in sterilized containers before and after fermentation (FSA1–5 and FSB1–5, respectively). Samples were kept in iceboxes during transportation. Three replicates were collected to analyze fermentation quality and chemical composition. For sequencing analysis, DNA/RNA Shield (Zymo Research) as protectant was added to the samples to avoid DNA degradation.

Fermentation quality

Measurement of pH and titratable acidity

Sterile water (225 g) was added to 25 g feed, followed by mixing at 150 rpm for 30 min in a homogenizer. A pH meter was used to measure the pH. Total titratable acidity was measured using an automatic titrator (809 Titrator; Metrohm, Herisau, Switzerland).

Microbiological analysis

The number of microorganisms was measured using the plate count method and reported as cfu/g fresh matter (FM). Feed samples (25 g) were shaken well with 225 g distilled water and 10-fold dilutions were prepared in 0.85% sodium chloride solution. For each dilution, 1 mL of suspension was spread on agar plates of selective media. LAB was enumerated on de Man, Rogosa, and Sharpe agar (Oxoid, Nepean, ON, Canada) following anaerobic incubation at 37 °C for 48 h in an anaerobic workstation (DG250; Don Whitley Scientific, West Yorkshire, UK). Coliform bacteria were counted on blue light broth agar (Nissui-Seiyaku, Tokyo, Japan) after aerobic incubation at 30 °C for 48 h. Molds were counted on potato dextrose agar (CM0139; Oxoid) that had been acidified to pH 3.5 with sterilized lactic acid following aerobic incubation at 30 °C for 48 h.

Chemical mycotoxin analysis

A number of the fermented feed samples were dried in a forced-air oven at 60 °C. After 48 h, they were removed and weighed to calculate the moisture content of the fermented feed. Crude protein (CP) contents were analyzed with the Horwitz and Latimer method. Mycotoxins, including aflatoxin B1, vomitoxin, and zearalenone, were detected using ultra performance liquid chromatography–electrospray ionization–quadrupole time-of-flight mass spectrometry (UPLC–ESI–QTOF MS) (Waters, Milford, MA, USA). Sample preparation, UPLC, and QTOF-MS conditions were previously described (Bao et al., 2016). The chemical composition of each sample was tested three times, and the results were expressed as the mean $\pm$ standard deviation.

Composition of the total microbial and live bacterial communities of fermented feeds

PMA treatment for detecting live bacterial communities

All of the feed samples were treated with PMA using a described method (Erkus et al., 2016). Briefly, PMA was dissolved in 20% dimethyl sulfoxide to generate a 20 mM PMA stock solution and stored at –20 °C in the dark. An adequate amount of PMA stock solution was added to 1 mL feed suspensions diluted 10-fold for a final concentration of 30 μ M. Subsequently, the tubes were incubated at room temperature for 5 min in the dark with occasional mixing, and triplicate samples were light-exposed to a light-emitting diode photosensitive reactor for 10 min to activate and photolyze the PMA. Following photo-induced cross-linking of PMA, cells were pelleted at $10,000 \times g$ for 10 min prior to DNA extraction.

DNA extraction and PCR amplification

A total of 20 samples were collected and divided into four groups: before fermentation and no PMA treatment (CA), before fermentation and PMA treatment (PA), after fermentation and no PMA treatment (CB), and after fermentation and PMA treatment (PB). DNA was extracted using the Omega DNA isolation kit (Omega Bio-Tek, D5625-01, Norcross, GA, USA) according to the manufacturer's instructions. DNA purity and concentration were evaluated using 0.8% agarose gel electrophoresis and optical density using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The full-length 16S and 28S rRNA genes were amplified by PCR for barcoded SMRT sequencing. The 16S rRNA gene of bacteria was amplified using the universal forward 27F (5'-GAGAGTTTGATCCTGG CTCAG-3') and reverse 1541R (5'-AAGGAGGTGATCCAGCC GCA-3') primers. The 28S rRNA gene of fungi targeted at the domain fungi was amplified with the forward primer KITS28SF (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer KITS28S-R (5'-TCCTGAGGGAACTTCG-3'). These primers contained a set of 16-nucleotide barcodes. The PCR conditions were as follows: 95 °C for 4 min and 28 cycles of 95 °C for 1 min, 60 °C for 45 s, and 72 °C for 1 min, followed by a final cycle at 72 °C for 7 min (7220 Thermal Cycler; Applied Biosystems, Foster City, CA, USA) (Hu et al., 2015).

SMRT sequencing and data analysis

A PacBio RS II instrument (Pacific Biosciences, Menlo Park, CA, USA) was used to sequence the bacterial and fungal amplicons. The PCR amplification and sequence preprocessing controls were performed as described previously (Mosher et al., 2013; Zheng et al., 2016). The protocol RS_ReadsOfInsert.1 was applied to process the raw data, which are available in the SMRT Portal version 2.7. The filtering parameter was 5, minimum predicted accuracy was 90, and the minimum and maximum read lengths of the inserts were 1,400 and 1,800, respectively. The high-quality sequences were extracted using the Quantitative Insights into Microbial Ecology package (version 1.7), and the sequences were aligned under 100% clustering of sequence identity to obtain representative sequences using PyNAST and UCLUST software (Caporaso et al., 2009; Edgar, 2010). Using UCLUST, the unique sequence set was classified into operational taxonomic units (OTUs) under the threshold of 97% identity (Lozupone et al., 2006). The Ribosomal Database Project II databases were applied to determine the taxonomy of the OTUs at a minimum bootstrap threshold of 80% (Cole et al., 2007). Chao1, rarefaction estimators, Shannon–Wiener, and Simpson's diversity were computed to evaluate alpha diversity. A de novo taxonomic tree was constructed employing a representative OTU set in FastTree for downstream analysis, including the beta diversity calculation (Price et al., 2009). Both weighted and unweighted calculations were performed for principal coordinate analysis (PCoA). Graphs were generated with the R package (version 3.1.2) and Origin software (version 8.5). To assess the microbiota structure of different samples, both weighted and unweighted calculations were performed for PCoA based on the UniFrac distances derived from the phylogenetic tree (Lozupone et al., 2005). The linear discriminant analysis effect size (LEfSe) method based on a normalized relative abundance matrix was employed to identify significant differences between feed samples (Segata et al., 2011).

Statistical analysis

Statistical analyses were performed with SAS software (SAS version 9.0; SAS Institute Inc., Cary, NC, USA), and statistical significance was tested with the analysis of variance test.

Differences in the relative abundances of taxonomic groups at the phylum, genus, and species levels between samples were evaluated with the Mann–Whitney test with adjustment for multiple testing by the Benjamini–Hochberg method. Adjusted *P*-values of <0.05 were considered statistically significant.

Nucleotide sequence accession numbers

The sequence data reported in this study have been deposited in the Metagenomic Rapid Annotations using Subsystems Technology database (accession no. mgp83637).

Results

Fermented feed characteristics

The fermented silage was opened after 3 d, the texture was uniform, the color and luster were maize-yellow, and the flavor was fragrant. The pH, titratable acidity, chemical composition, and microbial population of non-fermented and fermented silage are shown in Table 1. Silage pH decreased to below 4.3 in all of the samples, and titratable acidity ranged from 28 ± 1.06 °T to 362 ± 3.55 °T. During the course of fermentation, the moisture content slightly decreased, while the CP content slightly increased. The population sizes of LAB, mold, and coliform based on FM were approximately 10^6 , 10^5 , and 10^3 cfu/g, respectively. After 3 d of anaerobic fermentation, the mean LAB value increased significantly to 10^9 cfu/g FM, whereas the mean amounts of mold and coliform were significantly reduced to levels below the detection limit.

Sequencing coverage and estimation of bacterial and fungal diversity

Sequence abundance and diversity

In total, 92,275 and 82,897 reads were obtained for the bacterial and fungal microbiomes of feed samples, respectively. The Shannon diversity curves but not rarefaction curves of bacteria and fungi for all of the samples reached the saturation phase, which indicates that the sequence depth obtained was adequate for all samples (Supplementary Figure S1). The Shannon index, Simpson diversity index, Chao1, and observed species of each sample were used to evaluate the species richness and diversity (Figure 1 and Supplementary Table S1). The Mann–Whitney test

was applied to evaluate significant differences in the bacterial and fungal richness and diversity between the before and after fermentation and PMA treatment samples. After fermentation and PMA treatment, the bacterial indicators were significantly lower than before fermentation and PMA treatment ($P < 0.001$), whereas the mean values of the fungal indicators in the CB group before fermentation were significantly higher than in the CA groups ($P < 0.001$). These results suggest that the abundance and diversity of bacteria and fungi in the feed before fermentation were significantly higher than those after fermentation. This is mainly because anaerobic fermentation and low pH inhibit many microorganisms.

Changes in bacterial composition following silage fermentation and PMA treatment

From the five pre-fermentation samples, 13 phyla, 143 genera, and 272 species of bacteria were identified. Major phyla with a relative abundance >0.5% included Firmicutes (73.70%), Proteobacteria (20.86%), Cyanobacteria (2.57%), Actinobacteria (1.51%), and Bacteroidetes (0.57%). However, from the five post-fermentation samples, 2 phyla, 21 genera, and 36 species of bacteria were identified, including only 2 phyla, Firmicutes (99.13%) and Proteobacteria (0.86%), which exhibited significant differences in the CA and CB groups. At the genus level, nine major genera (average relative abundance of >1%) were detected in the CA group, namely *Bacillus* (36.86%), *Paenibacillus* (17.39%), *Lactococcus* (7.63%), *Pantoea* (5.81%), *Pseudomonas* (4.47%), *Cronobacter* (3.63%), *Alkaliphilus* (3.62%), *Enterococcus* (2.31%), and *Psychrobacter* (1.08%) (Supplementary Figure S2). Only three bacterial genera with relative abundances >1.0% were detected in the CB group, namely *Lactobacillus* (91.06%), *Staphylococcus* (3.85%), and *Bacillus* (3.69%). With the exception of *Bacillus*, the remaining genera in the CA group were almost undetectable in the CB group, which were inhibited by LAB inoculants. A total of 5.13% and 0.50% of the bacterial sequences could not be identified at the genus level in the CA and CB groups, respectively. Thirteen species had a relative abundance >1% in the CA group, namely *Bacillus cereus* (22.68%), *Lactococcus piscium* (5.94%), *B. circulans* (4.07%), *B. flexus* (3.68%), *Alkaliphilus oremlandii* (3.56%), *Cronobacter sakazakii* (3.24%), *B. altitudinis* (3.17%), *Pantoea agglomerans* (2.66%), *Pseudomonas fragi* (2.47%), *Paenibacillus barcinonensis* (1.81%), *P. amylolyticus* (1.53%), *Lac. lactis* (1.07%), and *Psychrobacter cibarius* (1.01%). Except for *Psychrobacter cibarius*, the relative abundances of the other species decreased significantly in the CB group (Figure 2, and Supplementary Figure S2A). *Lactobacillus plantarum* (88.94%), *Staphylococcus gallinarum* (1.48%), and *B. amyloliquefaciens* (1.21%) were the dominant species in the CB group, and their relative abundances were significantly higher than those of the CA group (Figure 2).

From the five pre-fermentation samples with PMA treatment (PA group), 12 phyla, 96 genera, and 159 species of bacteria were identified. Firmicutes (87.77%) and Proteobacteria (11.55%) were still the major phyla, but their relative abundances were significantly higher compared with the samples before PMA treatment (CA group). Compared to the CA group, 3 phyla, 78 genera, and 191 species of bacteria were not detected in the PA group, and 2 phyla, 31 genera, and 68 species were only detected in the PA group. The relative abundances of some species exhibiting significant changes following PMA treatment are shown in Figure 2. The relative abundances of *Lactococcus piscium*, *Lac. lactis*, *B. flexus*, *P. agglomerans*, and *P. fragi* were significantly decreased following PMA treatment. We deduced that these species existed in the raw feed material, and died during the course of processing, storage, and transport. Moreover, the relative

Table 1. Fermentation characteristics and chemical composition of pre-fermented and post-fermented feeds

Item	Pre-fermented feeds	Post-fermented feeds
Fermentation quality		
pH value	6.4 ± 0.07	4.3 ± 0.12
Titratable acidity, T	28 ± 1.06	362 ± 3.55
Chemical composition		
Moisture content, %	36 ± 0.89	35 ± 1.15
CP, g/100g	24.11 ± 0.76	26.18 ± 0.92
Mycotoxin composition		
Aflatoxin B1, ppb	3.26 ± 0.01	ND
Vomitoxin, ppm	3.74 ± 0.05	2.18 ± 0.08
Zearalonone, ppm	0.58 ± 0.08	ND
Microbial population		
(log ₁₀ cfu/g FM)		
LAB, CFU/g	$(4.08 \pm 0.17) \times 10^6$	$(2.20 \pm 0.08) \times 10^9$
Mold, CFU/g	$(1.95 \pm 0.09) \times 10^5$	<10
Coliform, MPN/g	≥1100	<3

Figure 1. Boxplots of the α -diversity indexes of bacteria (A) and fungi (B) in feed samples.

abundances of *B. cereus*, *B. circulans*, *A. oremlandii*, *C. sakazakii*, *P. barcinonensis*, and *P. amylolyticus* were significantly increased following the PMA treatment. From the five post-fermentation samples with PMA treatment (PB group), 2 phyla, 18 genera, and 34 species of bacteria were identified. Firmicutes (99.81%) and Proteobacteria (0.18%) were the major phyla, and their relative abundances were not significantly different between the CB and PB groups. Compared with the CB group, 9 genera and 14 species of bacteria were not detected in the PB group, and 6 genera and 12 species were only detected in the PB group. The relative abundance of *L. plantarum* was increased to 94.93% following PMA treatment, whereas the relative abundances of *B. flexus*, *B. altitudinis*, *P. agglomerans*, *P. calida*, and *Lac. lactis* were decreased to nearly undetectable levels in the PB group.

Changes in fungal composition following silage fermentation

In total, 4 phyla, 40 genera, and 56 species of fungi were identified in the five pre-fermentation samples of the CA group, and 4 phyla, 20 genera, and 25 species of fungi were identified in the CB group. The major fungal phyla were Ascomycota and Basidiomycota and their relative abundances were significantly different in the CA (64.80% and 19.42%, respectively) and CB (98.16% and 1.05%, respectively) groups. The major fungal genus with a relative abundance >1.0% was *Aspergillus* (55.30%) in the CA group, and 43.26% of the fungal sequences could not be identified at the genus level (Supplementary Figure S2). In the CB group, *Wickerhamomyces* (75.46%), *Candida* (4.84%), and *Aspergillus* (2.39%) were the dominant fungal genera. The relative abundance of *Wickerhamomyces* and *Candida* were increased

Figure 2. Heat map of the relative abundance of 15 bacteria species detected in feed samples with significant differences.

Table 2. The relative abundance of eight fungal genera detected in feed samples with significant differences

Fungal genus	CA	CB	P-value
<i>Aspergillus</i>	55.298 ± 5.21	2.393 ± 1.26	0.0079
<i>Alternaria</i>	0.132 ± 0.03	0.022 ± 0.02	0.0119
<i>Candida</i>	0.010 ± 0.01	4.842 ± 4.12	0.0119
<i>Chrysosporium</i>	0.062 ± 0.04	0.006 ± 0.01	0.0178
<i>Eurotium</i>	0.395 ± 0.18	0.052 ± 0.06	0.0079
<i>Emericella</i>	0.055 ± 0.02	0.000 ± 0.00	0.0075
<i>Wickerhamomyces</i>	0.049 ± 0.03	75.456 ± 5.31	0.0079
<i>Xeromyces</i>	0.448 ± 0.16	0.003 ± 0.01	0.0097

significantly following fermentation with LAB, but the relative abundance of *Aspergillus* decreased following fermentation (Table 2). Because most of the fungal sequences could not be identified at the species level, we did not analyze the relative abundance of fungal species in this study.

Comparison of the bacterial and fungal community structure following silage fermentation and PMA treatment

To compare the bacterial and fungal communities, we performed weighted and unweighted UniFrac PCoA analysis based on the OTU abundance table. As shown in Figure 3A and B, bacterial data points were largely separated in relation to feed fermentation and PMA treatment status by both unweighted (principal component PC1 and PC2 accounted for 28.89% and 9.05% of the total variance, respectively) and weighted (PC1 and PC2 accounted for 86.71% and 10.43% of the total variance, respectively) UniFrac analysis. Principal coordinates based on the unweighted (PC1 and PC2 accounted for 45.50% and 10.57% of the total variance, respectively) (Figure 3C) and weighted (PC1 and PC2 accounted for 97.36% and 1.54% of the total variance,

respectively) (Figure 3D). The UniFrac distances revealed fungal structural differences, as the symbols representing the two groups were separated. These results suggested that the bacterial and fungal community structures exhibited large differences after fermentation and PMA treatment.

The bacteria taxonomic groups that are differentially abundant among the four groups were further identified using LEfSe analysis with $\alpha = 0.05$, linear discriminant analysis (LDA) score of at least 4, and relative abundance greater than 0.1. A total of 41 significantly enriched taxa were identified across two phyla; 21, 10, 3, and 7 taxa were enriched in the CA, CB, PA, and PB groups, respectively (Figure 4A and B). At the genus level, microbiota of the CA group were differentially enriched with the genera *Bacillus*, *Paenibacillus*, *Cronobacter*, *Alkaliphilus*, *Pantoea*, and *Enterococcus*; the CB group was enriched with *Lactococcus* and *Pseudomonas*, whereas the PA and PB groups were enriched with *Staphylococcus* and *Lactobacillus*, respectively. At the species level, the CA group microbiome was characterized by an abundance of *B. cereus*, *B. circulans*, *C. sakazakii*, and *A. oremlandii*, the CB group microbiome was characterized by an abundance of *Lac. piscium*, *B. altitudinis*, *P. barcinonensis*, and *P. amylolyticus*, whereas *L. plantarum* and *L. casei* were enriched in the PB group.

Discussion

Fermented concentrated feed has been widely recognized as ideal feed in the animal industry, as it is nutritious, flavorful, palatable, and safe. In this study, in addition to analyzing the quality of fermented feed using conventional indicators such as pH, chemical analysis, and the formation of mycotoxins, we focused on elucidating and comparing the viable bacterial and fungal communities of fermented feed samples produced by the addition of LAB additives using SMRT sequencing technology combined with PMA pretreatment.

During the fermentation of concentrated feed, LAB can grow rapidly and produce organic acids, resulting in a drop in pH and

Figure 3. UniFrac principal coordinate analysis (PCoA) of the bacterial (A, B) and fungal (C, D) communities in feed samples. Each symbol represents the feed microbiota of one sample; sample group is represented by the respective color.

an increase in the titratable acidity of feed samples. A low pH is favorable, as more pathogenic microorganisms are inhibited and the feed is more stable. In particular, three mycotoxins decreased upon fermentation, and aflatoxin B1 and zearalenone were not detected in feed samples after fermentation (Table 1). Mycotoxins are toxic secondary metabolites produced by filamentous fungi (molds). Aflatoxin B1 is generally produced by strains of the genus *Aspergillus*, and T-2, zearalenone, and vomitoxin are associated with fungi that belong to the genus *Fusarium* (Pittet, 1998; Roigé et al., 2009). The relative abundance of the genus *Aspergillus* was $55.30 \pm 5.20\%$ in pre-fermentation feed samples, which dropped to $2.39 \pm 1.26\%$ in post-fermentation feed (Table 2). This suggests that *Aspergillus* strains are inhibited by the acidic and anaerobic environment produced by LAB additives. This could explain the decrease in mycotoxin concentration in the feed samples following fermentation. We did not detect the genus *Fusarium* in all of the samples by SMRT sequencing, which may also reflect low abundance. Moreover, these observations were in accordance with the observed microbial counts, as less molds and coliforms were detected after fermentation, and the number of LAB exceeded 2.20×10^9 cfu/g FM in the fermented feed samples (Table 1).

The microbial population of feeds may affect the quality and storage stability of feeds, as well as the health of animals. In our

study, bacterial and fungal microbiomes (272 bacterial and 56 fungal species) were detected in the raw materials. After 3 d of fermentation with LAB additives, the abundance and diversity of bacteria and fungi were significantly lower compared with the raw materials based on the Shannon index, Simpson diversity index, Chao1, and observed species of each sample (Figure 1 and Supplementary Table S1). Furthermore, the UniFrac PCoA analysis showed that the bacterial and fungal community of the post-fermentation group was clearly separated from those of the pre-fermentation group (Figure 3), which indicates significant changes in the bacterial community after fermentation. Through further analysis of different species, we found that the relative abundances of 12 species were significantly decreased and those of three species were significantly increased following fermentation (Figure 2). Among these, five species, *B. flexus*, *B. altitudinis*, *Pantoea agglomerans*, *Pseudomonas fragi*, and *Lac. lactis*, died because they were not detected in post-fermentation feeds with PMA treatment. Eight species (relative abundance < 0.2%) were still present as live bacteria: *B. cereus*, *Lac. piscium*, *B. circulans*, *A. oremlandii*, *C. sakazakii*, *P. barcinonensis*, *P. amylolyticus*, and *B. amyloliquefaciens*. Moreover, *L. plantarum* was the dominant species in the PB group, because it was the main strain in our LAB additives. *B. cereus* is a spore-forming foodborne pathogen, which is widespread in nature and frequently isolated from soil, dairy

Figure 4. Linear discriminant analysis effect size (LEfSe) and linear discriminant analysis (LDA) based on operational taxonomic units characterize the bacteria of the four groups. (A) Taxonomic cladogram produced from LEfSe analysis. Significant discriminant taxon nodes of the CA, CB, PA, and PB groups are represented by green, red, purple, and blue, respectively, while non-discriminant taxon nodes are represented by yellow. Branch areas are shaded according to the highest-ranked variety for that taxon. (B) Horizontal bar chart showing discriminant taxa. Significant discriminant taxa of the CA, CB, PA, and PB groups are represented by red, green, blue, and purple, respectively. The LDA score indicates the level of differentiation among the four groups of feed samples.

products, and growing plants (Stenfors Arnesen et al., 2008). It is not surprising that corn, soybean meal, and wheat bran have high relative abundances of this strain. However, when allowed access to mammalian tissues, *B. cereus* is an opportunistic pathogen that may cause severe local or systemic infections (Kotiranta et al., 2000). *B. circulans*, a psychrotrophic strain, is the main limitation and cause of failure to meet extended shelf-life (Carlin et al., 2000). Strain *B. circulans* PB7 was reported to be a probiotic supplement in feed for *Catla catla* fingerlings (Bandyopadhyay and Mohapatra, 2009). *C. sakazakii* is an opportunistic pathogen, which is associated with severe illness and high mortality in neonates and infants (Hunter and Bean, 2013). This suggests that the anaerobic conditions and antibacterial substances inhibited the growth of those pathogens, but were not able to kill them. One mechanism by which LAB additives shape the feed microbiota during fermentation is their ability to produce antibacterial substances (Hu et al., 2015). Hence, we inferred that LAB additives may play key roles in rapid fermentation and inhibiting pathogens. In addition, three species, *L. plantarum*, *B. amyloliquefaciens*, and *S. gallinarum*, were increased following fermentation. Clearly, the prevalent species in the fermented feed were highly dependent on the LAB additives. *B. amyloliquefaciens* nearly undetectable in the PB group, which indicated the strains of this species died after fermentation. The significantly lower abundance and diversity of bacteria and fungi may lead to the slight increase in the relative abundance of *B. amyloliquefaciens*. *S. gallinarum* has been isolated from chickens, the saliva of healthy human adults, and the blood of patients (Devriese et al., 1983; Yu et al., 2008). Although *S. gallinarum* is generally not pathogenic, it has been reported as an opportunistic pathogen for chickens and humans in some cases (Tibra et al., 2010). Therefore, this species should not be ignored. In conclusion, as additives, *L. plantarum* rapidly grew and became the

dominant bacteria. These results are in accordance with previous silage studies (Bao et al., 2016; Zheng et al., 2017).

Little information regarding fungal communities in fermented feeds is available, although the presence and growth of mycotoxigenic fungi have been extensively studied in silage (Streit et al., 2012). In our study, 40 and 20 genera of fungi were identified based on 28S rDNA sequencing in the CA and CB groups, respectively. The most notable decreases in relative abundance were observed in the genera *Aspergillus*, *Eurotium*, *Xeromyces*, and *Chrysosporium* (Figure 3, Supplementary Figure S2B). With the exception of the genus *Aspergillus*, the other genera were not detected after fermentation. However, strains of *Wickerhamomyces* and *Candida* were strongly increased and became the dominant fungal genera in the CB group. *Aspergillus* spp. was the most prevalent mold isolated from the fermented feed, which could produce aflatoxins and ochratoxin A and harm the health of the animals (Li and Yoshizawa, 2000; Roigé et al., 2009). A strong reduction in this genus was also noted during feed fermentation, which proved the antifungal efficacy of the LAB additives. *W. anomalus* is widespread in natural environments and frequently isolated from cereal-related sources (Daniel et al., 2011). *W. anomalus* has the ability to survive adverse conditions and to flourish in stressful environments such as low water activity, and osmotic and pH stresses, as well as in the presence of LAB (Fredlund et al., 2002; Vrancken et al., 2010). There are no data suggesting that *C. sojae*, a xylose-consuming yeast initially isolated from defatted soybean flakes (Nakase et al., 1994), can cause infections in humans and other animals. Thus, the most notable decreases in the relative abundance of these species have no effect on feed quality. It is worth mentioning that the percentage of sequences that could not be assigned to the genera and species level was high, particularly

in the CA group (43.26% and 93.17% of the fungal sequences, respectively), which suggests that a large number of new or poorly characterized fungi are present in the feed samples. Little information on the fungal communities in fermented feeds and the limitations of culture-based approaches may be the main reasons for the limited database resources of the 28S rRNA gene. Further work is required to identify and assign functions to these organisms during feed fermentation. Recent research has also demonstrated the presence of new fungal genera and species in home composts (Langarica-fuentes et al., 2015).

Currently, high-throughput sequencing technologies have shown tremendous promise in analyzing microbial community composition and diversity from foods, intestines, and other diverse environments. However, these approaches offer an opportunity to comprehensively detect not only uncultured and rare microbial communities but also dead microbial cells. Thus, these methods are unable to actually reflect the live microbial communities present during fermentation. For this reason, we combined PMA pretreatment with SMRT sequencing technology to assess the viable bacterial population present in fermented feed. Our data have shown that this is a viable method for assessing the quality of fermented feed.

This is the first report of the use of PacBio SMRT sequencing technology combined with PMA treatment to evaluate changes in the viable bacterial and fungal composition of fermented feeds with LAB additives. The four additive LAB inoculants had positive effects on the fermentation of concentrated feed. With the exception of reducing mycotoxin content, the LAB additives regulated and improved the bacterial and fungal compositions of fermented feed. Therefore, large-scale fermentation of feeds may be useful to promote growth and maintain animal health. In addition, coupling PMA treatment with PacBio SMRT sequencing technology in this study provided accurate live microbiota profiling data, which will allow for the detection of live microbial communities in diverse environments and samples in the future.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Figure S1. Rarefaction analysis (A, C) and Shannon diversity (B, D) estimates of the single-molecule real-time sequencing reads of bacteria (A, B) and fungi (C, D) in feed samples. Lines with colors represent samples from different groups.

Figure S2. Relative abundances and diversity of bacteria (A) and fungi (B) in the feed samples at the species level.

Table S1. Sample information, sequence abundance, and microbial diversity in feeds samples

Acknowledgment

This study was funded by the Natural Science Foundation of Inner Mongolia (Inner Mongolia Autonomous Region; grant no. 2018MS03041), Excellent Young Scientist Foundation of Inner Mongolia Agricultural University of China (Inner Mongolia agricultural university; grant no. 2017XYQ-4), and the University for Fostering Distinguished Young Scholars (Inner Mongolia agricultural university; grant no. 2017XJQ-2).

Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

- Ahlberg, S. H., V. Joutsjoki, and H. J. Korhonen. 2015. Potential of lactic acid bacteria in aflatoxin risk mitigation. *Int. J. Food Microbiol.* 207:87–102. doi:10.1016/j.ijfoodmicro.2015.04.042
- Amado, I. R., C. Fuciños, P. Fajardo, N. P. Guerra, and L. Pastrana. 2012. Evaluation of two bacteriocin-producing probiotic lactic acid bacteria as inoculants for controlling listeria monocytogenes in grass and maize silages. *Animal Feed Sci. Technol.* 175(3–4):137–149. doi:10.1016/j.anifeedsci.2012.05.006
- Bandyopadhyay, P. and P. K. D. Mohapatra. 2009. Effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets: on growth, nutritional quality and immunity of *Catla catla* (Ham.). *Fish Physiol. Biochem.* 35:467–478. doi:10.1007/s10695-008-9272-8
- Bao, W., Z. Mi, H. Xu, Y. Zheng, Y. K. Lai, H. Zhang, and W. Zhang. 2016. Assessing quality of *Medicago sativa* silage by monitoring bacterial composition with single molecule, real-time sequencing technology and various physiological parameters. *Sci. Reports* 6:28358. doi:10.1038/srep28358
- Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. Desantis, G. L. Andersen, and R. Knight. 2009. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26:266–267. doi:10.1093/bioinformatics/btp636
- Carlin, F., C. Choma, R. Pasqualini, A. Braconnier, and C. Nguyen-the. 2000. Sporeforming bacteria in commercial cooked, pasteurised and chilled vegetable purees. *Food Microbiol.* 17:153–165. doi:10.1006/fmic.1999.0299
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, A. S. Kulamsyedmohideen, D. M. Mcgarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje. 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res.* 35:169–72. doi:10.1093/nar/gkl889
- Daniel, H. M., M. C. Moons, S. Huret, G. Vrancken, and L. De Vuyst. 2011. *Wickerhamomyces anomalus* in the sourdough microbial ecosystem. *Antonie Van Leeuwenhoek* 99:63–73. doi:10.1007/s10482-010-9517-2
- Devriese, L. A., B. Poutrel, R. Kilpper-Balz, and K. H. Schleifer. 1983. *Staphylococcus gallinarum* and *Staphylococcus caprae*, two new species from animals. *Int. J. Syst. Bacteriol.* 33:480–486. doi:10.1099/00207713-33-3-480
- Dhama, K., V. Verma, P. M. Sawant, R. Tiwari, R. K. Vaid, and R. S. Chauhan. 2011. Applications of probiotics in poultry: enhancing immunity and beneficial effects on production performances and health – a review. *J. Immunol. Immunopath.* 13:1–19.
- Dunier, L., S. Xu, J. Long, C. Elekwachi, Y. Wang, K. Turkington, R. Forster, and T. A. Mcallister. 2017. Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. *BMC Microbiol.* 17:50. doi:10.1186/s12866-017-0947-0
- Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461. doi:10.1093/bioinformatics/btq461
- Erkus, O., V. C. de Jager, R. T. Geene, A. I. Van, L. Hazelwood, S. A. van Hijum, M. Kleerebezem, and E. J. Smid. 2016. Use of propidium monoazide for selective profiling of viable microbial cells during Gouda cheese ripening. *Int. J. Food Microbiol.* 228:1–9. doi:10.1016/j.ijfoodmicro.2016.03.027
- Francis, G., H. P. S. Makkar, and K. Becker. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199:197–227. doi:10.1016/S0044-8486(01)00526-9
- Fredlund, E., U. Druvefors, M. E. Boysen, K. J. Lingsten, and J. Schnürer. 2002. Physiological characteristics of the biocontrol yeast *Pichia anomala* J121. *FEMS Yeast Res.* 2:395–402. doi:10.1016/S1567-1356(02)00098-3
- Fujimoto, J., K. Tanigawa, Y. Kudo, H. Makino, and K. Watanabe. 2011. Identification and quantification of viable *Bifidobacterium breve* strain Yakult in human faeces by using strain-specific

- primers and propidium monoazide. *J. Appl. Microbiol.* 110:209–217. doi:[10.1111/j.1365-2672.2010.04873.x](https://doi.org/10.1111/j.1365-2672.2010.04873.x)
- Hu, X., W. Hao, H. Wang, T. Ning, M. Zheng, and C. Xu. 2015. Fermentation characteristics and lactic acid bacteria succession of total mixed ration silages formulated with peach pomace. *Asian-Australas. J. Anim. Sci.* 28:502–510. doi:[10.5713/ajas.14.0508](https://doi.org/10.5713/ajas.14.0508)
- Hunter, C. J., and J. F. Bean. 2013. Cronobacter: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. *J. Perinatol.* 33:581–585. doi:[10.1038/jp.2013.26](https://doi.org/10.1038/jp.2013.26)
- Kotiranta, A., K. Lounatmaa, and M. Haapasalo. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect.* 2:189–198. doi:[10.1016/s1286-4579\(00\)00269-0](https://doi.org/10.1016/s1286-4579(00)00269-0)
- Langarica-Fuentes, A., G. Fox, and G. D. Robson. 2015. Metabarcoding analysis of home composts reveals distinctive fungal communities with a high number of unassigned sequences. *Microbiology* 161:1921–1932. doi:[10.1099/mic.0.000153](https://doi.org/10.1099/mic.0.000153)
- Li, F. q., and T. Yoshizawa. 2000. Alternaria mycotoxins in weathered wheat from China. *J. Agric. Food Chem.* 48:2920–2924. doi:[10.1021/jf0000171](https://doi.org/10.1021/jf0000171)
- Li, J., Y. Zheng, H. Xu, X. Xi, Q. Hou, S. Feng, L. Wuri, Y. Bian, Z. Yu, L. Y. Kwok, et al. 2017. Bacterial microbiota of Kazakhstan cheese revealed by single molecule real time (SMRT) sequencing and its comparison with Belgian, Kalmykian and Italian artisanal cheeses. *BMC Microbiol.* 17:13. doi:[10.1186/s12866-016-0911-4](https://doi.org/10.1186/s12866-016-0911-4)
- Liener, I. E., J. Wiseman, and D. J. A. Cole. 1990. Naturally occurring toxic factors in animal feedstuffs. *Feedstuff Evaluation* 377–394. doi:[10.1016/B978-0-408-04971-9.50028-7](https://doi.org/10.1016/B978-0-408-04971-9.50028-7)
- Lozupone, C., M. Hamady, and R. Knight. 2005. UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* 241:184–186. doi:[10.1186/1471-2105-7-371](https://doi.org/10.1186/1471-2105-7-371)
- Lozupone, C., M. Hamady, and R. Knight. 2006. UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* 7:371. doi:[10.1186/1471-2105-7-371](https://doi.org/10.1186/1471-2105-7-371)
- McEniry, J., P. O’Kiely, N. J. Clipson, P. D. Forristal, and E. M. Doyle. 2010. Assessing the impact of various ensilage factors on the fermentation of grass silage using conventional culture and bacterial community analysis techniques. *J. Appl. Microbiol.* 108:1584–1593. doi:[10.1111/j.1365-2672.2009.04557.x](https://doi.org/10.1111/j.1365-2672.2009.04557.x)
- Mosher, J. J., E. L. Bernberg, O. Shevchenko, J. Kan, and L. A. Kaplan. 2013. Efficacy of a 3rd generation high-throughput sequencing platform for analyses of 16S rRNA genes from environmental samples. *J. Microbiol. Meth.* 95:175. doi:[10.1016/j.mimet.2013.08.009](https://doi.org/10.1016/j.mimet.2013.08.009)
- Mosher, J. J., B. Bowman, E. L. Bernberg, O. Shevchenko, J. Kan, J. Korlach, and L. A. Kaplan. 2014. Improved performance of the PacBio SMRT technology for 16S rDNA sequencing. *J. Microbiol. Meth.* 104:59. doi:[10.1016/j.mimet.2014.06.012](https://doi.org/10.1016/j.mimet.2014.06.012)
- Muck, R. E. 2010. Silage microbiology and its control through additives. *Rev. Bras. Zootecn.* 39:183–191. doi:[10.1590/S1516-35982010001300021](https://doi.org/10.1590/S1516-35982010001300021)
- Nakase, T., M. Suzuki, M. Takashima, Y. Miyakawa, K. Kagaya, Y. Fukazawa, and K. Komagata. 1994. *Candida sojae*, a new species of yeast isolated from an extraction process of water-soluble substances of defatted soybean flakes. *J. Gen. Appl. Microbiol.* 40:161–169. doi:[10.2323/jgam.40.161](https://doi.org/10.2323/jgam.40.161)
- Nocker, A., C. Y. Cheung and A. K. Camper. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Meth.* 67: 310–320. doi:[10.1016/j.mimet.2006.04.015](https://doi.org/10.1016/j.mimet.2006.04.015)
- Nocker, A., T. Richter-Heitmann, R. Montijn, F. Schuren, and R. Kort. 2010. Discrimination between live and dead cell-sin bacterial communities from environmental water samples analyzed by 454 pyrosequencing. *Int. Microbiol.* 13:59–65. doi:[10.2436/20.1501.01.111](https://doi.org/10.2436/20.1501.01.111)
- Nocker, A., K. E. Sossa, and A. K. Camper. 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *J. Microbiol. Methods* 70:252–260. doi:[10.1016/j.mimet.2007.04.014](https://doi.org/10.1016/j.mimet.2007.04.014)
- Pittet, A. C. 1998. Natural occurrence of mycotoxins in food and feeds – an updated review. *Revue Méd. Vét.* 49: 479–492. doi:[doi:10.1117/12.454434](https://doi.org/10.1117/12.454434)
- Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26:1641–1650. doi:[10.1093/molbev/msp077](https://doi.org/10.1093/molbev/msp077)
- Roigé, M. B., S. M. Aranguren, M. B. Riccio, S. Pereyra, A. L. Soraci, and M. O. Tapia. 2009. Mycobiota and mycotoxins in fermented feed, wheat grains and corn grains in Southeastern Buenos Aires Province, Argentina. *Rev. Iberoam. Micol.* 26:233–237. doi:[10.1016/j.riam.2009.03.003](https://doi.org/10.1016/j.riam.2009.03.003)
- Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi:[10.1186/gb-2011-12-6-r60](https://doi.org/10.1186/gb-2011-12-6-r60)
- Stenfors Arnesen, L. P., A. Fagerlund, and P. E. Granum. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* 32:579–606. doi:[10.1111/j.1574-6976.2008.00112.x](https://doi.org/10.1111/j.1574-6976.2008.00112.x)
- Streit, E., G. Schatzmayr, P. Tassis, E. Tzika, D. Marin, I. Taranu, C. Tabuc, A. Nicolau, I. Aprodu, O. Puel, et al. 2012. Current situation of mycotoxin contamination and co-occurrence in animal feed—focus on Europe. *Toxins (Basel)*. 4:788–809. doi:[10.3390/toxins4100788](https://doi.org/10.3390/toxins4100788)
- Tibra, N. K., S. Jalali, A. K. Reddy, R. Narayanan, and R. Agarwal. 2010. Traumatic endophthalmitis caused by *Staphylococcus gallinarum*. *J. Med. Microbiol.* 59(Pt 3):365–366. doi:[10.1099/jmm.0.011551-0](https://doi.org/10.1099/jmm.0.011551-0)
- Vaishampayan, P., A. J. Probst, M. T. La Duc, E. Bargoma, J. N. Benardini, G. L. Andersen, and K. Venkateswaran. 2013. New perspectives on viable microbial communities in low-biomass cleanroom environments. *ISME J.* 7:312–324. doi:[10.1038/ismej.2012.114](https://doi.org/10.1038/ismej.2012.114)
- Vibhute, V. M., R. R. Shelke, S. D. Chavan, and S. P. Nage. 2011. Effect of probiotics supplementation on the performance of lactating crossbred cows. *Vet. World* 4:557–561. doi:[10.5455/vetworld.2011.557-561](https://doi.org/10.5455/vetworld.2011.557-561)
- Vrancken, G., L. De Vuyst, R. Van der Meulen, G. Huys, P. Vandamme, and H. M. Daniel. 2010. Yeast species composition differs between artisan bakery and spontaneous laboratory sourdoughs. *FEMS Yeast Res.* 10:471–481. doi:[10.1111/j.1567-1364.2010.00621.x](https://doi.org/10.1111/j.1567-1364.2010.00621.x)
- Yang, J., Y. Cao, Y. Cai, and F. Terada. 2010. Natural populations of lactic acid bacteria isolated from vegetable residues and silage fermentation. *J. Dairy Sci.* 93:3136–3145. doi:[10.3168/jds.2009-2898](https://doi.org/10.3168/jds.2009-2898)
- Yao, G., J. Yu, Q. Hou, W. Hui, W. Liu, L. Y. Kwok, B. Menghe, T. Sun, H. Zhang, and W. Zhang. 2017. A perspective study of koumiss microbiome by metagenomics analysis based on single-cell amplification technique. *Front. Microbiol.* 8:165. doi:[10.3389/fmicb.2017.00165](https://doi.org/10.3389/fmicb.2017.00165)
- Yu, D., Y. Chen, Y. Pan, H. Li, M. A. McCormac, and Y. W. Tang. 2008. Case report: *Staphylococcus gallinarum* bacteremia in a patient with chronic hepatitis B virus infection. *Ann. Clin. Lab. Sci.* 38:401–404. doi:[10.1684/abc.2008.0270](https://doi.org/10.1684/abc.2008.0270)
- Zheng, M. L., D. Z. Niu, D. Jiang, S. S. Zuo, and C. C. Xu. 2017. Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. *J. Appl. Microbiol.* 122:1456–1470. doi:[10.1111/jam.13456](https://doi.org/10.1111/jam.13456)
- Zheng, Y., X. Xi, H. Xu, Q. Hou, Y. Bian, Z. Yu, L. Y. Kwok, W. Zhang, Z. Sun, and H. Zhang. 2016. Using PacBio long-read high-throughput microbial gene amplicon sequencing to evaluate infant formula safety. *J. Agric. Food Chem.* 64:6993–7001. doi:[10.1021/acs.jafc.6b01817](https://doi.org/10.1021/acs.jafc.6b01817)