The dynamics of the bacterial communities developed in maize silage

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Summary

Ensilage provides an effective means of conserving summer-grown green forage to supply as winter feed to ruminants. The fermentation process involved in the ensilage process relies on lactic acid bacteria (LAB). Here, 16S ribosomal DNA amplicon pyrosequencing was used to follow the dynamic behaviour of the LAB community during the ensilage of maize biomass, with a view to identify the key species involved in the process. The biomass used for ensilage was a single-cross maize hybrid, harvested at the milk-line stage. The crop was grown at three contrasting locations. Aspects of the physico-chemical composition of the material and the LAB species present were sampled at 0, 3, 6, 14, 21 and 32 days after ensilage was initiated. In all three cases, members of the Leuconostocaceae family dominated the epiphytic bacterial community, notably Leuconostoc and Weissella, but some variation was noted in the abundance of certain Leuconostocaceae and Lactobacillaceae species, as well as that of some Acetobacteraceae, Enterobacteriaceae and Moraxellaceae species. The constellation of the microbiome which developed during the ensilage process differed markedly from that of the epiphytic one, with Lactobacillaceae, particularly Lactobacillus and Pediococcus spp. dominating. The abundance of heterofermentative Leuconostocaceae spp. in the epiphytic community and the extent of the transition from hetero- to homo-fermentation during the initial ensilage period are important factors in determining silage quality.

Introduction

In most temperate regions, green fodder is seldom available throughout the year. The two commonest means of conserving summer-grown plant biomass to provide winter feed to ruminant animals during winter are drying to form hay and fermentation to form silage (Charmley, 2001). For the latter process, biomass is macerated at a crop growth stage chosen to maximize the dry matter (DM) content of the material (McDonald, 1991; Filya, 2004). The chopped plant materials are then compacted into large stacks or clamp silos using a roller or tractor, covered with a plastic sheet and carefully sealed to keep plant material away of atmospheric oxygen. Shortly after ensiling the residual oxygen present in the silo is consumed by plant cells and microbes associated with the ensiled material, and therefore, an anaerobic condition is quickly established. The material is then fermented anaerobically, largely through the activity of a range of lactic acid bacteria (LAB). The drop in pH of the silage which results from the production by LAB of lactic and acetic acid protects the silage from spoilage (McDonald, 1991; Weinberg and Muck, 1996).

Ensilage is a natural and spontaneous process that largely depends on anaerobic microbial fermentation. At the time of ensilage, the plant material harbours a range of microorganisms (the “epiphytic community”; Muck, 2013), but the fermentation process favours the multiplication of lactic acid bacteria (LAB; Ennahar et al., 2003; Yang et al., 2010; Eikmeyer et al., 2013; McGarvey et al., 2013) largely belonging to the genera Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Streptococcus and Leuconostoc (McDonald, 1991; McGarvey et al., 2013; Muck, 2013). In some cases, the
representation of LAB present does not effectively establish the required anaerobic and acidic conditions, leading to spoiling through the activity of other microorganisms (Woolford, 1990; McDonald, 1991; Muck, 2010). Attempts have been made to compensate for the low count of LAB in the epiphytic community and also to improve the fermentation efficiency, quality and aerobic stability of the silage by applying inoculant of laboratory-expanded LAB to the ensiled plants (McDonald, 1991; Filya, 2003; Holzer et al., 2003; Filya et al., 2006; Muck, 2010; Eikmeyer et al., 2013). Thus, among a number of other factors, the constitution of the epiphytic community is an important determinant of silage quality. A knowledge of which LAB species are key to the production of healthy silage would aid the formulation of silage inoculants designed to bolster the natural epiphytic community.

The composition of the silage microbiome has been explored using various microbiological and DNA-based techniques (Langston and Bouma, 1960; Ennahar et al., 2003; Parvin and Nishino, 2009; McEniry et al., 2010; Yang et al., 2010; McGarvey et al., 2013). In general, these approaches are not powerful enough to identify species present in low abundance, some of which may nevertheless be critical for optimal fermentation. However, the power of current DNA sequencing technologies now allows a much greater level of resolution than has been available in the past. For example, the microbial communities associated both with grass-based silage inoculated with Lactobacillus buchneri (Eikmeyer et al., 2013) and with manyflower silvergrass supplemented with microalgae (Li et al., 2015) have been identified via the deployment of current sequencing technologies. The objective of the present study was to characterize the microbiome associated with ensiled maize biomass both before and during the ensilage process.

Results

Physico-chemical analysis of the silage

At day 0 (prior to ensilage), the samples varied significantly ($P < 0.006$) with respect to DM: the biomass produced at the Isfahan site had the highest DM (26.8%) and that from the Qazvin site the lowest (23.7%; Fig. 1A). Over the initial ensiling period, the Isfahan samples retained their DM content more effectively than did those from either Qazvin or Gorgan; the latter had lost about 19% of their DM by day 32. Overall, silage samples collected from Gorgan displayed the same trend in DM reduction as that of Qazvin (Fig. 1A). The liquor pH of all the samples declined significantly ($P < 0.0001$), averaging 3.9 by day 3 and remaining relatively constant thereafter (with the exception of the Qazvin sample, which experienced a rise at day 32; Fig. 1B). With respect to NDF content, there was a significant difference between the Isfahan (56.3%) and Qazvin (60.1%) samples on day 0 (Fig. 1C). While the NDF content of the Qazvin samples remained relatively stable during the ensilage process (falling by just 4.8%), that of the Isfahan samples fell by 16.5% by day 32 (Fig. 1C). The ADF content also changed during the course of the ensilage process: the Gorgan samples were the least affected (−3.7%) and the Isfahan samples the most affected (−16.0%; Fig. 1D). The LA content of silages was sharply increased three days after the ensilage was initiated and continued to increase with storage time (Fig. 1E). The Isfahan samples showed the highest LA content compared to other two silage samples after 32 days of ensiling ($P < 0.002$). The ammonia content (averaging 1.1%) was comparable among the three sets of materials at day 0, and the effect of the ensilage process was to raise it significantly: for the Qazvin samples, the increase between days 0 and 32 was 4.6-fold, while the increase for the Gorgan samples was more modest (3.3-fold; Fig. 1F). The total OM content of silages samples was quite stable throughout the ensilage process ($P < 0.059$, Fig. S1). The overall conclusion was that among the three sites, the Isfahan-grown material produced the highest quality silage.

16S ribosomal DNA amplicon sequencing

The pyrosequencing yielded 143 405 raw reads of mean length 404 nt. Around 65% of the sequences (93 263) was retained after the filtration step: this set had a somewhat higher mean read length (472 nt). The clustering algorithm sorted the sequences into 6459 OTUs, of which 227 were identified by the ChymeraSlayer algorithm as likely chimeric and so were discarded. Once the low-abundance OTUs and those considered to reflect sequence artefacts had been removed from the analysis, 842 distinct OTUs (representing 78 756 sequences) remained for use in the subsequent diversity analysis. The details of number of reads generated per sample before and after quality filtering along with the number of OTUs detected in each sample before and after chimera detection and low abundant OTU filtering are presented in Table S1.

Diversity analysis of the silage microbiome

A rarefaction analysis (Fig. S2) suggested that the sequencing depth achieved was sufficient to have captured the majority of the microbial diversity present in the materials both before and after ensilage. An estimation of species richness based on the Chao1 diversity index (Table 1) confirmed this conclusion. At day 0, the Isfahan and Gorgan samples harboured the fewest microbial...
species (Table 1). Even so, the estimated species richness present in these two samples indicated that the observed phylotypes covered over 80% of the microbial diversity. The Qazvin samples exhibited the greatest species richness both before and after ensilage, but the observed phylotypes only covered 73–83% of the diversity. Species richness tended to rise during the early period of ensilage (days 3–21), but had tailed off by day 32. The Shannon diversity parameter behaved in a similar manner, increasing up to day 3 and remaining fairly constant thereafter. At day 0, the Isfahan and Gorgan samples displayed the lowest Simpson indices (0.72 and 0.85, respectively) indicating a lower community evenness (Table 1). In contrast, the goods coverage, which estimates the proportion of the bacterial OTUs represented in each given sample, ranged from 0.95 to 0.99 (Table 1). The Isfahan and Gorgan samples at day 0 had a coverage of, respectively, 0.99 and 0.98, suggesting that a near complete set of the high abundance species had been sampled.

For the purposes of a beta diversity analysis, a calculation of pair-wise similarities between the samples was made, using both weighted and unweighted Unifrac phylogenetic metrics; the results were visualized using PCoA plots (Fig. 2). The phylogenetic composition based on the unweighted Unifrac metric takes into account the presence and absence of OTUs: it was quite uniform across the three sampling locations at day 0 (Fig. 2A). The Isfahan origin ensiled biomass had a distinct microbial composition and appeared clustered in the PCoA plot. By day 14, the composition of the Gorgan and Qazvin samples became rather similar. Implementing the weighted Unifrac metrics, in contrast, showed that the composition of the ensiled material became uniform over time irrespective of the material’s geographical origin (Fig. 2B). Based on this analysis, the composition of the Qazvin sample on day 0 was clearly different from that of the other two sites.

**Taxonomic composition of the silage microbial community**

Of the 842 OTUs identified, 752 (89.3%, 74 114 reads) were associated with *Firmicutes* species 46 (5.4%, 3344 reads) with *Proteobacteria* species, with the other 44 (5.2%, 1298 reads) remaining unannotated. At the family level, sequences were allocated to the *Lactobacillaceae* (71.7%), *Leuconostocaceae* (22.1%), *Acetobacteraceae* (2.5%), *Enterobacteriaceae* (0.9%), *Moraxellaceae* (0.8%) and *Streptococcaceae* (0.1%). The distribution of families on day 0 varied from site to site (Fig. 3). *Leuconostocaceae* species predominated, especially the
genera *Leuconostoc* and *Weissella*. Site-to-site differences related for the most part to the relative abundance of *Lactobacillaceae*, *Acetobacteraceae*, *Enterobacteriaceae* and *Moraxellaceae*. The microbial community present at day 0 was heavily dominated by members of the *Leuconostocaceae* family, which accounted for an average across the samples of 78.8% of the species present. The Qazvin samples showed the greatest diversity at day 0, with *Leuconostocaceae* and *Lactobacillaceae* species accounting for, respectively, > 58% and > 12%, while both *Enterobacteriaceae* and *Moraxellaceae* species accounted for > 10%; *Acetobacteraceae* and *Streptococcaceae* species were also represented, albeit at a low frequency. The Gorgan sample was dominated by the *Leuconostocaceae* (80%) and *Acetobacteraceae* (15%) and lacked any representation from *Moraxellaceae*, while

| Sampling location | Sampling day | Observed species | Chao1   | Shannon | Simpson | Goods coverage |
|-------------------|-------------|-----------------|---------|---------|---------|----------------|
| Gorgan            | 0           | 114             | 137.0   | 4.05    | 0.85    | 0.98           |
|                   | 3           | 233             | 335.8   | 5.28    | 0.91    | 0.95           |
|                   | 6           | 261             | 340.1   | 5.74    | 0.94    | 0.95           |
|                   | 14          | 295             | 388.2   | 6.17    | 0.94    | 0.95           |
|                   | 21          | 285             | 374.7   | 6.15    | 0.95    | 0.95           |
|                   | 32          | 261             | 336.9   | 5.98    | 0.95    | 0.96           |
| Isfahan           | 0           | 77              | 95.3    | 3.07    | 0.72    | 0.99           |
|                   | 3           | 185             | 242.9   | 5.35    | 0.92    | 0.97           |
|                   | 6           | 182             | 228.9   | 5.41    | 0.93    | 0.98           |
|                   | 14          | 185             | 215.2   | 5.38    | 0.93    | 0.98           |
|                   | 21          | 188             | 210.2   | 5.30    | 0.91    | 0.97           |
|                   | 32          | 190             | 222.7   | 5.43    | 0.93    | 0.97           |
| Qazvin            | 0           | 228             | 311.2   | 5.89    | 0.95    | 0.96           |
|                   | 3           | 284             | 363.6   | 6.14    | 0.94    | 0.95           |
|                   | 6           | 276             | 339.4   | 6.14    | 0.94    | 0.96           |
|                   | 14          | 271             | 344.1   | 6.38    | 0.97    | 0.96           |
|                   | 21          | 280             | 340.6   | 6.40    | 0.97    | 0.96           |
|                   | 32          | 187             | 222.8   | 6.07    | 0.97    | 0.98           |

Fig. 2. Beta diversity analysis. The PCoA plots presented report the phylogenetic separation between the various silages, taking into account either OTU presence/absence ((A) unweighted Unifrac metric) or OTU relative abundance ((B) weighted Unifrac metric). Only the first two components which explained higher variations are shown. Blue triangles represent Isfahan silage samples, red squares represent Gorgan silages, and orange circles represent Qazvin silages.

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the Isfahan material was overwhelmingly associated with Leuconostocaceae species (98.8%), with neither Acetobacteraceae nor Moraxellaceae species present (Fig. 3); the abundance of the latter fell from 80.6% to 1.5% in the Gorgan silage, from 98.8% to 19.6% in the Isfahan silage and from 58.8% to 0.6% in the Qazvin silage. The abundance of both the Acetobacteraceae and Enterobacteriaceae was also diminished. At the genus level, most of the Lactobacillaceae representatives were assigned to Lactobacillus, with only a small representation of Pediococcus species (79 reads). In all samples, the abundance of Lactobacillus rose throughout the ensilage process. The commonest species present were L. brevis and L. paralimentarius, and only a minor number of OTUs were assigned to L. plantarum and L. vaginalis.

RT-PCR quantification of major LAB species contributing to the ensilage process
To further validate data obtained by amplicon pyrosequencing, we applied RT-PCR to monitor the dynamics of some LAB species that are known as critical components of silage microbial community. For this analysis, primers were selected to target intergenic spacer region of 16S-23S rRNA gene of L. reuteri and L. acidophilus and recA gene of L. brevis, L. buchneri, L. plantarum and P. pentosaceus. RT-PCR analysis showed that the count of LAB such as L. acidophilus, L. reuteri and L. buchneri was significantly lower in the Isfahan silage compared to that in the Gorgan and Qazvin silages and likely have minor contribution in silage quality (Fig. 4). The Qazvin biomass that produced the lowest quality silage was associated with higher representation of L. reuteri and L. buchneri and significantly lower representation of P. pentosaceus. Interestingly, P. pentosaceus was detected at comparable level in all biomasses before ensiling (day 0), while significantly increased in abundance in the Isfahan silage following the ensilage process and decreased steadily in that of the other two sites with storage time (Fig. 4). RT-PCR data also showed that L. brevis, L. plantarum and P. pentosaceus are among dominant LAB of maize epiphytic community.

Predicted metabolic potential of silage metagenome
PICRUSt enabled to identify major metabolic pathways associated with the ensilage process. The predicted metagenome of silage was annotated into 328 KO functional categories, in which many of them were not linked to the silage fermentation. The ensiling process was associated with depletion of pathways for nitrotoluene and styrene degradation, cell motility and secretion, and

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arginine and proline metabolism (Fig. 5). Importantly, ensiling was characterized by higher representation of pathways for ethylbenzene, naphthalene and xylene degradation as well as pathways for the biosynthesis of glycosphingolipid and bile acids. In addition, comparing metagenomes associated with maize biomasses obtained from different growing environments showed that the Isfahan silage had higher representation for propionate metabolism and lower representation for metabolism of xenobiotics, glycerophospholipid and cellular antigens (Fig. S4).

Discussion

Differences in growing environment at the three sites were reflected in the contrasting levels of DM measured in the samples prior to ensilage. The DM content of the biomass strongly influences the ensilage process, because the LABs responsible for fermentation require moisture for their growth and reproduction (McDonald, 1991; Hu et al., 2009). Differences were also observed in the rate of DM loss during the ensilage process: silages in which DM loss is limited tend to be those where lactic acid fermentation is most effective (McDonald et al., 1973; McDonald, 1991). Silages which are initiated from biomass with a high water content generally develop a lower pH because of the higher concentration of water soluble sugars and more extensive fermentation (McDonald, 1991). Here, differences in the DM content had little discernible effect on the rate of decline in the pH during the ensilage period, which implied that the differences in DM content were not great enough to affect the temporal behaviour of the pH. The fermentation response of NDF and ADF suggested that the ensilage process improved the nutritional quality of the Isfahan silage much more than the other two silages. The amount of ammonia generated also varied: high levels may reflect excessive protein breakdown or amino acid de-amination, and is usually associated with clostridial activity (McDonald, 1991; Driehuis and Oude Elferink, 1991; Hu et al., 2009).

Fig. 4. Real-time PCR quantification of LAB contributing in the maize silage fermentation. RT-PCR was run using species-specific primers targeting intergenic spacer region of 16S-23S rDNA and/or recA gene. For each species, a standard curve was constructed using 10-fold serial dilution of plasmid containing the target PCR amplicon. Data are presented on the basis of log2 absolute count of bacteria detected in 2 ng of metagenomic DNA.
Fig. 5. Functional prediction of maize silage metagenome using PICRUST (Langille et al., 2013). Box and whisker plots show the abundance of KEGG orthologs predicted to differ between the metagenome of maize biomass before (day 0) and 3, 6, 14, 21 and 32 days post-ensilage. The functional metagenomes were calculated from OTUs picked against the GREENGENES database (version 13.5) using PICRUST. The pathway abundance values were estimated based on proportion of sequences assigned to each pathway and normalized to the total number of genes present in a particular pathway for each sampling day. Only significant KEGG pathways which were identified by One-way ANOVA and Tukey’s post hoc test using the STAMP statistical package (Parks et al., 2014) are displayed. A P-value < 0.05 was considered statistically significant.
Overall, the indications were that in terms of silage quality (higher lactic acid concentration, lower pH and higher nutritional value), the biomass harvested at Isfahan was superior to the others.

With respect to the pre-existing microbial community prior to the initiation of the ensilage process, the Isfahan material appeared to be less diverse than the other two (Table 1). The composition of the epiphytic community, as demonstrated by 16S ribosomal DNA sequencing, emphasized the dominance of Leuconostocaceae species, particularly the genera Leuconostoc and Weissella. The extent of this dominance (98% in the Isfahan biomass, 80% in the Gorgan biomass and 58% in the Qazvin biomass) appeared to be correlated with the final silage quality. Leuconostoc and Weissella are obligatory heterofermentative LAB species associated with numerous forage crops and silages (Cai et al., 1998; Ennahar et al., 2003; Yang et al., 2010). It is well established that these microbial species are critical for the normal fermentation and aerobic stability of silage (McDonald, 1991). The abundance of epiphytic LAB determines the adequacy of fermentation, contributes in silage quality and determines whether to apply bacterial inoculation to silage (McDonald, 1991; Lin et al., 1992b; Yang et al., 2010). Pediococci, Leuconostoc, Streptococci and Enterococci are all responsible for the lactate fermentation necessary to create the anaerobic acidic environment required for the growth and multiplication of LAB (Cai et al., 1998).

The variously sourced biomass was associated with a differential abundance of members of Acetobacteraceae, Enterobacteriaceae, Lactobacillaceae and Moraxellaceae. Two of these families (Acetobacteraceae and Moraxellaceae) were not represented in the Isfahan biomass, which produced the highest quality silage, and only harboured a limited abundance of Lactobacillaceae (0.49%) and Enterobacteriaceae (0.24%). In contrast, the Qazvin material, which produced the poorest quality silage, housed an abundance of Erwinia spp. (Enterobacteriaceae), Lactobacillus and Pediococcus spp. (Lactobacillaceae) and Acinetobacter spp. (Moraxellaceae). The latter are non-LAB, aerobic, non-fermenting, gram-negative bacteria, sometimes associated not just with maize silage (Li and Nishino, 2011), but also form a component of the lettuce epiphytic community (Weiss et al., 2015). Erwinia spp., along with other gamma-proteobacteria, colonize alfalfa (McGarvey et al., 2013), lettuce (Weiss et al., 2015), citrus (Yang et al., 2001) biomass, but are not generally associated with the ensilage process. Here, the abundance of Enterobacteriaceae fell significantly during the early period of the ensilage, thereafter remaining low, an observation which was taken to indicate their lack of importance for silage fermentation. Acetobacteraceae spp. were present in the Gorgan biomass at an appreciable abundance (15%), but not in that from the other two sites. Members of this family including Gluconobacter oxydans have been exploited as an additive to maize silage, as they encourage the development of silages with a reduced lactic to acetic acid ratio and a low ammonia concentration (Queiroz et al., 2013). The representation in the population of Acetobacteraceae was strongly decreased over the first three days of ensilage, suggesting that these microbes play at best a minor role in maize silage formation.

The ensilage process shifted the composition of the bacterial community from the heterofermentative LAB Leuconostocaceae to species belonging to the Lactobacillaceae which contained both homo- and heterofermentative microorganisms. The majority of the latter were classified to the genus Lactobacillus, with a few (< 1%) assigned to the genus Pediococcus. The shift from Leuconostocaceae to Lactobacillaceae LAB was incomplete in the Isfahan silage at day 3: the abundance of Lactobacillaceae increased from 0.49% at day 0 to 79% at day 3. In contrast, in the Gorgan and Qazvin silages, their representation was close to 100% at this time. The Isfahan silage showed the highest relative abundance of Leuconostocaceae (19.6%, 8.4%, 0.9%, 3% and 2.9% at, respectively, days 3, 6, 14, 21 and 32), which were uncommon in the other two silages. According to Li et al. (2015), Lactobacillus spp. quickly come to dominate in manyflower silvergrass silage, while McGarvey et al. (2013) have also observed a similar shift from gamma-proteobacteria to homofermentative LAB in alfalfa silage. Similarly, the abundance of Lactobacillus spp. increases, while that of Lactococcus spp. decreases during grass ensilage (Eikmeyer et al., 2013). Obligate heterofermentative LAB are thought to be critical for biomass fermentation, especially in warmer climates (Muck, 2013); their role is to initiate fermentation by transforming soluble carbohydrates into lactic and acetic acid, thereby lowering the pH of the silage. As the pH falls, obligate heterofermentative LAB are disadvantaged, driving the shift towards homofermentative LAB (McDonald, 1991). The end-point quality and nutritional value of silage are strongly dependent on a rapid establishment of both a low pH and a sufficiently anaerobic environment (Weinberg and Muck, 1996), which is promoted by an abundant representation of LAB in the epiphytic community. Here, we found that the abundance of heterofermentative LAB belonging to the family Leuconostocaceae is significantly higher in epiphytic community associated with the Isfahan silage (98.8%) and notably lower in that of the Qazvin silage (58.8%). Thus, the higher quality of the Isfahan silage can be partly attributed to the favourable constellation of its epiphytic community.
Heterofermentative LAB convert one mole of glucose into one mole of lactic acid, one mole of CO₂ and a mole of either ethanol or acetic acid (Muck, 2010). Under anaerobic conditions, certain LAB have the ability to transform lactic acid to acetic acid (Elferink et al., 2001). The acetic acid produced by LAB is thought to inhibit the growth of yeasts, improving the silage’s stability during storage and feeding, and minimizing nutrient loss during its long-term storage (Moon, 1983; Hu et al., 2009; Li and Nishino, 2013). Most LAB used as inoculants for silage are homofermentative, producing two moles of lactic acid from one mole of glucose (Ennahar et al., 2003; Filya, 2003; Muck, 2010). At the species level, the LAB present in the various maize silages data indicate that L. brevis is one of the dominant LAB of maize epiphytic community, an observation that is in accordance with previous RT-PCR data (Stevenson et al., 2006).

Ensilage was associated with significantly higher prevalence of L. brevis during the initial days of ensiling (days 3 and 6) particularly in the Isfahan and Qazvin silages and remained at high level in the Isfahan silage and dropped to its initial level in the Qazvin silages thereafter (Fig. 4). These data suggest that the higher abundance of L. brevis is likely required for maintaining the quality of silages as the storage time of the ensiled material is extended. These results are somewhat in accord with previous data that indicate that L. brevis tends to populate later during the ensilage process (Lin et al., 1992a; Stevenson et al., 2006).

RT-PCR showed that the abundance of homofermentative L. plantarum was quite high in the epiphytic community of maize biomass irrespective of the material’s geographical origin and increased with the storage time particularly in the Isfahan silage. However, based on 16S rDNA sequencing data, OTUs affiliated to this species were present at a low abundance in the Isfahan and Gorgan silages, and this may indicate that the majority of OTUs belonging to this species skipped assignment at the species level. It has been reported that inoculating biomass with L. plantarum has a beneficial effect on silage quality (Cai et al., 1998; Hu et al., 2009). The presence of this species also appears to improve the aerobic stability of silage because it produces antifungal substances such as phenyl lactic and 4-hydroxy-phenyl lactic acids (Lavermicocca et al., 2000). The population of L. plantarum was positively correlated with the LA concentration \( r = 0.5, P = 0.03 \) and negatively with the pH \( r = -0.5, P = 0.02 \) and ADF content \( r = -0.73, P = 0.002 \), suggesting an important role for this bacterium in silage fermentation and fibre digestibility.

The prevalence of homofermentative P. pentosaceus in the epiphytic community of maize biomass obtained from different growing location was quite comparable. However, ensiling was associated with a dramatic rise in its abundance in the Isfahan silage (peaked at day 3) but not in the other two silages, suggesting a key role for this species in efficient fermentation of maize biomass. A strong positive correlation was observed between the DM content of the ensiled material and the population of P. pentosaceus \( r = 0.82, P = 5.6e-06 \), suggesting that the population of this species likely helped to maintain the DM content of the maize biomass during the ensilage process. P. pentosaceus is widely used in commercial inoculants which are applied to improve silage fermentation (McDonald, 1991). The results of RT-PCR indicate that the population of Lactobacilli such as L. reuteri, L. acidophilus and L. buchneri showed inverse correlation with the silage quality. Interestingly, L. reuteri showed a positive correlation with the NDF \( r = 0.63, P = 0.003 \) and the pH \( r = 0.54, P = 0.018 \) of the ensiled material, suggesting that this bacterium has a negative impact on maize silage fermentation. L. buchneri is a heterofermentative LAB that is frequently used as an inoculant for improving aerobic stability of silage (Holzer et al., 2003; Hu et al., 2009; Eikmeyer et al., 2013). Inoculation of grasses with L. buchneri is known to result in higher concentration of acetic acid, lower concentration of lactic acid, higher pH and higher concentration of alcohols such as propanediol and ethanol (Hu et al., 2009; Eikmeyer et al., 2013). Here we found that the population of L. buchneri has a strong negative correlation with the DM content of the ensiled material \( r = -0.85, P = 8.7e-07 \), suggesting that the presence of this species is likely associated with higher DM loss. This finding is in accord with the previous study that showed that DM loss increases with increasing the inoculation level of L. buchneri above \( 10^5 \) cfu g \(^{-1} \) (Driehuis et al., 1999). This DM loss could be explained in part by the mode of fermentation performed by this bacterium, which is a heterofermentative LAB. In heterofermentative fermentation for every mole of acetic acid formed, an equivalent molecule of carbon dioxide is generated (McDonald, 1991); therefore, a considerable loss of DM would be expected with heterofermentative fermentation (Wilkinson and Davies, 2013).

The pattern of gene abundance in the microbial community of maize biomass obtained from different growing sites, as predicted by PICRUSt, suggested the enrichment of genes for propionate metabolism in the Isfahan silage. Propionic acid is widely used as an additive to the ensiled material to restrict the growth of moulds and yeasts (Oladosu et al., 2016). The high concentration of propionate is known to associate with improved aerobic stability, less spoilage and limited mycotoxin formation in corn silage (Kung et al., 1998). These data suggest a potential link between propionate metabolism and silage...
quality. Moreover, functional metagenome prediction highlighted that the Isfahan silage was also benefited from less representation of pathways for metabolism of xenobiotics and glycerophospholipid.

Comparing the predicted metagenome of maize biomass before and after ensiling demonstrated the higher representation of pathways for degradation of toxic compounds such as ethylbenzene, xylene and naphthalene in the silage community, suggesting that degradation of toxic compounds could be an important function of microbes involved in silage fermentation. Ensiling also led to an increased metabolism of primary and secondary bile acids, suggesting a potential link between bile acid metabolism and silage fermentation. In human, bile acids appear to play a critical role in regulating gut bile acid metabolism and silage fermentation. Ensiling also led to an increased metabolism of primary and secondary bile acids, suggesting a potential link between bile acid metabolism and silage fermentation. In human, bile acids appear to play a critical role in regulating gut bile acid metabolism and silage fermentation. Ensiling also led to an increased metabolism of primary and secondary bile acids, suggesting a potential link between bile acid metabolism and silage fermentation. Ensiling also led to an increased metabolism of primary and secondary bile acids, suggesting a potential link between bile acid metabolism and silage fermentation. Ensiling also led to an increased metabolism of primary and secondary bile acids, suggesting a potential link between bile acid metabolism and silage fermentation.
Microbial cell recovery and DNA extraction

Ten grams of silage samples per silo was homogenized in 30 ml of dissociation buffer (100 mM of Tris-HCl, 50 mM of EDTA, 150 mM of NaCl, pH 8.0) for 30 min. The mixture was vigorously mixed every 5 min and then passed through a mesh filter. The filtrate was collected in a sterile container and centrifuged at 8,000 × g for 10 min at 4°C. The resulting pellet was washed twice in the same buffer and re-centrifuged (8,000 × g, 10 min, 4°C), and the pellet was resuspended in 1.4 ml of stool lysis buffer (ASL buffer), a component of the QiaAmp®DNA Stool Mini kit (Qiagen, Valencia, CA, USA). DNA was extracted according to the manufacturer’s protocol for isolating DNA from stools for pathogen detection.

PCR amplification

The V1-V3 hypervariable region of the 16S ribosomal RNA was PCR-amplified using the universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG)/534R (5'-ATTACCCTGCGGTGCTGG), which yields a ~500 bp amplicon suitable for pyrosequencing using Roche 454 FLX titanium chemistry. To barcode the PCR products, a unique multiple identifier 10 nt sequence was added to the 5’ end of the 534R primer followed by the 454 A-key primer sequence (5'-CCATCTCATCCCTGCGTGTCTCC GAICTCGA). The B-key primer sequence (5'-CCTATC CCTGTGTGCCCTGCTCC) was appended to the 5’ end of 27F. PCRs (50 µl) were run in triplicate: each reaction comprised 25 µl of PCR master mix (Thermo Fisher Scientific, www.thermofisher.com), 0.5 µM of each primer, 30 ng of microbial DNA and 22 µl of double-distilled water. The PCR regime consisted of an initial 94°C/5 min denaturation, followed by 30 cycles of 94°C/30 s, 56°C/40 s, 72°C/30 s, and finished with a 72°C/7 min extension. Following their electrophoresis, the amplicons were recovered from an agarose gel using a Qiaquick™Gel Extraction kit (Qiagen), quantified fluorometrically and pooled in equimolar fashion before being subjected to pyrosequencing on a 454 GS FLX titanium sequencer (Roche, www.roche.com) based at the Beijing Genome Institute (www.bgi.com). The raw sequence data have been deposited in the NCBI short read archive (SRA).

Sequence analysis

The 16S rDNA gene sequences were analysed using the qiime pipeline version 1.9.1 (Caporaso et al., 2010b), as described previously with minor modification (Gharachahi et al., 2015). Sequences shorter than 200 nt and longer than 1000 nt were discarded, along with those which contained ambiguous bases, those having a mean quality score < 25, those containing runs of six or more of the same base, those containing a missing qual score and those including > 2 mismatches from the primer sequences. The remaining sequences were then clustered, using uclust software (Edgar, 2010), into operational taxonomic units (OTUs), based on a criterion of > 97% sequence similarity. The most abundant sequence in each OTU was selected as being representative, and these sequences were then aligned against the greengenes core set (gg_13_8) (DeSantis et al., 2006), using the PyNAST aligner set with a minimum sequence identity of 75% (Caporaso et al., 2010a). Taxonomies were assigned to each OTU using the Ribosomal Database Project naïve Bayesian classifier (Wang et al., 2007), applying a minimum confidence value of 0.8. The alignments were filtered to remove gaps and hypervariable regions and to define conserved and non-conserved positions using a Lane mask (Caporaso et al., 2010a). A phylogenetic tree representing the relationship between OTUs was constructed from the filtered alignment using fasttree software (Price et al., 2009). Chimeric OTUs were detected using chimeraSlayer (Haas et al., 2011) and removed from the OTU table. Low-abundance OTUs (representing < 0.01% of the sequences) were discarded. To avoid heterogeneity resulting from unequal sampling, diversity indices were calculated at a sequencing depth corresponding to the read count of the sample containing the smallest set of sequences. Rarefaction plots and alpha diversity indices including chao1, shannon, simpson, goods coverage and observed species were calculated using the core_diversity_analyses.py script within the qiime pipeline (Caporaso et al., 2010b). For a beta diversity analysis, weighted and unweighted UniFrac phylogenetic distance matrices were constructed using the OTU table and the phylogenetic tree as input. Principal coordinate analysis (PCoA) plots were used to visualize phylogenetic relationships (Lozupone and Knight, 2005).

Quantitative real-time PCR analysis (RT-PCR)

To monitor population dynamics of some major LAB contributing in silage fermentation process, a quantitative PCR analysis was performed on same DNA extracts used for amplicon-based 16S rDNA sequencing. The species included were Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus plantarum and Pediococcus pentosaceus. Primers targeting intergenic spacer region of 16S-23S rRNA genes were used for quantification of
L. acidophilus and L. reuteri as described previously (Haarman and Knol, 2006). Primers previously designed to
target recA gene were used for species-specific quantification of L. brevis, L. buchneri, L. plantarum and
P. pentosaceus (Stevenson et al., 2006). The sequence of
primers used in this study is presented in Table S2. Each primer pair was first used to amplify the correspond-
ing target amplicon using either genomic DNA of the iso-
lated strain (for L. Plantarum and L. buchneri) or pooled
DNA extract of all silage samples as template. PCR was
run in a 25 μl reaction containing 2.2 μl of 25 mM MgCl₂,
2.5 μl of 10 × reaction buffer, 2.0 μl of dNTP mix
(10 mM), 1 μl of each primer (10 μM), 0.5 μl of Taq DNA
polymerase (5 U μl⁻¹), 2.0 μl of template DNA (20–
30 ng μl⁻¹ of genomic DNA) and 13.8 μl of nuclease-free
water. The resulting PCR product was gel-purified using
Qiagen® Gel Extraction kit (Qiagen). The gel-purified
DNA fragments were TA-cloned into the pGEM-T Easy
vector using the pGEM-T Easy cloning kit (Promega,
Madison, Wisconsin, USA) following the manufacturer’s
instruction. Plasmid contacting the target amplicon was
fluorometrically and used in 10-fold serial dilu-
tion (1 × 10⁸ to 1 × 10¹) for the generation of standard
curve suitable for absolute quantification of the corre-
sponding species. RT-PCR was performed by a Bio-
FACT® 2X Real-Time PCR SYBR Green Master Mix
using the MyiQ™ single colour Real-Time PCR Detection
System (Bio-Rad, Hercules, CA, USA). Standards and
samples were assayed in a 20 μl of reaction mixture con-
taining 10 μl of master mix, 1 μl of each primer (10 μM),
7 μl of nuclease-free water and 1 μl of DNA template
(2 ng). The amplification program was 95°C for 15 min,
40 cycles at 95°C for 10 s, 59–64°C for 20 s and 72°C for
20 s and a final extension at 72°C for 5 min. For each
species, standards and samples were run on the same
plate in triplicate. All standard curves were carefully
curated to meet the required standard of efficiency
(95% < efficiency < 115% and R² > 0.99). To determine
the specificity of amplification, the melting curve of each
amplicon was generated by slow heating from 60 to 95°C
(alternating 0.5°C increments held for 5 s), with fluo-
rescence collection at 0.5°C intervals.

Metagenome functional prediction based on 16S rDNA
sequence data

The functional potential of microbial community of silage
samples prepared from maize biomasses grown in three
contrasting sites was inferred via Phylogenetic Investiga-
tion of Communities by Reconstruction of Unobserved
States (PICRUSt; Langille et al., 2013). For this analysis,
quality filtered sequences were subjected to closed-reference
OTU picking based on 97% sequence similarity
using GREENGENES OTUs (v. 13.5) as a template. The OTU
table was normalized for 16S rRNA gene copy number
using normalize_by_copy_number.py script implemented
in the PICRUSt software. The final metagenome prediction
was performed using predict_metagenomes.py script.
The metagenome table was then collapsed at level 3 of
functional hierarchy and subjected to statistical analysis
using the STAMP statistical package (Parks et al., 2014).
One-way analysis of variance (one-way ANOVA) and
Tukey’s honest significant post hoc test were used to
identify Kyoto Encyclopedia Genes and Genomes (KEGG)
pathways and categories differed in abundance in
the ensiled material between the three sampling sites and
six storage time points.

Statistical analysis

The data were subjected to analyses of variance using
the General Linear Model procedure implemented in SAS
v9.2 software (SAS institute, Cary, NC, USA). Statistically
significant differences between means were calculated
using Duncan’s multiple range test. To identify OTUs
whose abundance differed significantly between the three
sampling sites, an analysis of variance was conducted, in
which the P-value was adjusted by the Bonferroni correc-
tion. A spearman correlation analysis was performed
between absolute abundance of species obtained by RT-
PCR and some physico-chemical properties of silages
using the observation_metadata_correlation.py script
implemented in the Qiime pipeline. Differences were con-
sidered to be statistically significant when the P-value was
< 0.05.

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Conflict of interest

None declared.

References

Barker, S.B., and Summerson, W.H. (1941) The colorimetric
determination of lactic acid in biological material. J Biol
Chem 138: 535–554.
Cai, Y., Benno, Y., Ogawa, M., Ohmomo, S., Kumai, S.,
and Nakase, T. (1998) Influence of Lactobacillus spp.
from an inoculant and of Weisella and Leuconostoc spp.
from forage crops on silage fermentation. Appl Environ
Microbiol 64: 2982–2987.
Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis,
T.Z., Andersen, G.L., and Knight, R. (2010a) PyNAST: a
flexible tool for aligning sequences to a template align-
ment. Bioinformatics 26: 266–267.
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Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al. (2010b) QILME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335–336.

Charmley, E. (2001) Towards improved silage quality-a review. Can J Anim Sci 81: 157–168.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., et al. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72: 5069–5072.

Driehuis, F., and Oude Elferink, S.J. (2000) The impact of the quality of silage on animal health and food safety: a review. Vet Q 22: 212–216.

Driehuis, F., Elferink, S.J., and Spoelstra, S.F. (1999) The impact of propionic acid on the fermentation, aerobic stability, and ruminal degradability of maize silage. J Appl Microbiol 87: 583–594.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460–2461.

Eikmeyer, F.G., Ko, L. Jr (2009) The effect of nitrogen level on the fermentation, aerobic stability of corn silages stored at high moisture content. J Dairy Sci 92: 3907–3914.

Elferink, S.J.O., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F., and Driehuis, F. (2001) Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri. Appl Environ Microbiol 67: 125–132.

Ennahar, S., Cai, Y., and Fujita, Y. (2003) Phylogenetic diversity of lactic acid bacteria associated with paddy rice silage as determined by 16S ribosomal DNA analysis. Appl Environ Microbiol 69: 444–451.

Filya, I. (2003) The effect of Lactobacillus buchneri and Lactobacillus plantarum on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. J Dairy Sci 86: 3575–3581.

Filya, I. (2004) Nutritive value and aerobic stability of whole crop maize silage harvested at four stages of maturity. Anim Feed Sci Technol 116: 141–150.

Filya, I., Suci, E., and Karabulut, A. (2006) The effect of Lactobacillus buchneri on the fermentation, aerobic stability and ruminal degradability of maize silage. J Appl Microbiol 101: 1216–1223.

Gharechahi, J., Zahiri, H.S., Noghabi, K.A., and Salekdeh, G.H. (2015) In-depth diversity analysis of the bacterial community resident in the camel rumen. Syst Appl Microbiol 38: 67–76.

Haarman, M., and Knol, J. (2006) Quantitative real-time PCR analysis of fecal lactobacillus species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 72: 2359–2365.

Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G., et al. (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 21: 494–504.

Holzer, M., Mayrhuber, E., Danner, H., and Braun, R. (2003) The role of Lactobacillus buchneri in forage preservation. Trends Biotechnol 21: 282–287.

Hu, W., Schmidt, R.J., McDonell, E.E., Klingerman, C.M., and Kung, L. Jr (2009) The effect of Lactobacillus buchneri 40788 or Lactobacillus plantarum MTD-1 on the fermentation and aerobic stability of corn silages ensiled at two dry matter contents. J Dairy Sci 92: 3907–3914.

Islam, K.B., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., et al. (2011) Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. Gastroenterology 141: 1773–1781.

Kakiyama, G., Pandak, W.M., Gillevet, P.M., Hylemon, P.B., Heuman, D.M., Daita, K., et al. (2013) Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. J Hepatol 58: 949–955.

Kung, L. Jr, Sheperd, A.C., Smagala, A.M., Endres, K.M., Bessett, C.A., Ranjit, N.K., and Glancey, J.L. (1998) The effect of preservatives based on propionic acid on the fermentation and aerobic stability of corn silage and a total mixed ration. J Dairy Sci 81: 1322–1330.

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., et al. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31: 814–821.

Langston, C.W., and Bourna, C. (1960) A study of the microorganisms from grass silage. II. The Lactobacillii. Appl Microbiol 8: 223–234.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., and Gobbetti, M. (2000) Purification and characterization of novel antifungal compounds from the sourdough Lactobacillus plantarum strain 21B. Appl Environ Microbiol 66: 4084–4090.

Li, Y., and Nishino, N. (2011) Monitoring the bacterial community of maize silage stored in a bunker silo inoculated with Enterococcus faecium, Lactobacillus plantarum and Lactobacillus buchneri. J Appl Microbiol 110: 1561–1570.

Li, Y., and Nishino, N. (2013) Effects of ensiling fermentation and aerobic deterioration on the bacterial community in Italian ryegrass, guinea grass, and whole-crop maize silages stored at high moisture content. Asian Australas J Anim Sci 26: 1304–1312.

Li, L., Sun, Y., Yuan, Z., Kong, X., Wao, Y., Yang, L., et al. (2015) Effect of microalgae supplementation on the silage quality and anaerobic digestion performance of Manyflower silvergrass. Bioresour Technol 189: 334–340.

Lin, C., Bolsen, K.K., Brent, B.E., and Fung, D.Y.C. (1992a) Epiphytic lactic acid bacteria succession during the presilage and ensiling periods of alfalfa and maize. J Appl Bacteriol 73: 375–387.

Lin, C., Bolsen, K.K., Brent, B.E., Hart, R.A., Dickerson, J.T., Feyertherm, A.M., and Aimutis, W.R. (1992b) Epiphytic microflora on alfalfa and whole-plant corn. J Dairy Sci 75: 2484–2493.

Lozupone, C., and Knight, R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71: 8228–8235.

McDonald, P. (1991) The Biochemistry of Silage. Chichester, UK: John Wiley & Sons Ltd.

McDonald, P., Henderson, A.R., and Raithon, I. (1973) Energy changes during ensilage. J Sci Food Agric 24: 827–934.

McEniry, J., O’Kiely, P., Clipson, N.J., Forristal, P.D., and Boyle, E.M. (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.

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McGarvey, J.A., Franco, R.B., Palumbo, J.D., Hnasko, R., Stanker, L., and Mitloehner, F.M. (2013) Bacterial population dynamics during the ensiling of Medicago sativa (alfalfa) and subsequent exposure to air. *J Appl Microbiol* 114: 1661–1670.

Moon, N.J. (1983) Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. *J Appl Bacteriol* 55: 453–460.

Muck, R.E. (2010) Silage microbiology and its control through additives. *Rev Bras Zootecnia* 39: 183–191.

Muck, R. (2013) Recent advances in silage microbiology. *Agric Food Sci* 22: 3–15.

Oladosu, Y., Rafii, M.Y., Abdullah, N., Magaji, U., Hussin, G., Ramli, A., and MIah, G. (2016) Fermentation quality and additives: a case of rice straw silage. *Biomed Res Int* 2016: 7985167.

Parks, D.H., Tyson, G.W., Hugenholtz, P., and Beiko, R.G. (2009) FastTree: inferring large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 26: 1641–1650.

Queiroz, O.C., Arriola, K.G., Daniel, J.L., and Adesogan, A.T. (2013) Effects of 8 chemical and bacterial additives on the quality of corn silage. *J Dairy Sci* 96: 5836–5843.

Ridlon, J.M., Kang, D.J., Hylemon, P.B., and Bajaj, J.S. (2014) Bile acids and the gut microbiome. *Curr Opin Gastroenterol* 30: 332–338.

Stevenson, D.M., Muck, R.E., Shinners, K.J., and Weimer, P.J. (2006) Use of real time PCR to determine population profiles of individual species of lactic acid bacteria in alfalfa silage and stored corn stover. *Appl Microbiol Biotechnol* 71: 329–338.

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. (1991) Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci* 74: 3583–3597.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73: 5261–5267.

Weatherburn, M.W. (1967) Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem* 39: 971–974.

Weinberg, Z.G., and Muck, R. (1996) New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiol Rev* 19: 53–68.

Weiss, A., Scheller, F., Oggenfuss, M., Walsh, F., Frey, J.E., Drissner, D., and Schmidt, H. (2015) Analysis of the bacte-rial epiphytic microbiota of oak leaf lettuce with 16s ribosomal rna gene analysis. *J Microb Biotechnol Food Sci* 5: 271.

Wilkinson, J.M., and Davies, D.R. (2013) The aerobic stability of silage: key findings and recent developments. *Grass Forage Sci* 68: 1–19.

Woolford, M.K. (1990) The detrimental effects of air on silage. *J Appl Bacteriol* 68: 101–116.

Yang, C.H., Crowley, D.E., Borneman, J., and Keen, N.T. (2001) Microbial phyllosphere populations are more complex than previously realized. *Proc Natl Acad Sci USA* 98: 3889–3894.

Yang, J., Cao, Y., Cai, Y., and Terada, F. (2010) Natural populations of lactic acid bacteria isolated from vegetable residues and silage fermentation. *J Dairy Sci* 93: 3136–3145.