

Long-read 16S amplicon analyses and improved cultivation techniques as joined approach for the identification of viable bacterial populations in silage

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Abstract

Aims: Lactic acid bacteria (LAB) are crucial for the quality and preservation of silage. Exploration and isolation of the key microbiome are important for improving fermentation processes. While cultivation-dependent methods enable the isolation of LAB, they often overlook organisms with challenging cultivation requirements. Next-generation sequencing provides cultivation-independent microbiome insights. In contrast to commonly used methods, long-read sequencing platforms like the Pacific Biosciences (PacBio) single-molecule real-time (SMRT) platform offer species-level resolution. This study aims to investigate the benefits of a combined cultivation-dependent and -independent approach for silage microbiome analyses.

Methods and results: The common isolation medium for LAB was supplemented with sterile pressed juice from plant material improving the cultivation conditions. PacBio amplicon sequencing provided an almost complete and cultivation-independent picture of the bacterial community. The use of mock communities and a live/dead discriminating treatment of the samples revealed that the analysis can be misleading if appropriate controls are not performed.

Conclusion: Growth of plant-associated microorganisms can be supported by a plant juice containing isolation medium, allowing isolation of the dominant LAB from silage. Microbial dynamics can be represented using long-read sequencing. However, the use of controls such as a live/dead discrimination and mock communities is essential for the reliability of the data.

Impact Statement

The results of this work show that the main microbiota of silage can be isolated using improved cultivation conditions, which is important for the study of bacterial processes in silage and for the development of effective inoculants. Furthermore, it is demonstrated, how long-read sequencing methods can distort the picture of the bacterial composition in silage and how this can be avoided by using appropriate techniques and controls.

Keywords: microbiome; amplicon sequencing; isolation; lactic acid bacteria; agriculture microbiology

Introduction

Silage is an important feedstuff for ruminants that can be fed throughout the year, whereby the demand of high-quality silage continues to increase (Wilkinson and Lee 2018, Wilkinson and Muck 2019). The quality of the silage depends on many factors during all four phases of ensiling (field, ensiling, storage, and feed-out phase). The epiphytic lactic acid bacteria (LAB) or the optional addition of inoculants influence both the pH drop in the fermentation phase and the aerobic stability during the feed-out phase by their production of organic acids and other metabolites (Muck et al. 1991, Okoye et al. 2023). A fast lactic acid built-up during fermentation inhibits undesirable microbiota (Bolsen et al. 1996). Other organic acids, especially acetic acid, can delay aerobic spoilage by inhibiting yeast growth (Weinberg and Muck 1996, Danner et al. 2003). Furthermore, undesirable microorganisms can be inhibited by the production of bacteriocins by certain LAB (Vandenberg 1993, Müller et al. 1996). Another advantage of us-

ing microbial additives is the resulting improvement in animal performance (Oliveira et al. 2017). Additives to improve silage preservation have been available for decades and continue to evolve through extensive research (Yitbarek and Tamir 2014, Muck et al. 2018). Despite the existence of effective inoculants, new possibilities for understanding bacterial processes that can influence the ensiling process are constantly emerging (Okoye et al. 2023). Therefore, the study of the silage microbiome remains essential for the further development of effective inoculants.

Next-generation sequencing (NGS) is a valuable tool for understanding the microbiome of different habitats. A large number of studies have analyzed the microbiome of various silage samples using the Illumina MiSeq platform or Ion Torrent technique by sequencing the hypervariable regions of the 16S rRNA gene V3-V4 or only V4, resulting in a resolution of the operational taxonomic units (OTU) only to the genus level (Kraut-Cohen et al. 2016, Mogodiniyai Kasmaei et al.

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2017, Ni et al. 2017, Romero et al. 2017). In contrast to these short-read sequencing technologies, the sequencing methods offered by Pacific Biosciences (PacBio) and Oxford Nanopore allow maximum read lengths of 300 kilobases and more than four megabases, respectively (Hu et al. 2021), covering the entire 16S rRNA gene sequence. One of the major limitations of these methods has been their higher error rates, but as these technologies become more accurate, they are gaining in popularity (Hu et al. 2021, Karst et al. 2021). PacBio single-molecule real-time (SMRT) sequencing technology generates highly accurate circular consensus sequences (CCS) of individual 16S rRNA genes, characterized by multiple reads of the same sequence to improve results (Mosher et al. 2014). More recent studies have shown that using these full-length sequencing technologies can give insights into the silage microbiome up to species level (Bao et al. 2016, Du et al. 2021, Li et al. 2021). This can be advantageous as members of the same genus can exhibit a high degree of heterogeneity. For instance within the genus *Listeria* only a few species are pathogenic (Orsi and Wiedmann 2016), of which *Listeria monocytogenes* is infectious for humans and animals, including ruminants, and therefore undesirable in silage (Driehuis et al. 2018). Furthermore, species can be assigned to new genera, as recently done during the reclassification of the lactobacilli (Zheng et al. 2020). Those taxonomic reassignments may confound the data interpretation if sequence assignment is limited to the genus level. In order to obtain optimal results that exclude the sequencing of dead cells, a prior treatment with propidium monoazide (PMA) of the samples can be performed. This live/dead discrimination is possible as PMA can be covalently linked to DNA of cells with compromised membranes and inhibit PCR amplification (Nocker et al. 2007).

Apart from the analyses of the silage microbiome by cultivation-independent NGS techniques, the cultivation of microorganisms from silage is essential for the characterization of metabolic performance and growth requirements of the microbial key players. Last but not least also for description of new microbial taxa from this habitat, the isolation of viable strains is indispensable. The generation of isolates includes classical microbiological plating methods using deMan-Rogosa-Sharpe (MRS) medium in case of LAB. This complex medium contains glucose as only carbohydrate. While homofermentative and facultative heterofermentative LAB ferment hexoses to lactic acid as sole product, obligate heterofermentative LAB produce side products like ethanol and CO₂ using the phosphoketolase pathway (Kandler 1983, Pahlow et al. 2003). However, fermentation of pentoses results in the production of lactic and acetic acid as well as additional ATP and for some heterofermentative LAB even poor growth on glucose has been shown (Richter et al. 2001, Maicas et al. 2002, Richter et al. 2003, Zaunmüller et al. 2006). For these reasons, growth on pentoses or other nutrients from silage plants may be preferred to growth on hexoses. It is therefore questionable whether MRS medium is sufficient to isolate the full diversity of LAB, as strains with different growth requirements may be overlooked.

The aim of this study is to investigate whether plating methods can reveal the entire composition of the relevant silage microbiome. For this purpose, several silage samples of different crops and ensiling durations are analyzed. For these analyses, not only the conventional MRS medium is used to generate isolates, but a modified variant containing additives of the pressed juice of the respective plant. This is intended

to overcome the above-mentioned deficiencies of the MRS medium by providing additional carbon sources and other nutrients from the natural habitat. Full-length amplicon sequencing methods, which provide a cultivation-independent picture of the bacterial community, are used to check whether the improved cultivation technique produced a more complete picture of the viable microbial communities in silage samples. PMA treatment and analysis of mock communities are conducted to investigate the reliability of the data obtained from PacBio sequencing of silage samples.

Materials and methods

Silage crop and preparation

Grass and alfalfa were grown at Campus Frankenforst of the Rheinische Friedrich-Wilhelms-Universität Bonn (Königswinter, Germany; 50°42'50.1"N 7°12'24.9"E). Grass and alfalfa were harvested on 2 May 2024 and 4 June 2024, respectively. After harvesting, the crops were chopped to a length of 8 mm and ensiled in 1.5 l jars in triplicates.

Media production

The isolation medium used was deMan-Rogosa-Sharpe medium (Carl Roth, Karlsruhe, Germany) supplemented with pressed plant juice (MRSPJ) from the same plant that was used for ensiling. Subcultivation was conducted on both MRSPJ and MRS agar. For production of plant juice, crops were harvested 1 week before ensiling, chopped to a theoretical length of 8 mm, and pressed in a pressing plunger. The plant juice was sterilized by autoclaving and the liquid was separated from precipitates by centrifugation at 3220 × g for 15 min in a sterile centrifuge tube and then stored in a sterile bottle. For MRSPJ production, double-concentrated MRS was diluted 1:1 (v/v) with sterile plant juice.

Microbial analysis

A 10 g of each replicate of a silage sample were pooled in a stomacher bag and filled up to 300 g total weight with Ringer solution (Merck, Darmstadt, Germany) and homogenized in a Stomacher Star Blender LB 400 (VWR, Darmstadt, Germany). A serial dilution from this suspension was plated on MRSPJ medium of the corresponding plant and incubated anaerobically using the Millipore Anaerocult A (Merck, Darmstadt, Germany) at 30°C for 5 days. After incubation, at least 90 and up to 110 isolates from each silage sample were identified by sequencing the 16S rRNA gene. Here, either all colonies on a plate were identified or, if the number of colonies was too high, an area was marked and all colonies contained were analyzed. This was to prevent certain colony morphologies from being favorably selected.

DNA extraction, PMA treatment, and PCR

DNA extraction from isolates, PCR of the 16S rRNA gene and sequencing, as well as DNA extraction from complex silage samples and propidium monoazide (PMA) treatment were performed as previously described (Weber et al. 2019) with some modifications. Isolates were identified by 16S rRNA gene sequencing of the unpurified PCR products in a 96-well plate. For amplicon sequencing of the 16S rRNA genes from silage samples, 2 ml of the above-mentioned sample homogenate were taken from the stomacher bag, and PMA treat-

Table 1. Investigated silage samples are listed.

crop	Sample name	Ensiling duration	pH	PMA treatment
Alfalfa	A-T1	43 hours	6.0	No
	A-T1*			Yes
	A-T2*	1.5 weeks	5.4	Yes
	A-T3*	2 months	5.1	Yes
Grass	G-T1	22 hours	5.1	No
	G-T2	1 week	4.5	No
	G-T3	2 months	4.2	No

Important information, including crop type, ensiling duration, and pH, is given. The asterisk indicates whether the sample has been PMA-treated.

ment was performed using a final concentration of 100 $\mu\text{mol l}^{-1}$. Cells were lysed for DNA extraction using 180 μl of lysis buffer [20 mmol l^{-1} Tris HCl (pH 8.2), 2 mM EDTA, and 1.2% Triton X-100] containing lysozyme (20 mg ml^{-1}), and 9 μl of mutanolysin was added (stock solution: 1000 U ml^{-1} in water).

Mock communities

Mock communities were created containing isolates from grass silage (grass silage community, GC) and from alfalfa silage (alfalfa silage community, AC) as a control for the PacBio analysis. Each of them was prepared in two variants. Firstly, liquid cultures of the isolates were prepared, adjusted to a uniform OD, and mixed in equal parts, from which the DNA was extracted. Secondly, DNA of the pure cultures was extracted separately and the concentrations of the DNA were determined using the Gen5 Take3 module in the BioTek Epoch microplate spectrophotometer (Agilent Technologies, Waldbronn, Germany), and then extracted DNA was mixed in equal concentrations. This was done to visualize influences on quantification of sequencing data such as DNA extraction.

16S Amplicon sequencing and data processing

Sequencing of the 16S rRNA gene Amplicon in complex samples was done by Biomarker Technologies (BMK) GmbH (BMKGENE, Münster, Germany) using the PacBio Sequel II platform in the CCS sequencing mode. The bioinformatic analyses described below were also performed by BMKGENE. Raw CCS sequences were identified from the barcode sequences using lima version 1.7.0. Primer removal was performed by cutadapt version 1.9.1 (Martin 2011) and CCS sequences were filtered based on their length (1200–1650 bp). UCHIME version 4.2 (Edgar et al. 2011) was used to remove chimeric sequences and CCS sequences with an identity of at least 97% were clustered into OTUs by USEARCH version 10 (Edgar 2013). OTUs were filtered with a threshold of 0.005%. QIIME 2 (version 2020.06) (Bolyen et al. 2019) was applied for feature annotation and abundance determination by a combined method of Bayesian classifier (Bokulich et al. 2018) and BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990) using SILVA (Release 138) (Quast et al. 2013) as database.

Finalized data was received listing all OTUs and their abundances in the samples. These data were then post-processed manually. Based on the amplicon sequencing data of the samples and the mock communities, a lower threshold for the percentages was set, and all results below 0.07% were removed. This value was set to limit overestimation of the OTUs, but still capture important representatives of the silage sam-

ples. The 16S rRNA gene copy number was taken from the database *rrnDB* (Stoddard et al. 2015), and was used for recalculation of the OTU proportions based on 16S rRNA gene copy numbers of the detected taxa.

Results

Cultivation-dependent and cultivation-independent methods were used to analyze various grass and alfalfa silage samples. The samples were analyzed either during ensiling and/or at the end of ensiling to ensure that a broad spectrum of the microbial community was covered. Silage samples and their characteristics are listed in Table 1. To highlight restrictions of cultivation-independent methods, PMA-treated and -untreated samples and mock communities were analyzed using PacBio SMRT technology. Because isolation techniques can favor certain bacteria, an optimized medium was prepared, and long-read amplicon sequencing, which provides high-resolution identification up to species level, was used to control for successful isolation of key microbiota.

PMA treatment reveals altered microbial composition of amplicon sequencing data

To exclude the 16S rRNA genes of dead cells from sequencing, a PMA treatment was conducted on alfalfa samples. To receive insights into how the PMA treatment might influence the sequencing results, the untreated sample A-T1 is compared to the treated sample A-T1*. As shown in Table 2, the treatment resulted in different microbial ratios and composition of the same sample. Organisms with a relative abundance of at least 10% were considered dominant/main representatives. While in the untreated sample A-T1 *Weissella hellenica* (W. *hellenica*) makes up the largest proportion, accounting for 46%, it is greatly reduced by the PMA treatment, resulting in a new composition of dominant species in A-T1*. *Weissella cibaria*, whose proportion is increasing, remains part of the main representatives, and *Leuconostoc mesenteroides* and *Pantoea vagans* are added. A total of nine species from sample A-T1 are excluded by the PMA treatment. Although there is a total reduction of OTUs by the PMA treatment from 26 in A-T1 to 20 in A-T1*, the exclusion of dead cells permits the detection of new species of low abundance, as these have thus exceeded the threshold value. This is the case for *Enterococcus moraviensis*, *Providencia alcalifaciens*, and *Pseudoclavibacter helvolus*, which only appear in sample A-T1*.

Table 2. Comparison of the PacBio results of PMA-untreated (A-T1) vs. -treated (A-T1*) samples.

Species	A-T1	A-T1*
<i>Weissella hellenica</i>	45.95	9.72
<i>Weissella cibaria</i>	16.62	23.39
<i>Leuconostoc mesenteroides</i>	8.55	14.94
<i>Pantoea vagans</i>	7.30	20.19
<i>Lactococcus lactis</i>	6.33	2.77
<i>Kluyvera intermedia</i>	3.18	9.48
<i>Erwinia rhapontici</i>	2.93	8.00
<i>Serratia proteamaculans</i>	1.89	5.88
<i>Xanthomonas translucens</i>	1.66	0.94
<i>Weissella minor</i>	1.06	1.02
<i>Stenotrophomonas rhizophila</i>	0.88	–
<i>Pseudomonas graminis</i>	0.64	0.76
<i>Lactococcus garvieae</i>	0.48	0.11
<i>Pseudomonas fluorescens</i>	0.35	0.62
<i>Enterococcus mundtii</i>	0.34	0.35
<i>Enterococcus casseliflavus</i>	0.30	0.58
<i>Sphingomonas faeni</i>	0.30	–
<i>Acidovorax valerianellae</i>	0.20	–
<i>Latilactobacillus sakei</i>	0.17	0.18
<i>Kosakonia cowanii</i>	0.15	0.57
<i>Comamonas jiangduensis</i>	0.15	–
<i>Paenibacillus chinensis</i>	0.14	–
<i>Blautia obeum</i>	0.12	–
<i>Weissella soli</i>	0.12	–
<i>Exiguobacterium sibiricum</i>	0.10	–
<i>Methylobacterium adhaesivum</i>	0.09	–
<i>Enterococcus moraviensis</i>	–	0.13
<i>Providencia alcalifaciens</i>	–	0.07
<i>Pseudoclavibacter helvolus</i>	–	0.26
Total number of OTUs	26	20

The abundance of identified OTUs is presented in percentage. Bold numbers indicate main representatives.

Incorrect quantification of taxa abundances and taxa counts in mock communities

Mock communities consisting either of grass isolates (GC) (Table 3) or alfalfa isolates (AC) (Table 4) were analyzed to assess the reliability of the microbial diversity and composition obtained from the PacBio data. The species were contained in equal parts, with the ratios being set either by OD or by DNA concentration. Firstly, a selective detection of species in the mock communities was observed. Independent of sample preparation, the species *Latilactobacillus curvatus* (*Lt. curvatus*), *Leuconostoc citreum*, and *Leuconostoc pseudomesenteroides* remained undetected by amplicon sequence analyses. Apart from that, there was a considerable overestimation of the OTU counts in the mock communities. Of all OTUs identified in GC and AC, the genuine members of the communities comprised only between 7.7% and 27.6% of total OTU counts. However, their relative abundances made up the majority of the samples. In contrast, the relative abundances of the artificial OTUs were low with the highest percentage share of 0.31% recorded for *Blautia obeum* in AC (Table 4). Nevertheless, this considerable overestimation of OTU count was taken into account when analyzing the PacBio data of the actual silage samples, as it might artificially increase silage diversity. To approximate the true diversity, a lower threshold value has been set for the percentages. As the dominant species, *Lentilactobacillus buchneri* (*Ln. buchneri*) and *Lentilactobacillus parabuchneri* (*Ln. parabuchneri*) based on the cultivational approach of sample G-T3 accounted for <0.31% according to the PacBio re-

sults (Table 6), the lower threshold value was set to include these species. After processing the PacBio data by applying the threshold value and the 16S copy number correction, the shares of *Ln. buchneri* and *Ln. parabuchneri* accounted for 0.75%, showing a high discrepancy between the cultivation-dependent and -independent results. Similar discrepancies between the expected and actual results of the PacBio data were observed in the mock communities. There were considerable differences in the proportions of the genuine members that should be equally represented (Tables 3–4). Instead of approximately equal proportions of the representatives, single members were overrepresented in each mock community, with shares ranging from 44.75% to 66.25%. It should be noted, however, that no correction of 16S rRNA gene copy number was performed for the mock communities because the copy numbers were unknown for many of the species listed here.

Key silage microbiota can be identified using conventional cultivation methods

The microbial composition obtained by cultivation techniques and the composition determined by full-length sequencing of the 16S rRNA gene of various grass and alfalfa silages were compared. Contrary to the alfalfa samples, the grass samples were not PMA treated, as this method was introduced later in the course of the project.

PacBio sequencing provided an almost complete picture of the bacterial composition, whereas selective cultivation conditions resulted in the composition of only members of the *Lactobacillales*. For a direct comparison of the isolates obtained from the pressed plant juice containing MRSPJ-agar with the 16S amplicon sequencing data, only those species from amplicon sequencing that were identified as members of the *Lactobacillales* were considered in this comparison. These accounted for 81%–99% of the taxa. The PacBio analysis revealed the microbial composition up to species level and the dominating microorganisms (at least 10%) were considered as most relevant of the respective phase of ensiling. Due to the high resolution of the PacBio data, it was shown that indeed in all cases the dominant species could be isolated by using microbial plating methods (Tables 5–8). Only in the sample G-T3 (Table 6), the organism *Leuc. mesenteroides* could not be found on agar plates but seemed to be dominating according to the PacBio data. In contrast, this bacterium could be isolated in the earlier phases T1 and T2 of ensiling (Table 5).

Analysis of the mock communities revealed that several species in these communities were not detected in the PacBio sequencing approach. In accord with this, the isolation approach detected single species, which were not covered by the sequencing approach on the same sample (Tables 5–8). These were *Leuc. citreum* (G-T1), *Lt. curvatus* (G-T1, G-T2), and *Leuc. pseudomesenteroides* (G-T1, G-T2), which were members of the mock communities but were not detected by amplicon sequence analyses of these mock communities. Further undetected species were *Leuconostoc miyukkimchii* (G-T1), *Lentilactobacillus kefir* (G-T3), *Enterococcus durans* (A-T3*), *Lentilactobacillus buchneri* (A-T3*), *Pediococcus parvulus* (A-T3*), *Secundilactobacillus oryzae* (A-T3*), and *Weissella paramesenteroides* (A-T3*). In the cases of *Lt. curvatus* and *Secundilactobacillus oryzae* (*S. oryzae*) from G-T2 (Table 5) and A-T3* (Table 8), respectively, these were

Table 3. Amplicon sequencing results of the mock community GC.

Species	Mock community member	Equal OD	Equal DNA conc.
<i>Latilactobacillus sakei</i>	X	44.75	1.48
<i>Weissella koreensis</i>	X	13.75	5.60
<i>Leuconostoc kimchii</i>	X	12.65	12.96
<i>Weissella hellenica</i>	X	10.71	0.06
<i>Weissella cibaria</i>	X	9.86	11.02
<i>Levilactobacillus brevis</i>	X ^a	7.04	–
<i>Leuconostoc mesenteroides</i>	X	0.77	0.50
<i>Lactiplantibacillus plantarum</i>	X	0.32	0.48
<i>Companilactobacillus musae</i>	X ^b	0.01	1.43
<i>Romboutsia ilealis</i>		0.01	0.02
<i>Blautia luti</i>		0.01	0.01
<i>Ligilactobacillus murinus</i>		0.01	0.01
<i>Limosilactobacillus reuteri</i>		0.01	6.8E-03
<i>Pediococcus pentosaceus</i>		0.01	3.4E-03
<i>Faecalibacillus intestinalis</i>		0.01	3.4E-03
<i>Lactococcus lactis</i>	X ^b	7.5E-03	66.25
<i>Eubacterium hallii</i>		7.5E-03	0.02
<i>Dorea longicatena</i>		7.5E-03	0.01
<i>Streptococcus salivarius</i>		7.5E-03	0.01
<i>Lactobacillus johnsonii</i>		3.7E-03	6.8E-03
<i>Blautia massiliensis</i>		3.7E-03	3.7E-03
<i>Carnobacterium maltaromaticum</i>		3.7E-03	3.4E-03
<i>Enterobacter ludwigii</i>		3.7E-03	3.4E-03
<i>Ruminococcus bromii</i>		3.7E-03	3.4E-03
<i>Aerococcus viridans</i>		3.7E-03	–
<i>beta proteobacterium AAP121</i>		3.7E-03	–
<i>Delftia acidovorans</i>		3.7E-03	–
<i>Lacticaseibacillus casei</i>		3.7E-03	–
<i>Neorhizobium galegae</i>		3.7E-03	–
<i>Lactobacillus intestinalis</i>		–	0.01
<i>Staphylococcus xylosus</i>		–	0.01
<i>Bifidobacterium adolescentis</i>		–	6.8E-03
<i>Rahnella aquatilis</i>		–	6.8E-03
<i>Comamonas piscis</i>		–	3.4E-03
<i>Companilactobacillus alimentarius</i>		–	3.4E-03
<i>Enterococcaceae</i>		–	3.4E-03
<i>Loigolactobacillus coryniformis</i>		–	3.4E-03
<i>Pseudomonas graminis</i>		–	3.4E-03
<i>Pseudomonas syringae</i>		–	3.4E-03
<i>Ruminococcus torques</i>		–	3.4E-03
<i>Saccharopolyspora rosea</i>		–	3.4E-03
<i>Stenotrophomonas rhizophila</i>		–	3.4E-03
<i>Turicibacter sanguinis</i>		–	3.4E-03
Unassigned		–	3.4E-03
<i>Vagococcus</i> sp. bfs11-15		–	3.4E-03
<i>Weissella minor</i>		–	3.4E-03
Number of OTUs		29	40
Proportion of OTUs of genuine members		27.6%	22.5%

^apresent only in equal OD mock community.

^bpresent only in equal DNA conc. mock community and indicated in the corresponding column by bold numbers. The abundance of identified OTUs is presented in percentage. Genuine members of the mock communities are labeled in the second column by an X and indicated by bold numbers. All identified OTUs are listed, without correction of the 16S rRNA gene copy number and the OTU numbers using the threshold value of 0.07. Mock community GC consisted of 11 (equal OD) and 12 (equal DNA conc.) strains from different species. Each strain represented 9.1% or 8.3% of the total mock community, respectively. *Latilactobacillus curvatus*, *Leuconostoc citreum*, and *Leuconostoc pseudomesenteroides* were part of the mock community but not detected by amplicon sequencing.

even dominating representatives according to the cultivation-dependent method.

Medium optimization allows the cultivation of additional species

Plating was carried out on MRSPJ agar to provide additional nutrients from the natural habitat of the microorganisms. This was done to avoid overlooking important representatives in the silage with special nutritional requirements, e.g. hetero-

fermentatives. A total of 1012 isolates comprising 28 species were sequenced from MRSPJ-agar from 10 silage samples. Of these, only eight isolates comprising two species were not able to grow on MRS agar, which means subcultivation on MRS was possible for 99.2% of isolates. One of the species that was not able to grow on MRS agar was *Lactobacillus acetotolerans* (*L. acetotolerans*), which turned out to be one of the dominating species in G-T3 according to the PacBio data (Table 6).

Table 4. Amplicon sequencing results of the mock community AC.

Species	Mock community member	Equal OD	Equal DNA conc.
<i>Weissella hellenica</i>	X	54.3	47.90
<i>Leuconostoc mesenteroides</i>	X	18.2	13.80
<i>Lactiplantibacillus plantarum</i>	X	14.1	15.20
<i>Pediococcus pentosaceus</i>	X	6.99	13.00
<i>Weissella cibaria</i>	X	6.08	8.36
<i>Lachnospiraceae bacterium</i>		0.03	0.1
<i>Latilactobacillus sakei</i>		0.03	0.02
<i>Blautia obeum</i>		0.04	0.31
<i>Faecalibacillus intestinalis</i>		0.03	0.08
<i>Ruminococcus bromii</i>		0.03	0.07
<i>Eubacterium hallii</i>		0.02	0.17
<i>Ruminococcus torques</i>		0.02	0.05
<i>Anaerostipes hadrus</i>		0.02	0.03
<i>Lactobacillus johnsonii</i>		0.02	4.08E-03
<i>Staphylococcus xylosus</i>		0.01	0.01
<i>Eubacterium rectale</i>		9.47E-03	0.06
<i>Ligilactobacillus murinus</i>		9.47E-03	0.03
<i>Kosakonia cowanii</i>		9.47E-03	4.08E-03
<i>Lactobacillus crispatus</i>		9.47E-03	–
<i>Dorea longicatena</i>		6.32E-03	0.11
<i>Romboutsia ilealis</i>		6.32E-03	0.1
<i>Faecalibacterium prausnitzii</i>		6.32E-03	0.05
Unassigned		6.32E-03	0.04
unclassified <i>Tyzzerella</i>		6.32E-03	0.02
<i>Vagococcus bubulae</i>		6.32E-03	0.02
<i>Streptococcus ilei</i>		6.32E-03	8.16E-03
<i>Aerococcus urinaeequi</i>		6.32E-03	4.08E-03
<i>Psychrobacter cibarius</i>		6.32E-03	4.08E-03
unclassified Archaea		6.32E-03	4.08E-03
uncultured rumen bacterium		6.32E-03	4.08E-03
<i>Bifidobacterium pseudocatenulatum</i>		3.16E-03	0.04
<i>Blautia phocaeensis</i>		3.16E-03	0.03
<i>Streptococcus salivarius</i>		3.16E-03	0.03
<i>Companilactobacillus nuruki</i>		3.16E-03	0.02
<i>Phocaeicola plebeius</i>		3.16E-03	0.01
<i>Streptococcus hyointestinalis</i>		3.16E-03	0.01
<i>Dialister massiliensis</i>		3.16E-03	8.16E-03
<i>Faecalibaculum rodentium</i>		3.16E-03	8.16E-03
<i>Levilactobacillus brevis</i>		3.16E-03	8.16E-03
<i>Vagococcus fessus</i>		3.16E-03	8.16E-03
<i>Pantoea vagans</i>		3.16E-03	4.08E-03
<i>Brevundimonas vesicularis</i>		3.16E-03	–
<i>Catenibacterium mitsuokai</i>		3.16E-03	–
<i>Chlamydia trachomatis</i>		3.16E-03	–
<i>Clostridium senegalense</i>		3.16E-03	–
<i>Enterococcus moraviensis</i>		3.16E-03	–
<i>Lactococcus lactis</i>		3.16E-03	–
<i>Loigolactobacillus coryniformis</i>		3.16E-03	–
<i>Microbacterium oleivorans</i>		3.16E-03	–
<i>Priestia aryabhatai</i>		3.16E-03	–
<i>Saccharopolyspora rosea</i>		3.16E-03	–
<i>Streptococcus galloyticus</i>		3.16E-03	–
Unclassified <i>Clostridia</i> UCG 014		3.16E-03	–
<i>Weissella minor</i>		3.16E-03	–
<i>Holdemanella bififormis</i>		–	0.02
<i>Lentilactobacillus diolivorans</i>		–	0.02
<i>Turicibacter sanguinis</i>		–	0.02
<i>Lactobacillus intestinalis</i>		–	0.01
<i>Vibrio anguillarum</i>		–	0.01
<i>Kluyvera intermedia</i>		–	8.16E-03
<i>Methylobacterium mesophilicum</i>		–	8.16E-03
<i>Pseudomonas fluorescens</i>		–	8.16E-03
<i>Serratia proteamaculans</i>		–	8.16E-03
<i>Acidovorax valerianellae</i>		–	4.08E-03
<i>Carnobacterium inhibens</i>		–	4.08E-03
<i>Delftia tsuruhatensis</i>		–	4.08E-03

Table 4. Continued

Species	Mock community member	Equal OD	Equal DNA conc.
<i>Erwinia rhapontici</i>	–		4.08E-03
<i>Exiguobacterium sibiricum</i>	–		4.08E-03
<i>Methyloceanibacter marginalis</i>	–		4.08E-03
<i>Paenibacillus chinensis</i>	–		4.08E-03
<i>Pantoea agglomerans</i>	–		4.08E-03
<i>Pediococcus cellicola</i>	–		4.08E-03
<i>Pseudoalteromonas nigrifaciens</i>	–		4.08E-03
<i>Pseudomonas graminis</i>	–		4.08E-03
<i>Saccharopolyspora hordei</i>	–		4.08E-03
<i>Sphingomonas phyllosphaerae</i>	–		4.08E-03
<i>Streptococcus danieliae</i>	–		4.08E-03
unclassified <i>Eubacterium</i>	–		4.08E-03
<i>coprostanoligenes</i> group			
Number of total OTUs		54	65
Proportion of OTUs of genuine members		9.3%	7.7%

The abundance of identified OTUs is presented in percentage. Genuine members of the mock communities are labeled in the second column by an X and indicated by bold numbers. All identified OTUs are listed, without correction of the 16S rRNA gene copy number and the OTU numbers using the threshold value of 0.07. Mock community AC consisted of six strains from different species. Each strain represented 16.7% of the total mock community. *Leuconostoc pseudomesenteroides* was part of the mock community but not detected by amplicon sequencing.

Table 5. Comparison of the microbial compositions of samples G-T1 and G-T2 obtained by Amplicon sequencing (PacBio) and by isolation on MRSPJ agar (cultivation).

Species	G-T1		G-T2	
	PacBio	Cultivation	PacBio	Cultivation
<i>Leuconostoc mesenteroides</i>	51.78	60.19	56.52	60.55
<i>Weissella hellenica</i>	19.95	18.52	12.88	2.75
<i>Latilactobacillus sakei</i>	15.58	8.33	20.94	17.43
<i>Lactococcus lactis</i>	4.85	1.85	3.73	0.92
<i>Weissella koreensis</i>	1.84	–	0.70	–
<i>Weissella cibaria</i>	1.68	1.85	1.13	0.92
<i>Leuconostoc kimchii</i>	4.08	5.56	3.20	–
<i>Leuconostoc citreum</i>	–	0.93	–	–
<i>Latilactobacillus curvatus</i>	–	1.85	–	11.01
<i>Leuconostoc pseudomesenteroides</i>	–	0.93	–	0.92
<i>Leuconostoc moryella</i>	–	0.93	–	–
<i>Lactiplantibacillus plantarum</i>	–	–	0.34	0.92
<i>Companilactobacillus musae</i>	–	–	0.27	0.92
<i>Levilactobacillus brevis</i>	–	–	0.21	3.67
Others	0.23	–	0.09	–

The abundance of identified OTUs is presented in percentage. Since all isolated species belonged to the Lactobacillales, the PacBio data is restricted to the members of that order to achieve a direct comparison of both methods. Bold numbers indicate main representatives.

Discussion

Long-read amplicon sequencing of complex samples provides high-resolution data that is clearly superior to short-read amplicon sequencing. Short-read sequencing methods only cover a small part of the nine hypervariable regions of the 16S rRNA gene and therefore can only adequately identify at genus level (Johnson et al. 2019). Sequencing the full-length 16S rRNA gene, however, allows for differentiation even between closely related species (Earl et al. 2018). The main disadvantage of long-read sequencing is the high error rate, which has already been overcome by improved technologies and bioinformatic tools, just as the cost efficiency and turnaround rate will continue to increase (Hu et al. 2021). Accordingly, using long-read sequencing methods, samples can be analyzed more accurately at species level, which may provide new insights into the dynamics of different silage types. As shown here, this was used to investigate whether isolation methods are capable of cap-

turing the main microbiota in silage. Cultivation-dependent methods are criticized for underestimating the proportion of microorganisms that are difficult to cultivate and therefore underestimate the microbiome diversity. Although this is undoubtedly true, this work has shown that microbial plating methods are sufficient to isolate the main representatives from silages (Tables 5–8). The comparison with the PacBio sequencing data emphasized that no dominating members in ensiling are excluded. The addition of plant juice to the medium was conducted to isolate additional bacteria, for which the MRS medium is not sufficient to cover their nutrient requirements. Although this resulted in generating only a small number (0.8%) of additional isolates not growing on MRS, it was possible to obtain *L. acetotolerans* using the MRSPJ medium, which was one of the main representatives of grass T3 according to the PacBio sequencing data. This species has special nutrient requirements, such as riboflavin, pantothenic acid, and folic acid for growth (Entani et al. 1986), all of which are

Table 6. Comparison of the microbial compositions of sample G-T3 obtained by Amplicon sequencing (PacBio) and by isolation on MRSPJ agar (cultivation).

Species	G-T3	
	PacBio	Cultivation
<i>Levilactobacillus brevis</i>	18.42	4.40
<i>Lentilactobacillus diolivorans</i>	17.22	13.30
<i>Lactobacillus acetotolerans</i>	16.07	6.70
<i>Leuconostoc mesenteroides</i>	10.50	–
<i>Lactiplantibacillus plantarum</i>	7.46	1.10
<i>Pediococcus parvulus</i>	7.43	4.40
<i>Pediococcus cellicola</i>	3.97	1.10
<i>Latilactobacillus sakei</i>	3.42	–
<i>Weissella hellenica</i>	3.03	–
<i>Lactobacillus helsingborgensis</i>	2.60	–
<i>Levilactobacillus parabrevis</i>	2.11	–
<i>Loigolactobacillus coryniformis</i>	1.45	–
<i>Companilactobacillus alimentarius</i>	1.41	–
<i>Leuconostoc kimchii</i>	1.06	–
<i>Companilactobacillus musae</i>	0.96	–
<i>Lentilactobacillus parabuchneri</i>	0.75	40.00
<i>Lentilactobacillus buchneri</i>	0.75	24.40
<i>Lentilactobacillus kefir</i>	–	4.40
Others	1.38	–

The abundance of identified OTUs is presented in percentage. Since all isolated species belonged to the Lactobacillales, the PacBio data is restricted to the members of that order to achieve a direct comparison of both methods. Bold numbers indicate main representatives. The species *Leuconostoc mesenteroides* is a main representative according to the PacBio data but was not isolated from the silage sample. The main representatives *Lentilactobacillus buchneri* and *Lentilactobacillus buchneri* according to the cultivation method are underrepresented in the PacBio data.

Table 7. Comparison of the microbial compositions of sample A-T2* obtained by Amplicon sequencing (PacBio) and by isolation on MRSPJ agar (cultivation).

Species	A-T2*	
	PacBio	Cultivation
<i>Leuconostoc mesenteroides</i>	72.79	40.00
<i>Weissella hellenica</i>	14.60	40.95
<i>Weissella cibaria</i>	4.86	14.29
<i>Weissella minor</i>	2.48	–
<i>Lactococcus lactis</i>	1.89	1.90
<i>Enterococcus mundtii</i>	0.88	–
<i>Latilactobacillus sakei</i>	0.81	–
<i>Companilactobacillus nuruki</i>	0.62	–
<i>Enterococcus casseliflavus</i>	0.56	–
<i>Pediococcus pentosaceus</i>	0.27	1.90
<i>Loigolactobacillus coryniformis</i>	0.14	–
<i>Lactobacillus</i> sp. TS3	0.11	–
<i>Leuconostoc pseudomesenteroides</i>	–	0.95

The abundance of identified OTUs is presented in percentage. Since all isolated species belonged to the Lactobacillales, the PacBio data is restricted to the members of that order to achieve a direct comparison of both methods. Bold numbers indicate main representatives.

contained in plants. Especially folic acid occurs in significant amounts in green leafy vegetables (Delchier et al. 2016) and cereal grasses (Qamar et al. 2018), supporting the hypothesis that supplementation with pressed plant juice allowed the cultivation of these additional species. The hypothesis outlined at the beginning that heterofermentative LAB in particular would profit from the addition of pressed juice, as it may provide them with the favored pentoses, could not be observed. Nevertheless, it is reasonable that pressed plant juice as a complex medium is rich in plant-specific nutrients supporting microorganisms with challenging cultivation conditions. The ex-

Table 8. Comparison of the microbial compositions of sample A-T3* obtained by Amplicon sequencing (PacBio) and by isolation on MRSPJ agar (cultivation).

Species	A-T3*	
	PacBio	Cultivation
<i>Leuconostoc mesenteroides</i>	43.04	20.91
<i>Levilactobacillus brevis</i>	16.63	18.18
<i>Loigolactobacillus coryniformis</i>	10.46	20.00
<i>Companilactobacillus nuruki</i>	9.62	1.82
<i>Weissella hellenica</i>	6.72	–
<i>Enterococcus mundtii</i>	4.23	–
<i>Pediococcus pentosaceus</i>	3.30	4.55
<i>Lactiplantibacillus plantarum</i>	2.94	9.09
<i>Latilactobacillus sakei</i>	1.48	–
<i>Weissella cibaria</i>	0.53	–
<i>Enterococcus casseliflavus</i>	0.45	–
<i>Lactococcus lactis</i>	0.24	–
<i>Pediococcus cellicola</i>	0.17	–
<i>Enterococcus durans</i>	–	1.82
<i>Lentilactobacillus buchneri</i>	–	5.45
<i>Pediococcus parvulus</i>	–	0.91
<i>Secundilactobacillus oryzae</i>	–	16.36
<i>Weissella paramesenteroides</i>	–	0.91
Others	0.19	–

The abundance of identified OTUs is presented in percentage. Since all isolated species belonged to the Lactobacillales, the PacBio data is restricted to the members of that order to achieve a direct comparison of both methods. Bold numbers indicate main representatives.

act composition cannot be predicted as it varies with different factors, such as crop, environmental growth conditions, and harvest time. In grass and alfalfa, the content of several nutrients, e.g. non-structural carbohydrates, fiber, and protein, differs according to the time of day and the year in which it is harvested (Pelletier et al. 2010, Testa et al. 2011).

Although in most cases the main microbiota could be isolated using microbial plating methods, this was not the case for *Leuc. mesenteroides* in G-T3. However, as it was possible to isolate this bacterium at an earlier stage of the same silage, it is conceivable that the high abundance of *Leuc. mesenteroides* in G-T3 according to the PacBio data derived from DNA of dead cells. This finding can be emphasized since the treatment with PMA shows changes in the ratio of the microbial composition, which is also supported by the work of Cao et al. (2021). It has been reported that only 24%–70% of cells in environmental samples can be considered as viable (Gasol et al. 1999, Yokomaku et al. 2000, Freese et al. 2006), and free DNA containing 16S rRNA genes deriving from dead cells may persist in the samples. During ensiling, the microbial composition varies drastically as a result of fundamental changes in the environmental conditions (transition from aerobic to anaerobic and pH drop) (Huang et al. 2021b, Du et al. 2022). This can lead to the accumulation of 16S rRNA genes from bacteria that are unable to survive these conditions, resulting in misleading amplicon sequencing data. Therefore, PMA treatment is essential to reflect microbial dynamics in the silage. As shown in Table 2, a contamination of the data derived from dead cells can lead to remarkably different results, giving a new picture of the silage microbiome. For instance, the untreated sample overestimated the abundance of *W. hellenica* almost 5-fold. Such overestimations in turn lead to an underestimation of the real members, such as *Pantoea vagans* (*Enterobacterales*), an undesirable bacterium in silage.

This gave the impression that the number of enterobacteria was decreasing more quickly than it actually was.

Moreover, certain species were neither detected in the silage samples nor in the mock communities. Nevertheless, these species were present in the samples, either because they had been isolated from them or because they had been included in the artificially created mock communities. In this study, OTU clustering was performed at a 97% identity level, which is a commonly used value. However, species are discriminated at a 16S rRNA gene similarity threshold of 98.7% (Kim et al. 2014), and closely related organisms may have even higher identities (Fox et al. 1992, Huang et al. 2021a). Based on a BLAST search using NCBI, it was found that the 16S sequence similarities between strains of the species *Leuc. citreum*, *Leuc. pseudomesenteroides*, and *Leuc. mesenteroides* were indeed higher than the 97% cut-off, as were those of *Lt. curvatus* and *Latilactobacillus sakei* (*Lt. sakei*). Therefore, these species were clustered in the same OTU and assigned as *Leuc. mesenteroides* and *Lt. sakei*, respectively. Nevertheless, this does not apply to *S. oryzae*, a main representative in sample A-T3* according to the cultivation method. It was recognized as non-existent in the PacBio data because its detected relative abundance was below the 0.07% threshold. Comparable deviations of the relative abundances from the expected results were observed in the case of *Ln. bucheri* and *Ln. parabuchneri* in G-T3 (Table 6). Such deviations could be due to inappropriate experimental factors, e.g. poorer DNA extractability compared to other species, insufficient primer specificity, and sub-optimal PCR conditions for the particular target sequence. The impact of different experimental factors on microbial community analyses using short-read sequencing methods has been investigated in several studies. While different DNA extraction methods can influence the results observed (Willner et al. 2012, Hong et al. 2024), the impact of PCR-related factors, such as primer specificity, seems to be more relevant (Fouhy et al. 2016). This is consistent with the findings of Baer et al. (2024), in which PCR had the greatest impact on variations of the microbial composition of PacBio amplicon sequencing data. Such factors may have led to an underrepresentation of the affected species in the cultivation-independent method. In addition, these organisms may favor the selected cultivation condition, which can lead to their overrepresentation in the cultivation-dependent approach. Of course, these influences can also have the opposite effect. All those experimental and bioinformatic factors mentioned above always influence the quantifiability of the microbiota, which is why both methods only give an estimation of the actual composition, but could not perfectly reflect the real situation. For microbiome analyses, it is therefore advisable to use both cultivation-dependent and -independent methods and to implement replicates for the latter. To reduce the effort of the cultivation-dependent method, isolates can be pre-grouped by morphological characteristics or by their fatty acid composition, as previously done by Weber et al. (2019). To promote isolation of representatives with slow growth rates, cultivation can be performed in a 96-well plate instead of agar plates, as described by Zhang et al. (2021).

In addition, an overestimation of OTUs was recorded in the mock communities, an observation that has been described before as contamination of reagents (Willner et al. 2012). Although relative abundance of these OTUs is low, microbial diversity is artificially inflated. It is therefore advisable to have mock communities as a control to assess the impact of ad-

ditional non-sample-associated and artificial OTUs. Although in this work the data was corrected by setting a threshold, the highest relative abundance of artificial OTUs that occurred was 0.31% for *Blautia obeum*, which is above the threshold. As a balance must be found between the exclusion of artificial OTUs and the inclusion of true members, low-abundance OTUs should be treated with caution.

Cultivation techniques as well as PacBio SMRT technology can both cover the main microbiota of silage samples, which is useful for isolate generation and microbiome analysis, respectively. Yet there are sources of error that can strongly influence the results of microbiome analyses (absence of species, detection of dead cells, overdetermination of OTUs). To approximate the real bacterial composition, methodological adjustments are advisable, comprising the bioinformatic analysis (e.g. OTU clustering cut-off), a PMA treatment, and the concomitant analysis of mock communities as a control for amplicon analyses. Furthermore, replicates of the amplicon sequencing and cultivation-dependent methods should be included in order to circumvent the insufficient quantitativity.

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Author contribution

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Data availability

The data underlying this article are available in the article.

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