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The bacterial and yeast microbiota in livestock forages in Hungary

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Abstract

Background Along bacteria, yeasts are common in forages and forage fermentations as spoilage microbes or as additives, yet few studies exist with species-level data on these fungi's occurrence in feedstuff. Active dry yeast and other yeast-based products are also common feed additives in animal husbandry. Here, we aimed to characterize both fermented and non-fermented milking cow feedstuff samples from Hungary to assess their microbial diversity in the first such study from Central Europe.

Results We applied long-read bacterial metabarcoding to 10 fermented and 25 non-fermented types of samples to assess bacterial communities and their characteristics, surveyed culturable mold and yeast abundance, and identified culturable yeast species. Fermented forages showed the abundance of Aerococcaceae, Bacillaceae, Brucellaceae, Lactobacillaceae, Staphylococcaceae, and Thermoactinomycetaceae, non-fermented ones had Cyanothecaceae, Enterobacteriaceae, Erwiniaceae, Gomontiellaceae, Oxalobacteraceae, Rhodobiaceae, Rickettsiaceae, and Staphylococcaceae. Abundances of bacterial families showed mostly weak correlation with yeast CFU numbers, only Microcoleaceae (positive) and Enterococcaceae and Alcaligenaceae (negative correlation) showed moderate correlation. We identified 14 yeast species, most commonly *Diutina rugosa*, *Pichia fermentans*, *P. kudriavzevii*, and *Wickerhahomyces anomalus*. We recorded *S. cerevisiae* isolates only from animal feed mixes with added active dry yeast, while the species was completely absent from fermented forages. The *S. cerevisiae* isolates showed high genetic uniformity.

Conclusion Our results show that both fermented and non-fermented forages harbor diverse bacterial microbiota, with higher alpha diversity in the latter. The bacterial microbiome had an overall weak correlation with yeast abundance, but yeasts were present in the majority of the samples, including four new records for forages as a habitat for yeasts. Yeasts in forages mostly represented common species including opportunistic pathogens, along with a single strain of *Saccharomyces* used as a feed mix additive.

Keywords *Saccharomyces*, Silage, Mycobiome, Yeast diversity

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Background

There is growing interest in the microbial composition of fermented forages as well as in yeast additives in animal husbandry. Silages are fermented, high-moisture feeds made from grains, grasses, and other green forages commonly used as a source of nutrients for ruminants [1, 2]. Silages are typically stored in airtight containers or silos reducing the risk of spoilage [2, 3], however, spoilage is relatively common upon opening, resulting in aerobic deterioration [3]. They may be inoculated with fermentation starter cultures and/or other additives or fermented solely by locally present microbes [4]. The term 'haylage' is in use for high dry matter silage made from hay, while fermented total mixed ration (FTMR) feeds are mixes of high-moisture by-products with dry feeds [5]. Non-fermented total mixed rations are also widely used (TMR) and both the fermented and non-fermented types have been shown to harbor diverse microbes. FTMRs promote a more diverse rumen microbiome and alter ruminal fermentation parameters [6].

The microbial composition of silage has mostly been studied using culture-based methods (mostly focusing on bacteria), and recently, by metagenomics and metabarcoding in a few studies. Dominant bacteria in the first phases of the ensiling process include species of Actinomycetales, Bacillales, Burkholderiales, Enterobacteriales, Lactobacillales, Pseudomonadales, Sphingomonadales, and Xanthomonadales [7, 8] and can be grouped metabolically into lactic acid bacteria (LAB), propionic acid bacteria (PAB), and others. LABs become dominant in later phases [8, 9]. Upon aerobic exposure, Bacillales and Xanthomonadales may become abundant once again, along with acetic acid bacteria (AAB), and fungi (yeasts and molds) [8–11].

Pathogenic spore-forming bacteria and *Listeria* [3, 9], along with various mold species such as mycotoxigenic *Aspergillus flavus* [12] and *A. fumigatus* [13], *Fusarium* spp., and *Penicillium* spp [10, 14]. have been reported as major hazard risks especially in poorly fermented silages.

Members of Saccharomycotina (ascomycetous yeasts) have been found to be the dominant fungi in silage after fermentation as well as after aerobic exposure, while they are usually present in very low abundance upon ensiling [8]. Yeasts, most importantly, but not exclusively lactate-assimilating yeasts (LAY) [15, 16], are often associated with aerobic deterioration. The main problems associated with yeasts in silages are elevating the pH (in the case of LAYs) [16], ethanol production [17], and volatile organic compound production [18]. We reviewed published literature on yeasts and yeast-like fungi occurring in silage and total mixed ration, and listed the recorded species in Table S1 using current taxonomy. Species of the genera *Candida*, *Kazachstania*, *Kluyveromyces*, *Pichia*, and *Saccharomyces* were most often recorded regardless of geographic setting. Merely 22 studies have assessed silage yeast species worldwide according to our literature review, focusing mostly on samples from Australia, Brazil, Canada, China, Israel, Italy, and USA, with no records so far from Central Europe, our current focus in this study. It is noted that recently, studies have also been carried out with the yeasts *S. cerevisiae* and *S. paradoxus* as silage starters [19], or with *Saccharomyces* and *Pichia* species exhibiting antagonistic effects to the growth of molds in silage [20–22].

Additionally, yeasts are not just found in fermented feedstuff but are also used in several yeast-based or compound direct-fed microbials (DFM), most commonly in the form of active dry yeasts (ADY) [19, 23–25] or in the form of inactivated yeasts [26]. Live yeast products may be considered animal probiotics if gut colonization is the supposed way of action, but the terminology is not necessarily consistent [24]. The so-called probiotic yeasts (*S. cerevisiae* var. '*boulardii*', *S. 'boulardii*') themselves are well-known and actively researched for human use, but their application in animal husbandry is also noteworthy among mammals [27], and other farmed animals as well. Furthermore, the use of yeast-based products (especially in the case of *S. cerevisiae*), e.g. yeast cell wall, yeast extracts, and *S. cerevisiae* fermentation products (SCFP) is widespread in animal feedstuff preparations [28].

The yeasts in silage and in animal forages are thus relatively minor, but important and under-researched spoilage microbes, while their use in feed concentrates and premixes is more pronounced and actively researched. In this study, our aim was to evaluate the bacterial and yeast microbiota of milking cow forages from Hungary, to extend knowledge on microbes occurring in silage and haylage fermentations in Central Europe, as well as on the microbiota of local non-fermented forages. We aimed to assess potential correlations between the bacterial and yeast microbiota, and to survey whether *Saccharomyces* occurs naturally or only as an additive in local forages. For bacteria, long-read metabarcoding was applied that captures a wide diversity and relative abundance of bacterial taxa. Yeasts were assessed with a conventional culture-based approach to circumvent methodological constraints associated with yeast identification in metabarcoding analyses [29] and to enable focus on viable and culturable yeast species that only represent a fraction of fungal cells and DNA in forages.

Methods

Silage and other forage samples

Feedstuff samples were collected from dairy companies in Hajdú-Bihar county, Eastern Hungary in 2020. Sampling into sterile velcro bags was performed from the various feedstuffs' uppermost layers (that were in use by the dairy farms at the time of sampling), with 10

parallel samples of ~500 g taken from of a single feedstuff sample. These were taken all form the upper layer, from equidistant portions, and were combined and transferred to our laboratory for homogenization as described by Adácsi et al. [30]. The samples were divided for total DNA isolation and for CFU determination and pre-culturing yeasts as described below. The companies were consulted about the origin and supplements included in the feedstuff. Samples, as detailed in Table 1, were listed into the following categories: 'corn', 'feed mix', 'hay', 'silage/haylage', and 'other'. Silages and haylages represented the 'fermented' feedstuff group, while the others were 'non-fermented' (fermentation was not involved in their production), except for a single fermented TMR sample falling in the 'feed mix' category.

Bacterial long-read 16 S metabarcoding of forage samples

Total DNA from the feedstuff samples was extracted by E.Z.N.A.® Soil DNA Kit and Macherey-Nagel (Düren, Germany) Genomic DNA From Soil kit following the manufacturers' protocols. 16 S metabarcoding was carried out using the 16 S long-read metabarcoding kit (SQK-16S024) of Oxford Nanopore Technologies (Oxford, UK) according to the manufacturer's instructions. In the first step of the library preparation a PCR was performed for the amplification of the 16 S rRNA gene target region (~1500 bp) and to add a unique barcode to each sample. DNA concentrations were quantified by Qubit™ fluorometer (Invitrogen, Waltham, MA). The initial DNA concentration was 10 ng per sample, the final PCR mix contained 25 µl LongAmp™ Hot Start Taq 2× Master Mix, 10 µl input DNA, 10 µl 16 S barcode primers (each) and 5 µl nuclease free water. The reaction was performed using a thermal cycler (Biometra TAdvanced, Analytik Jena, Jena, Germany) with the following PCR conditions: initial denaturation at 95 °C for 1 min, 25 cycles of 95 °C for 20 s, 55 °C for 30 s, 65 °C for 2 min, followed by a final extension step at 65 °C for 5 min. The amplicons were purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA) and eluted in 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. DNA concentration of the samples were quantified with a Nabi spectrophotometer (MicroDigital, Seongnam-si, South Korea). Approximately 100 fmol of combined library was loaded into an ONT SpotON flow cell. Super accurate basecalling and de-barcoding in the Guppy v6.4 software were performed after the sequencing, reads were identified to species level by using the software Emu [31] (default database of the software, a combination of rrnDB v5.6 and NCBI 16 S RefSeq from 17 September, 2020), along with abundance data. The pipeline was re-run for samples if necessary to reach at least 10 000 read counts. The sequencing files for metabarcoding are deposited under the BioProject number PRJNA992067.

Statistics and evaluation of 16 S metabarcoding data

Data processing, statistics, and the visualization of the results were carried out using the web-based platform MicrobiomeAnalyst [32]. First, a taxonomy file was created for the abundance data using Emu and species with unclear higher systematic status were manually edited to replace multiple identical "NA" entries shared by unrelated species with unique ones. Then MicrobiomeAnalyst was used to filter data (low count filter; minimum count 4 in in at least 50% of occurrences) and to apply total sum scaling and rarification of the data to the smallest library size. Stacked bar charts with abundance data were created with merging rare taxa (below 20 counts in rarified data). Alpha diversity was assessed using filtered data for families, using Chao1 diversity measure (total richness), with comparisons by Kruskal-Wallis test (as the alpha diversity values' distribution was not normal, Shapiro-Wilk test, $p<0.001$) with posthoc pairwise comparisons. Beta diversity was also assessed for families of Bacteria, with PCoA method, Bray-Curtis distance index, using pairwise PERMANOVA method. Clustering samples was performed for family data, with Bray-Curtis index and Ward clustering algorithm. For correlation analysis with yeast and mold CFU, the dairy feed concentrates containing added Saccharomyces active dry cells were excluded from the dataset, then the analysis was carried out on the level of families, with SparCC distance measure.

Yeast and mold colony forming unit (CFU) number determination

Samples from forages were taken with a sterile forceps, their weight measured, then samples were vigorously vortexed in 10 ml sterile water, decimal dilution series were prepared and spread to Dichloran Rose Bengal Chloramphenicol (DRBC) agar (VWR Chemicals, Solon, OH, USA), a standard selective medium used for enumeration of yeasts and molds in food and animal feeding stuffs. Plates were incubated at 25 °C and checked daily for mold and yeast colony numbers, then original CFU/g values were calculated based on dilution.

Yeast isolation and identification

Yeast colonies were isolated from CFU determination plates, or if no yeast was found, pre-culturing was applied before a repeated isolation attempt. For this, approximately 3–4 g of forage samples were placed in 250 ml sterile Erlenmeyer flasks in 100 ml of YPD (VWR Chemicals, pH 5.8) containing 0.01 mg/ml Chloramphenicol overnight with 180 rpm shaking at 28 °C. From the pre-cultures, 50 µL samples were plated onto two DRBC agar plates (VWR Chemicals) per sample and incubated until colonies of yeasts appeared (2–3 days at 30 °C). Colonies of each different morphotype from a single sample were subjected to one round of single-cell

Table 1 List of fermented and non-fermented forage samples analyzed in this work, along with composition, collection data, list of cultured yeast species, and yeast and mold CFU numbers

Group according to fermentation	Feedstuff group	Sample name	Collection date	Culturable yeast species	Yeast CFU/g	Mold CFU/g
Fermented	Silage-haylage	alfalfa_haylage_01	16.01.2020.	*Trichosporon aquatile*	< 1.5 × 10^01	1.5 × 10^01
Fermented	Silage-haylage	alfalfa_haylage_02	17.03.2021.	none	< 5 × 10^0	4.8 × 10^01
Fermented	Silage-haylage	alfalfa_haylage_03	05.05.2020.	*Kluyveromyces marxianus*	< 8 × 10^0	< 8 × 10^0
Fermented	Silage-haylage	corn_silage_01	24.02.2020.	*Kluyveromyces marxianus; Pichia fermentans; Pichia kudriavzevii*	< 5 × 10^0	< 5 × 10^01
Fermented	Silage-haylage	corn_silage_02	08.05.2020.	none	< 5 × 10^1	< 5 × 10^1
Fermented	Silage-haylage	corn_silage_03	10.07.2020.	*Pichia kudriavzevii*	1.09 × 10^02	< 5 × 10^1
Fermented	Silage-haylage	corn_silage_04	10.07.2020.	none	< 5 × 10^1	< 5 × 10^1
Fermented	Silage-haylage	rye_haylage_01	05.05.2020.	*Nakaseomyces glabratus*	< 1 × 10^1	< 1 × 10^1
Fermented	Silage-haylage	triticale_haylage_01	10.07.2020.	*Diutina rugosa*; *Pichia kudriavzevii*	< 1 × 10^1	< 1 × 10^1
Fermented	Feed mix	fermented total mixed ration = TMR_01	05.05.2020.	*Candida tropicalis*; *Pichia kudriavzevii*	2.05 × 10^03	2.71 × 10^03
Non-fermented	Hay	alfalfa_hay_01	05.05.2020.	*Nakaseomyces glabratus*; *Pichia fermentans*	3.86 × 10^03	4.07 × 10^03
Non-fermented	Hay	alfalfa_hay_02	08.05.2020.	*Diutina rugosa*	< 5 × 10^1	1.98 × 10^04
Non-fermented	Hay	alfalfa_hay_03	04.06.2020.	*Pichia fermentans*	2.15 × 10^04	2.92 × 10^03
Non-fermented	Hay	alfalfa_hay_04	10.07.2020.	*Pichia kudriavzevii*	6.00 × 10^02	1.60 × 10^03
Non-fermented	Hay	alfalfa_hay_05	10.07.2020.	*Pichia kudriavzevii*	6.25 × 10^02	3.70 × 10^03
Non-fermented	Hay	hay_01	05.05.2020.	*Diutina rugosa*; *Kluyveromyces marxianus*	1.00 × 10^03	< 1.00 × 10^03
Non-fermented	Hay	hay_02	13.08.2020.	*Trichosporon insectorum*	3.70 × 10^02	1.85 × 10^02
Non-fermented	Hay	sudangrass_01	08.05.2020.	*Diutina rugosa*; *Pichia kudriavzevii*	< 5 × 10^1	1.25 × 10^03
Non-fermented	Other	extracted sunflower meal = sunflower_01	04.06.2020.	*Meyerozyma carpophila*; *Pichia fermentans; Pichia kudriavzevii*	2.49 × 10^07	< 2 × 10^5
Non-fermented	Feed mix	feed for dairy cattle = dairy_feed_01	05.05.2020.	*Geotrichum candidum; Pichia kudriavzevii*; *Wickerhamomyces anomalus*	7.85 × 10^04	3.45 × 10^04
Non-fermented	Feed mix	feed for dairy cattle = dairy_feed_02	08.05.2020.	*Pichia kudriavzevii; Wickerhamomyces anomalus*	2.32 × 10^04	4.93 × 10^04
Non-fermented	Feed mix	feed for dairy cattle = dairy_feed_03	10.07.2020.	*Wickerhamomyces anomalus*	4.29 × 10^03	4.00 × 10^04
Non-fermented	Feed mix	feed for dairy cattle = dairy_feed_04	10.07.2020.	*Hyphopichia burtonii*; *Wickerhamomyces anomalus*	2.35 × 10^06	1.21 × 10^04
Non-fermented	Feed mix	feed for dairy cattle = dairy_feed_05	13.08.2020.	*Pichia kudriavzevii*	2.32 × 10^03	3.23 × 10^03
Non-fermented	Feed mix	feed concentrate for dairy cattle = dairy_concentrate_01	05.05.2020.	*Saccharomyces cerevisiae* (isolates FEEDY0001, FEEDY0002)	3.21 × 10^07	< 1 × 10^5
Non-fermented	Feed mix	feed concentrate for dairy cattle = dairy_concentrate_02	08.05.2020.	*Saccharomyces cerevisiae* (isolates FEEDY0003, FEEDY0004)	7.47 × 10^07	< 2 × 10^5
Non-fermented	Feed mix	feed concentrate for dairy cattle = dairy_concentrate_03	10.07.2020.	*Saccharomyces cerevisiae* (isolates FEEDY0005, FEEDY0006)	1.35 × 10^07	< 2 × 10^5
Non-fermented	Feed mix	feed concentrate for dairy cattle = dairy_concentrate_04	10.07.2020.	*Saccharomyces cerevisiae* (isolates FEEDY0007, FEEDY0008)	1.98 × 10^07	< 2 × 10^5
Non-fermented	Feed mix	minced corn kernels and rapeseed 50:50 mix = corn_rapeseed_01	04.06.2020.	*Pichia fermentans*	7.22 × 10^06	< 1 × 10^5
Non-fermented	Corn	corn kernels = corn_01	04.06.2020.	*Debaryomyces nepalensis*	7.64 × 10^03	6.37 × 10^02
Non-fermented	Corn	minced corn = corn_02	04.06.2020.	*Meyerozyma carpophila*; *Pichia fermentans*	1.97 × 10^04	1.52 × 10^04
Non-fermented	Corn	minced corn = corn_03	13.08.2020.	*Pichia fermentans*; *Wickerhamomyces anomalus*	4.44 × 10^05	3.51 × 10^04
Non-fermented	Corn	minced corn = corn_04	13.08.2020.	*Pichia kudriavzevii*	1.87 × 10^03	4.00 × 10^02
Non-fermented	Other	minced rapeseed = rapeseed_01	10.07.2020.	*Pichia kudriavzevii*	1.07 × 10^03	1.61 × 10^03

Table 1 (continued)

Group according to fermentation	Feedstuff group	Sample name	Collection date	Culturable yeast species	Yeast CFU/g	Mold CFU/g
Non-fermented	Other	soy_01	04.06.2020.	*Pichia fermentans*; *Pichia kudriavzevii*	1.59×10^07	<1×10^5

colony subculturing on YPD plates under the same conditions and saved as individual isolates into our collection at −70 °C in YPD+30% glycerol. Colony DNA for colony PCR tests was isolated according to Lõoke et al. [33] from the single-cell colonies and stored in 1×TE. These colony DNA samples were used for PCR amplification with the GoTaq Flexi Hot Start polymerase (Promega, Madison, WI, USA) of the variable region of the 26 S ribosomal large subunit of yeast with primers NL1 (GCATATCAA TAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTT CAAGACGG) [34]. The PCR products were subjected to capillary sequencing after PCR cleanup with the E.Z.N.A. cycle pure kit (Omega Bio-Tek, Norcross, GA, USA) with both primers by the sequencing core facility of the University of Debrecen. Sequenograms were manually checked and edited if needed, sequences were assembled from forward and reverse reads and the NCBI BLAST service was used for species identification, whereby the species with the closest hit in the NCBI GenBank was considered as a putative species identification, and then the sequence of the hit species' type strain was once again aligned to the query sequence using BLAST. A similarity of >99% with the type was considered a definitive species identification. Colonies were photographed after 10 days of incubation on YPD medium at 22 °C with a digital camera.

Genetic fingerprinting of *Saccharomyces* isolates

To assess whether *Saccharomyces* isolates represent different strains, we performed our recently developed interdelta and microsatellite fingerprinting multiplex PCR method using colony DNA. As described, we combined δ12–2, microsatellite (*YLR177w*, *YOR267c*), and as a control, ITS 1–4 primer pairs into a single PCR reaction [35, 36] and after gel electrophoresis (100 V, 2% agarose, 90 min) we compared the isolates to determine whether they are different, indicating the presence of genetically distinct strains. Furthermore, to survey karyotypes of *S. cerevisiae* isolates to amend fingerprinting, analysis was performed using 1% agarose gel (chromosomal grade, Bio-Rad, Hercules, CA, USA) by a counter-clamped homogenous electric field electrophoresis device (CHEF-Mapper; Bio-Rad). The following running parameters were used: run time 28 h, voltage 6 V/cm, angle 120°, temperature 14 °C and pulse parameters 60 to 120 s. As a control, the haploid *S. cerevisiae* 10–170 from the University of Debrecen, Department of Genetics and Applied Microbiology was used. After electrophoresis gels were stained with ethidium bromide and washed in sterile water for 48 h before photographing using UV-transillumination.

Results

Microbial communities in milking cow forages in Hungary

In this work, we recovered culturable yeasts from seven of the ten tested fermented forage samples, and from all of the 25 tested non-fermented feedstuffs (Figs. 1 and 2; Table 1). The yeasts isolates were saved to our collection and identified by sequencing the variable region of the large subunit of the rDNA (67 isolates altogether). GenBank accession numbers for Sanger sequencing results are listed in Table S2. Notably, *S. cerevisiae* was only recorded from dairy feed concentrates into which the manufacturer adds active dry yeast (ADY). Of the 13 other recorded species, *Diutina rugosa*, *Pichia fermentans*, *P. kudriavzevii*, and *Wickerhahomyces anomalus* were the most commonly recorded (each at least in four samples), and apart from the latter species, occurred both in fermented and non-fermented forages.

Yeast CFU/g values showed variation across the samples spanning many orders of magnitude. Non-fermented samples' yeast abundance ranged from $<5\times10^0$ to $\sim2–7\times10^7$, with highest yeast load in the extracted sunflower meal and soy samples, along with the dairy feed concentrates containing ADY. Apart from the former samples, only the corn rapeseed mix and the dairy_feed_04 sample had a CFU/g value exceeding 10^6. Fermented samples had very low yeast loads, all $<3\times10^3$ CFU/g. Mold CFU/g values showed markedly smaller variation and lower values: all non-fermented samples had $<5\times10^4$ molds per gram, fermented samples even had one order of magnitude smaller mold loads (Fig. 1; Table 1).

Bacterial community analysis as assessed by metabarcoding revealed the overall dominance of the phyla Bacillota, Pseudomonadota, and Cyanobacteria. On the level of Bacterial families, fermented forages showed the abundance of mostly Aerococcaceae, Bacillaceae, Brucellaceae, Lactobacillaceae, Staphylococcaceae, and Thermoactinomycetaceae (recorded from more than one sample with at least 10% abundance). Non-fermented forages harbored Cyanothecaceae, Enterobacteriaceae, Erwiniaceae, Gomontiellaceae, Oxalobacteraceae, Rhodobiaceae, Rickettsiaceae, and Staphylococcaceae most commonly (at

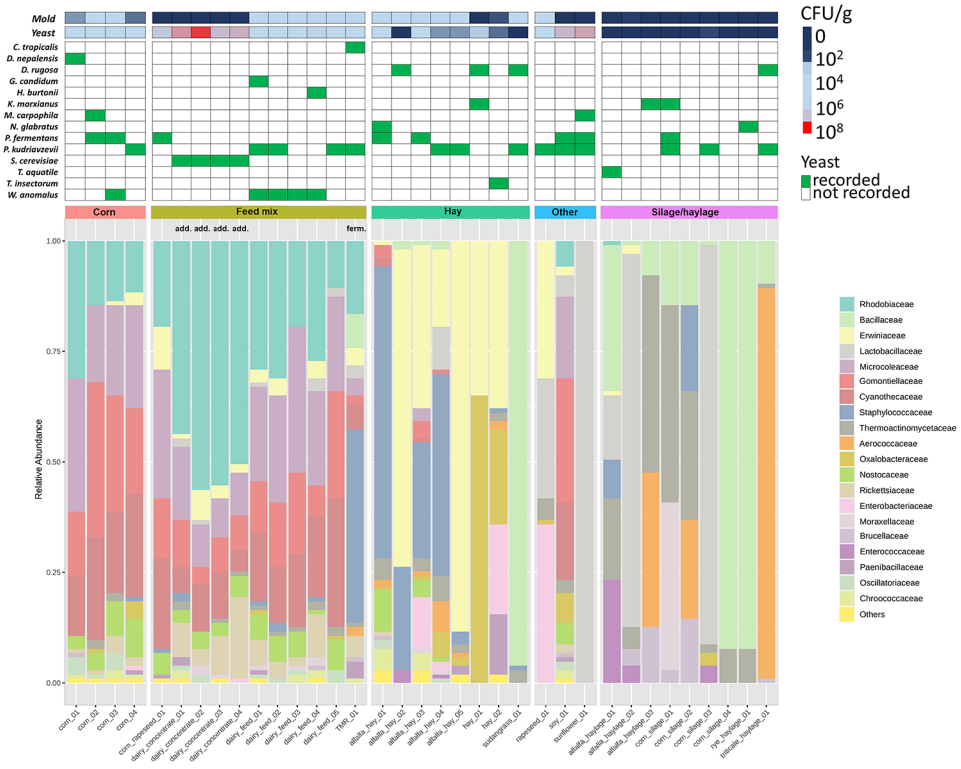

Fig. 1 Community composition for feedstuff samples. Major bacterial families and their relative abundances in feedstuff samples (each family is color-coded, very low-abundance taxa are excluded); on the top of stacked bar charts heat map shows mold and yeast CFU/g values, along with records of yeast species in table format. On the panels, dairy feed concentrates with added ADY are indicated ('add.'), and the single fermented sample in the feed mix group is also marked ('ferm.')

least 10% abundance in more than one sample) (Fig. 1). Bacterial family-level alpha diversity showed that the silage/haylage sample group had significantly lower diversity than corn or feed mix forages, and beta diversity differentiated between (silage/haylage and hay) and (corn, feed mix, and other) sample groups (Fig. 3). Family-level correlation analysis grouped most of the silage/haylage samples together with the sudangrass_01 sample, and in general, these fermented forages were more similar to hay samples than to corn or feed mix samples. The corn, corn-rapeseed, dairy feed mix and dairy feed concentrate samples formed a well-separated group in the correlation dendrogram (this group also contained the single soy sample), as shown on Fig. 1d. The dairy concentrates that contained *S. cerevisiae* were highly uniform in their bacterial composition (Figs. 1 and 3). The numerical data of bacterial abundance is uploaded to FigShare (https://doi.org/10.6084/m9.figshare.25285870.v1).

For testing correlations between yeast CFU numbers and bacterial community composition, the four samples containing ADY were excluded, since they contained added yeasts. Abundances of bacterial families showed mostly weak correlation with yeast CFU numbers, only Microcoleaceae (positive) and Enterococcaceae and Alcaligenaceae (negative correlation) abundances correlated with yeast CFU number with correlation coefficients exceeding 0.5 or −0.5, respectively (Fig. 4a). In the case of molds, correlations were weaker, none of the bacterial families correlated with mold CFU with coefficients > 0.5 or <– 0.5 (Fig. 4b).

Lack of genetic diversity among feedstuff *Saccharomyces* isolates

The species *S. cerevisiae* was recovered with high CFU numbers from four dairy concentrate samples and from none of the other sample types. These concentrates contained added active dry yeast and the occurrence of the species is thus artificial. The multiplex fingerprinting, targeting microsatellites and interdelta regions, did not differentiate between the eight *S. cerevisiae* isolates, named FEEDY0001–0008 (Table 1.) but resulted in a pattern clearly distinct from that of the probiotic yeast (Fig. 5a). The CHEF electrokaryotyping showed that no chromosomal length polymorphism was present among the isolates from four different feed samples (Fig. 5b) These results indicate that the same strain was present in the different samples and the isolates are genetically highly uniform.

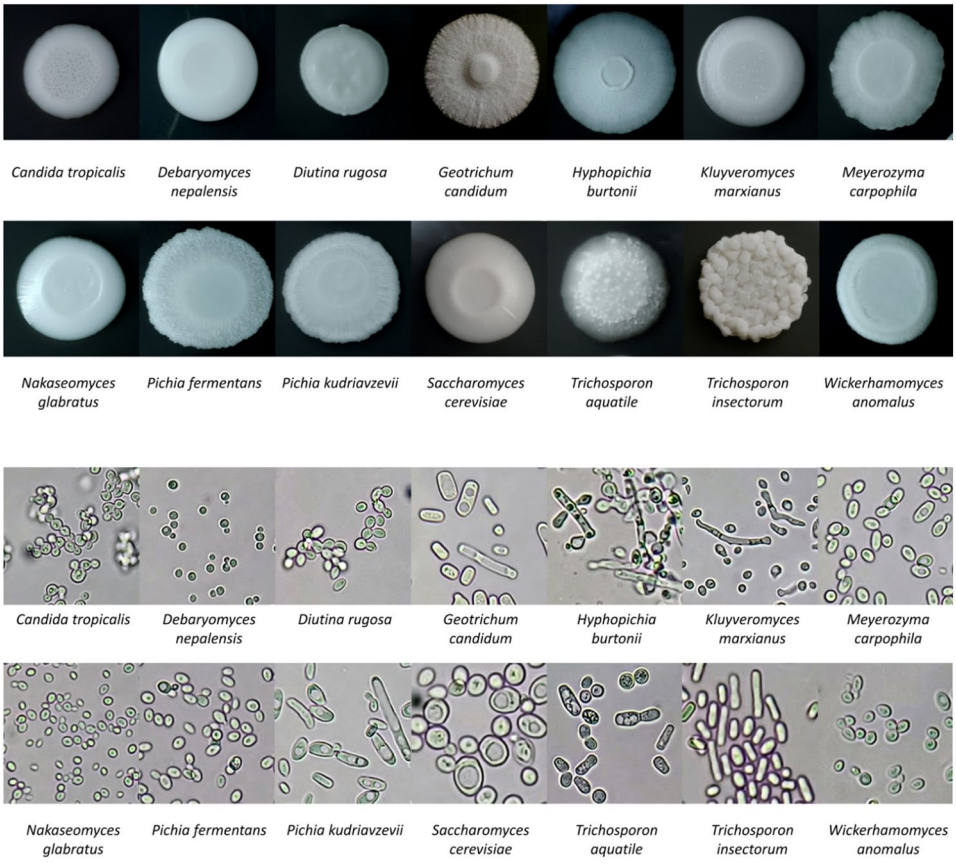

Fig. 2 Macro- (on top) and microphotographs (bottom) of representative strains of recorded yeast species on YPD medium after 10 days of growth. Images are not to scale

Discussion

In this study, we compared 10 fermented and 25 non-fermented milking cow feed samples from Hungary for their bacterial diversity with culture-independent Bacterial metabarcoding analysis and by comparing culturable yeasts, along with yeast and mold CFU loads in the samples. DNA-metabarcoding of bacteria has the advantage of capturing the widest taxonomic diversity including unculturable or difficult-to-culture bacteria, while at the same time unable to discard reads originating from dead cells and cell-free DNA in the samples. In the case of yeasts, our preliminary metabarcoding results with fungal ITS metabarcoding resulted in the over-abundance of plant saprophytic taxa that were highly likely to represent fungi originating from the plant material in feedstuff (data not shown in the text, but example of corn_silage_01 uploaded to FigShare, https://doi.org/10.6084/m9.figshare.26780077), and we obtained insufficient data on yeasts. As our focus in this work was the latter sub-group of fungi, we resorted to culture-based approaches, allowing us to only discuss species that were found to be in a viable and culturable form in the forages.

To our knowledge, this is the first study to report on yeast from silage samples from Central Europe and the second study to compare multiple feed samples from the European continent (following a study from Italy, as reviewed in Table S1). Our present work is also one of the first studies to report culturable yeasts from non-fermented milking cow forages. In general, our results showed that bacterial community composition correlated with feedstuff type (e.g. Figures 1 and 3), and yeasts and molds were minor or very minor contaminants apart from a few samples harboring more than a million live yeast cells per gram sample (Table 1; Fig. 1).

Bacterial communities consisted mostly of Bacillota, Pseudomonadota, and Cyanobacteria, the latter group may have been mostly represented in the form of feed additives or contaminants from soil and due to their photosynthetic nature, were likely no more viable or metabolically active in the forages. Fermented forages were dominated by Lactobacillaceae in only two cases, and in general, silages, haylages but also hay samples were not uniform in their bacterial microflora: individual samples often differed in the most abundant families of bacteria. Non-fermented corn forages and feed mixes harbored

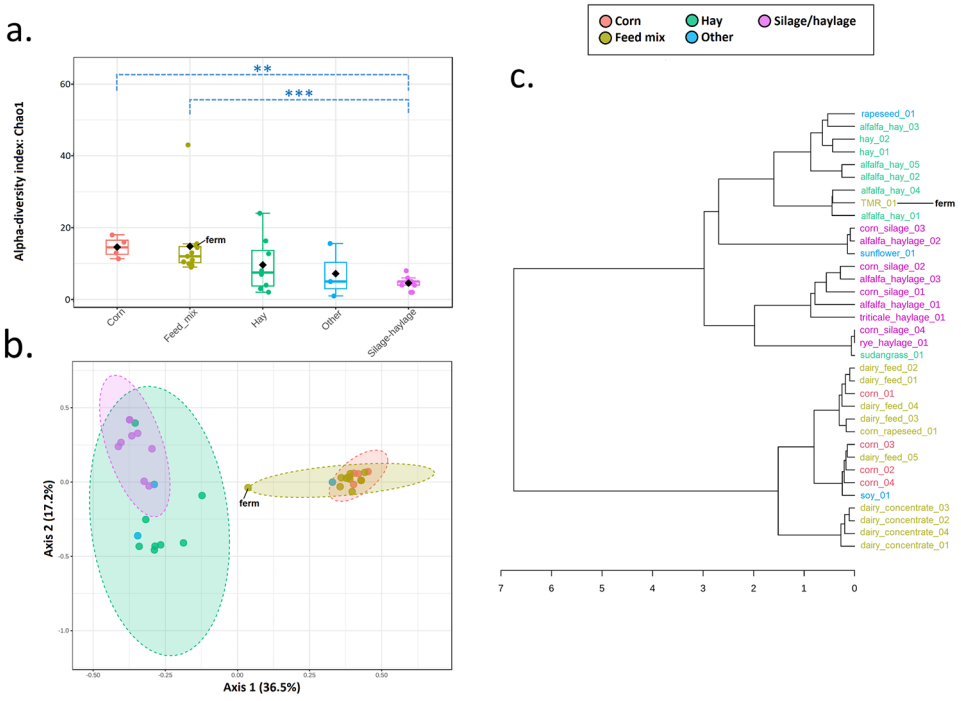

Fig. 3 Bacterial community comparisons of the samples. **a**: bacterial alpha-diversity on the level of families in the various sample groups (color-coded boxplots show alpha-diversity for all samples in a sample group). Significant differences among sample groups are indicated with blue asterisks (**: $p < 0.01$; ***: $p < 0.001$). **b**: beta diversity of bacterial families across sample groups in the form of Principal Coordinates Analysis, axes 1 and 2 shown, sample groups are color-coded. **c**: clustering analysis of all samples based on the abundance and occurrence of bacterial families, sample groups are color-coded. The single fermented sample in the feed mix group is also marked ('ferm')

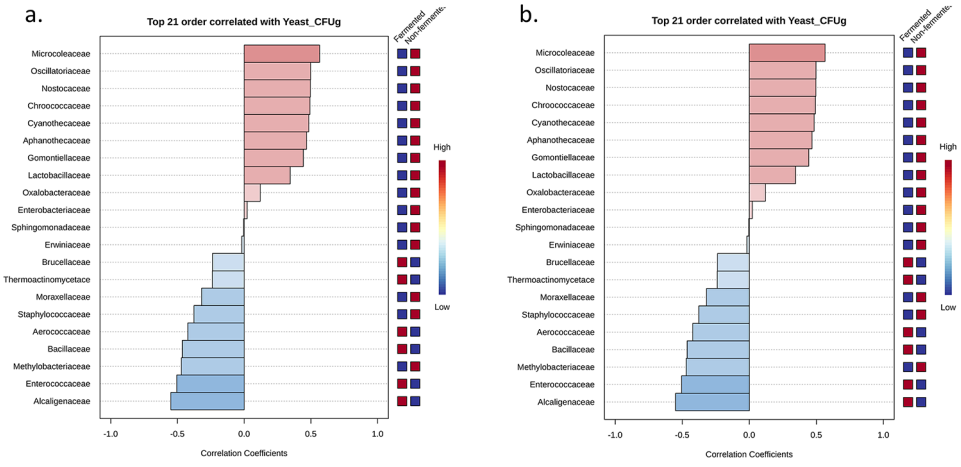

Fig. 4 Correlations of bacterial family abundance data and fungal colony forming unit (CFU) numbers in feed samples. Most correlated families shown, with correlation coefficients on x axis. The right panel shows feed types' heat map indicating relative abundances of the orders on the y axis in each sample type. **a**: Correlations of yeast CFU numbers. Samples with added active dry yeast excluded. **b**: Correlations of mold CFU numbers

a larger alpha diversity of bacterial families but differed less among samples in the same group (Figs. 1 and 3). The dairy concentrates that contained ADY *S. cerevisiae* were highly uniform in their bacterial microflora.

In fermented feedstuff (silage and haylage), we recorded *P. kudriavzevii* as the most common yeast, in line with trends observable worldwide (Table S1), and we report the occurrence of *T. aquatile* for the first time from silage worldwide. From fermented forages, we recorded eight species from ten samples, and three samples harbored no detectable yeasts. Among the non-fermented feedstuff samples, we recorded 15 species, with all 25 tested samples harboring at least one yeast species, although often with very low CFU numbers (Table 1). The most common species in such non-fermented feeds were *D. rugosa*, *P. kudriavzevii*, *P. fermentans*, *S. cerevisiae*, and

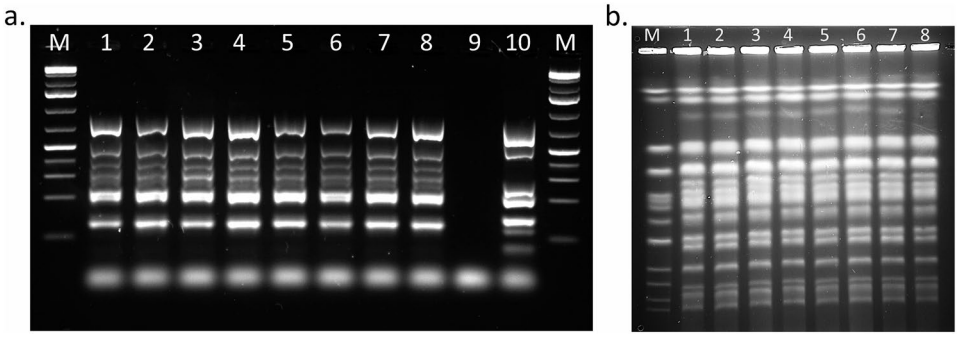

Fig. 5 Molecular genetic comparison of the cultured *S. cerevisiae* isolates from feedstuff. **a**: Multiplex PCR fingerprinting, the isolates are compared to the human and animal probiotic yeast *S.* var.'*boulardii*'. M: 1 kb Plus size marker. 1: FEEDY0001. 2: FEEDY0002. 3: FEEDY0003. 4: FEEDY0004. 5: FEEDY0005. 6: FEEDY0006. 7: FEEDY0007. 8: FEEDY0008. 9: negative control. 10: probiotic yeast strain PY0001. **b**: CHEF karyotyping of the isolates. M: *S. cerevisiae* haploid strain 10–170. 1: FEEDY0001. 2: FEEDY0002. 3: FEEDY0003. 4: FEEDY0004. 5: FEEDY0005. 6: FEEDY0006. 7: FEEDY0007. 8: FEEDY0008. Note the uniformity of band patterns for all Feed Yeast *Saccharomyces* samples

W. anomalus. The following species from non-fermented samples have not been recorded from forages according to our knowledge before: *D. nepalensis*, *M. carpophila*, *T. insectorum*. Notably, *S. cerevisiae*, was only found in dairy feed concentrates that contained artificially added active dry yeast as an ingredient. Although the species has been recorded from natural silage fermentations in Brazil, China, and the USA (Table S1), the presence of the species was thus not natural in the case of Hungarian samples. The isolates recovered by us were highly uniform genetically, by all probability representing the same strain used by manufacturers as live yeast additives. Although several manufacturers supplement their products with the *S.* var. '*boulardii*', our yeast samples were clearly different from the probiotic yeast (Fig. 5).

Our results showed that besides feed products that have active yeasts as a probiotic or gut microbiome modulating component, many other forages may contain live yeasts of various genera that can consequently enter the gastrointestinal tract in viable form. Whether these yeast species can colonize the GIT and modulate the local microbiome and animal health is a question beyond the scope of this study. However, several species recorded here have been isolated from the rumen of cows in earlier studies, *e.g. C. tropicalis* or *P. kudriavzevii* [37]. The occurrence of such yeasts in the GIT of cattle can plausibly linked to their presence in fermented forages (see Table S1), but according to our results, non-fermented feed might also serve as a means of colonization (Table 1). It must also be noted that several species recorded from forages in our study represent potential pathogens of ruminants and other mammals, e.g. the above-mentioned species [38], as well as *D. rugosa* [39]. It is also noteworthy that in a single study from a confined geographical region, we recorded around a quarter of all known ascomycetous and basidiomycetous yeast species known from forages worldwide in this study.

Conclusions

In this work, we reported on the bacterial microbiota and on culturable yeasts in milking cow forages for the first time form Central Europe. Bacterial microbiotas were dominated by three phyla and mostly week correlations were found with yeast or mold abundance. The species identified show that yeasts, although often not highly abundant in the samples, can be quite diverse and represent many species (approximately a quarter of all yeasts ever recorded from feedstuff were present in the samples) even in a confined geographic area. However, the most well-known yeast species, *S. cerevisiae* did not occur naturally in the tested fermented or non-fermented forages, but only as a single strain of yeast supplement across multiple total mixed rations. Recording and comparing *S. cerevisiae* samples from multiple regions from various feed supplements and probiotics may in the future reveal how diverse the baker's yeasts applied in animal husbandry are. Although bacteria are the most important players in the fermentation of forages, our results call attention to the lesser-known yeasts in these feedstuffs, and we also show that non-fermented feeds may harbor diverse species that includes ones known to be able to colonize and/or infect livestock.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Author contributions

Conceptualization, W.P.P, I.P.; methodology, W.P.P., K.M., B.N., H.V.R., A.I., E.H., Zs.A., T.P., B.B.; formal analysis, K.M., B.N., H.V.R., E.H., K.N.O.P., F.P., P.S., T.P.; resources, B.B., F.P., P.S., T.P.; data curation, K.M., E.H., W.P.P.; writing—original draft preparation, W.P.P., K.M.; writing—review and editing, W.P.P., I.P, T.P.; visualization, K.M., W.P.P.; supervision, W.P.P., I.P.; project administration, I.P.; funding acquisition, I.P., W.P.P. All authors reviewed the manuscript.

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Data availability
The datasets generated during the current study are available in the FigShare repository (10.6084/m9.figshare.25285870.v1: metabarcoding data) and in NCBI GenBank (OR250063 – OR250141: Sanger sequences).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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