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The Change in Microbial Diversity and Mycotoxins Concentration in Corn Silage after Addition of Silage Additives

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Abstract: In our study the presence of bacteria, yeast, and microscopic fungi was evaluated. Three forms of corn silage were made including silage without additive, silage with microbial additive (lactic acid bacteria), and silage with nutritional additive (urea). Silage additives were applied to the matter within the recommended dosage, then the matter was ensiled into plastic bags and stored at a constant temperature. After 5.5 months of storage, average samples for microbial and mycotoxins analysis were taken. From microbiological points, the plate count agar method for enumeration of total count of bacteria, lactic acid bacteria, enterococci, yeasts, and microscopic fungi and mass spectrometry for microbiota identification were used. In total, 43 species of bacteria and yeasts and 6 genera of microscopic fungi were identified from all samples of corn silages. The most isolated species were *Lentilactobacillus buchneri* and *Kazachstania exigua* from bacteria resp. yeasts and *Aspergillus* and *Penicillium* from microscopic fungi. Mycotoxins were determined by HPLC-MS/MS and divided into two groups as regulated and emerging. In the corn silages only *Fusarium* mycotoxins were observed. All corn silages, regardless of the addition of the additive, were the highest in nivalenol content. Deoxynivalenol and beauvericin with the highest concentrations were present in silage with urea. Although the mycotoxins content of the variants changed, these changes were not statistically significant. In general, addition of lactic acid bacteria *Lentilactobacillus buchneri* and *Lacticaseibacillus casei* and urea as silage additives affect the microbial diversity; however, the hygienic quality of whole crop corn silage was not negatively changed.

Keywords: diversity; microbiota; mycotoxin; microbial additive; urea; corn silage

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1. Introduction

The silage microbiota contains beneficial microorganisms such as lactic acid bacteria (LAB) and spoilage microorganisms such as mold and yeast [1]. Therefore, the abundance and species of epiphytic bacteria in harvested matter (before silaging) are crucial for the spontaneous fermentation process and microbial succession in silage production [2]. The members of the epiphytic microbiota and their variations are a critical factor in determining whether LAB inoculation was necessary for silage production [3].

The presence of mycotoxins has been confirmed worldwide in a variety of forages of plant origin, and several mycotoxins may be present in a single forage [4]. As discussed by Juan et al. [5], mycotoxins pose a serious threat to feed safety due to their negative impact

on consumer's health. Additionally, due to climate change, which could affect the degree of contamination of feedstuffs, concerns about feed safety and health are increasing. The potential of using preharvest models to predict risk from deoxynivalenol (DON) in wheat, fumonisin B1 in maize, and aflatoxins in maize in different continents are considered in the context of potential for adaptation to include climate-change scenarios. In addition, changes in post-weather conditions may lead to the development and growth of molds that have not been observed in the area and may become more important as warmer climatic conditions would be conducive to *Penicillium* and *Aspergillus*, respectively [6].

Dzuman et al. [7] argue that feed can be contaminated with a wide range of mycotoxins and in addition to the classical regulated mycotoxins such as aflatoxins, deoxynivalenol, ochratoxins, fumonisins, and zearalenone, these can also be emerging mycotoxins (enniatins, beauvericin, and moniliformin). Numerous analogues of enniatin, including types A, A1, B, B1, B2, B3, B4, D, E, F, and G, are known, according to Santini et al. [8]. Of these modified toxins, enniatins A, A1, B, and B1 are the most found in Europe, specifically in cereals [9], silages, and inoculated corn [10]. The maximum levels for these mycotoxins are currently not regulated by any legislation [11], as they are considered to be of minor importance in terms of their concentration in feed and food, as well as their toxic effect on humans and animals [12]. Although no limits have yet been set for enniatins, for other mycotoxins of the genus *Fusarium* such as deoxynivalenol (DON), T-2 toxinHT-2 toxin, fumonisins (FUM), and zearalenone (ZEA), the concentrations are regulated by the authorities [13].

Since the formation and development of mycotoxins is influenced by many factors (temperature, water activity, pH value, fungal strain), researchers are trying to figure out effective ways to improve the hygienic parameters of silages [14]. Generally, the most used are biological silage additives (inoculants), which in most cases have been able to reduce the mycotoxin content, but in some cases also to increase it [15,16]. Fabiszewska et al. [2] mentioned that mycotoxins such as deoxynivalenol, fumonisins, fusarium toxins, zearalenone, and ochratoxins can be eliminated by using strains of lactic acid bacteria. Additionally, mycotoxins content can be reduced by adding chemical additives [17], and some nutrients such as urea [16,17]. Moreover, during the silage fermentation process, urea is partially degraded to ammonia (acts as a buffer–alkaline environment), which has an antifungal effect [18,19]. Urea as a silage additive is mainly used for forages with carbohydrate character, is effective at inhibiting growth of molds and yeasts [20,21]. Furthermore, the addition of urea affects the nutritional value (by increasing NH_3-N, crude protein), but also the fermentation quality of silages (by boosting acetic, propionic and butyric acid, and by slowing pH decline) with decreasing nutrient losses and improved aerobic stability of silages [22].

Another option to decrease yeast and mold populations in silages is addition of facultatively and obligately heterofermentative bacteria, which are produced in addition to lactic acid, also acetic acid [18]. Inoculation by *L. plantarum* and *L. buchneri* would mitigate potential negative effects arising from fungal infestation by production of main fermentation compounds (acetic, propionic, and lactic acid) and bacteriocins [23]. Moreover, many species of bacteria and some specific fungi have been shown to enzymatically degrade mycotoxins, which are potentially promising candidates used to detoxify mycotoxins in feed and foods [24].

The hypothesis is regarding which types of silage additives are more effective against molds and mycotoxins formation and beneficial for silage microbiota during the fermentation process in whole crop corn silage. The aim of this study was to (a) determine the presence of microbiota and concentration of different mycotoxins in corn silage and (b) to determine the effect of silage additives on mycotoxin content for the improvement in silage hygienic quality and production of safe feeds for animals with the sustainable production of quality food for the human population

2. Materials and Methods

2.1. Ensilage of Corn Matter

In cooperation with the University Farm in Oponice, whole corn matter (hybrid FAO 480, dent grain type) was ensiled. The corn matter with a dry matter content of 39% at the milky-wax stage of the grain was harvested (harvested after 140 days after the sowing) with a self-propelled forage harvester (CLAAS, Omaha, Nebrasca, USA) and chopped to 2 cm of chopped length. The corn matter was ensiled in 3 variants and 3 repetitions: control (CONT /CONT1,2,3/, ALAB (additive on the base of lactic acid bacteria)/ALAB1,2,3/ and NAUR (nutritional additive: urea)/NAUR1,2,3/. The commercial additive in water-soluble powder on the base of lactic acid bacteria (*Lentilactobacillus buchneri* LN40177: obligately heterofermentative, *Lacticaseibacillus casei* LC32909: facultatively heterofermentative; min. lactic acid bacteria 1.1×10^{11} CFU/g) was applied using an applicator (Appli Pro Super Low Volume-Pioneer) directly on the forage harvester in a liquid state (10 mL/t) at a dose of 1 g/t of ensilaged matter. In the NAUR variant, a nutritional additive, urea, was applied to the untreated matter at a dose of 5000 g/t of ensilaged matter (applied manually in a solid state and subsequently homogenized by mixing with the matter). In the CONT control variant, the matter was ensiled without the addition of additives. The corn matter in all variants was ensiled into plastic bags (in one bag approx. 1.2 kg of ensilaged matter) in 3 repetitions using a vacuum packing machine MSW Motor Technics (Expendo Polska, Zielona Góra, Poland) and subsequently stored in the laboratory at a constant temperature (22 $\pm$ 2 °C). The plastic bags were opened, and average samples (n = 3) were taken for microbiological analysis after 5.5 months of storage. After pre-drying (at 60 °C), the dry matter content of the gravimetric method was determined at 103 $\pm$ 2 °C in corn silages. Laboratory samples of corn silages (n = 3) were subjected to mycotoxin analysis.

2.2. Microbiological Analyses

In the primary dilution of silage, 0.87% sterile saline with the quantity of 45 mL was used, to which 5 g of sample was added. Subsequently, serial dilutions (10^{-2} to 10^{-4}) were prepared, and 100 µL of them were applied to Tryptic Soya agar plates (TSA, Sigma-Aldrich®, St. Louis, USA) to determine the total number of bacteria. The presence of bacterial colonies was examined in the inoculated plates after the incubation period of 48–72 h at 30 °C.

Typical colonies of coliform bacteria were enumerated after 24–48 h (37 °C) of incubation on inoculated McConkey agar (MC, Sigma-Aldrich®, St. Louis, MO, USA) plates. Formation of typical colonies for enterococci was examined with the use of Enterococcus selective agar (ESA, Sigma-Aldrich®, St. Louis, MO, USA), whereas incubation time and temperature were the same as for coliform bacteria. Lactic acid bacteria were cultivated with the use of three different agars, specifically MRS (De Man, Rogosa and Sharpe agar), MSE (Mayeux, Sandine and Elliker), and APT (All Purpose TWEEN® agar, Sigma-Aldrich®, St. Louis, MO, USA). Inoculated plates were incubated under the anaerobic conditions for 72 h at 37 °C. For microscopic fungi and yeast identification, malt extract agar (MEA, Sigma-Aldrich®, St. Louis, MO, USA) and acid base indicator bromocresol green (Sigma-Aldrich®, St. Louis, MO, USA) (0.020 g/L) were used. The growth on inoculated plates was evaluated after 5 days of aerobic exposure and an incubation temperature of 25 °C. Because of the macroscopic morphological differences between the growing colonies, recultivation on TSA (Tryptic Soya agar, Oxoid, Basingstoke, UK) was completed. The cultivation of inoculated plates took place for 24 h at 30 or 25 °C for bacteria and yeasts, respectively. After the cultivation, the protein extraction was undertaken. One colony of each bacterial isolate was transferred into an Eppendorf tube and mixed with 300 µL of sterile water. After addition of ethanol (900 µL), the suspension was mixed and centrifuged (13,000× *g*, 2 min). After removal of supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial pellets were resuspended in 20–50 µL of formic acid (70%) and the same amount of acetonitrile. After centrifugation (2 min at 13,000× *g*), 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room

temperature. Then, 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany) and processed automatically after measurement. The logarithm of the score (log/score) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) $\geq$ 1.7 indicated identification at the genus level, log(score) $\geq$ 2.0 was set as the threshold for a match at the species level. Isolates with $\geq$2.0 were accepted as a correct identification. Further confirmation of microorganisms (the colonies from total microbial count, coliform bacteria, enterococci, lactic acid bacteria, fungi, and yeasts) was performed using MALDI-TOF MS Biotyper. Identification of selected colonies was examined after aerobic or anaerobic subculture on TSA agar overnight. The preparation of microbial isolates for MALDI-TOF MS analysis was previously published by Kačániová et al. [25] and realized according to the manufacturer´s extraction procedure (Bruker Daltonik, Bremen, Germany). Additionally, Singh et al. [26] published identification for fungal isolates. Identification was completed using MALDI-TOF MS Biotyper (Bruker Daltonics, Bremen, Germany) with Flex Control 3.4 software and Biotyper Realtime Classification 3.1 with BC specific software (Bruker Daltonics, Bremen, Germany).

2.3. Mycotoxin Analysis

Multimycotoxin analysis by HPLC-MS/MS (AT-SOP 31) based on EN 17280 in cooperation with the Romer Labs Diagnostic GmbH Austria was used for mycotoxin analysis. Samples (10 g) for mycotoxin analysis were extracted using 30 mL of extraction solution (mixture of acetonitrile/water 7:3). The sample was then centrifugated. The aliquot amount was diluted with eluent A of HPLC by factor 10. Before dilution, an internal standard of most mycotoxins was added. The diluted sample was injected to an HPLC system (Agilent -Agilent Technologies, Santa Clara, California, USA - 1260 infinity II binary pump with integrated degasser, column oven, and multisampler) with a column (Phenomenex Gemini C18/4.6.0 × 150 mm; 5 µm/). Data were acquired using LC-MS/MS at two mass transitions by using a C18 column and gradient program for eluents. Used eluents were: eluent A: 88.5% water, 10% MeOH, 1% acetic acid, 0.5% 1 m ammonium acetate; eluent B: 1.5% water, 97% MeOH, 1% acetic acid, 0.5% 1 m ammonium acetate. The method of internal standard for quantification was used. The calibration curve was used as the dependency of the concentration of analyte to ratio of the relevant analyte and the corresponding internal standard. The calibration was completed via external solvent calibration standards. The internal standards were 13C isotopic labeled standards, in which all 12C carbon atoms were replaced by 13C carbon. The internal standards were added to the injection sample in the autosampler prior to analysis. Identification followed SANTE 12089/2016 [27] (guidance document on identification of mycotoxins in food and feed) involving retention time and two product ions. In each batch, two internal control samples were running; results of the internal controls are recorded in a control chart.

The mycotoxin content was determined in the dry matter of the laboratory samples and subsequently converted to 88% dry matter. The mycotoxin content is presented in µg/kg. The determined mycotoxin profile is shown in Table 1.

Table 1. Mycotoxin groups and analytes analyzed by HPLC-MS/MS.

Mycotoxin Group	Analyte
Aflatoxins	Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2
Alternaria Metabolites	Alternariol
Aspergillus Metabolites	Gliotoxin, Sterigmatocystin
A-Trichothecene	Diacetoxyscirpenol, HT-2 Toxin, Neosolaniol, T-2 Toxin, T-2 Triol, T-2-Tetraol
B-Trichothecene	Deoxynivalenol, Deoxynivalenol-3-Glucoside, 15Acetoxyscirpenol, 15AcetylDeoxynivalenol, 3AcetylDeoxynivalenol, Nivalenol
Enniatins and Beauvericin	Beauvericin, Enniatin A, Enniatin A1, Enniatin B, Enniatin B1
Ergot Alkaloids	Agroclavine, alpha-Ergocryptine, alpha-Ergocryptinine, Dihydrolysergol, Elymoclavine, Ergine, Ergocornine, Ergocorninine, Ergocristine, Ergocristinine, Ergometrine, Ergometrinine, Ergosine, Ergotamine
Fumonisins	Fumonisin B1, Fumonisin B2, Fumonisin B3
Fusarium Metabolites	Fusarenon X, Moniliformin
Ochratoxins	Ochratoxin A, Ochratoxin B
Penicillium Metabolites	Mycophenolic Acid, Patulin, Penicillic Acid, Roquefortine C
Zearalenone + Metabolites	alpha-Zearalenol, beta-Zearalenol, Zearalanone

2.4. Statistical Evaluation of Results

The results were statistically evaluated using IBM SPSS 26.0 (Armonk, New York, NY, USA). The description statistics and differences between the variables were compared using a one-way ANOVA (Tukey test, $p < 0.05$). The correlation relationships between the molds and yeasts and determined mycotoxins were calculated using the Pearson correlation coefficient (r). The coefficient of determination was recalculated as the powered Pearson correlation coefficient (r^2).

3. Results and Discussion

3.1. Microbiota of Corn Silage

In the control samples, lactic acid bacteria ranged from 2.69 log cfu/g on APT to 5.11 log cfu/g on MRS, the numbers of coliform bacteria and enterococci were under the detection limit, the total number of microorganisms was 3.54 ± 0.43 log cfu/g and microscopic filamentous fungi was 2.73 ± 0.33 log cfu/g. In the silage with the additive on the base of lactic acid bacteria, lactic acid bacteria ranged from 2.83 log cfu/g on MSE to 5.05 log cfu/g on MRS, the numbers of coliform bacteria and enterococci were under the detection limit, total counts of microorganisms ranged from 3.23 to 3.51 log cfu/g and microscopic filamentous fungi ranged from 2.13 to 2.21 log cfu/g. In the silage with the nutritional addition, urea, lactic acid bacteria ranged from 4.14 to 4.21 log cfu/g, numbera of coliform bacteria and enterococci were under the detection limit, total number of microorganisms was 3.26 ± 0.22 log cfu/g, and microscopic filamentous fungi was 3.38 ± 0.45 log cfu/g (Table 2). Generally, the fermentation quality is largely influenced by the characteristic of the raw material and the epiphytic microorganisms on its surface [28]. Final feed quality is largely influenced by the species and numbers of dominant microorganisms in the fermentation process [29].

Table 2. Number of isolated groups of microorganisms in log cfu/g.

	MSE	MRS	APT	MC	EA	TSA	MEA
CONT1	ND	5.11	ND	ND	ND	3.89	2.84
CONT2	ND	5.08	ND	ND	ND	3.21	2.58
CONT3	ND	4.22	2.69	ND	ND	3.52	2.78
Σ CONT	ND	4.81 ± 0.48^{a}	2.69 ± 0.36^{a}	ND	ND	3.54 ± 0.43	2.73 ± 0.33^{a}
ALAB1	ND	5.05	3.21	ND	ND	3.46	2.21
ALAB2	ND	4.11	3.32	ND	ND	3.23	2.13
ALAB3	2.83	4.12	3.12	ND	ND	3.51	2.15
Σ ALAB	/	$4.43 \pm 0.49^{a,b}$	3.22 ± 0.21^{b}	ND	ND	3.40 ± 0.20	2.16 ± 0.16^{b}
NAUR1	ND	4.21	ND	ND	ND	3.34	3.83
NAUR2	ND	4.15	ND	ND	ND	3.25	3.41
NAUR3	ND	4.14	ND	ND	ND	3.18	2.89
Σ NAUR	ND	4.17 ± 0.13^{b}	ND	ND	ND	3.26 ± 0.22	3.38 ± 0.45^{c}
Total	/	4.47 ± 0.47	3.09 ± 0.34	/	/	3.40 ± 0.31	2.76 ± 0.60
p value	/	0.010	0.010	/	/	0.157	0.000

MSE—Mayeux, Sandine and Elliker; MRS—De Man, Rogosa and Sharpe agar; APT—All Purpose TWEEN® agar; MC—McConkey agar; ESA—Enterococcus selective agar; TSA—Tryptic Soya agar; MEA—malt extract agar; ND—not detected; *p* value—expresses the effect of the additives on microorganism groups; superscripts in the indexes indicate the significance of the results between the groups ($p < 0.05$); CONT—control (subsamples CONT1, CONT2, CONT3); ALAB—additive on the base of lactic acid bacteria (subsamples ALAB1, ALAB2, ALAB3); NAUR—nutritional additive: urea (subsamples NAUR1, NAUR2, NAUR3); Total—total mean regardless of additive application.

In our study, a total of 43 species of bacteria and yeast were identified (Table 3). The 17 families included Bacillaceae, Burkholderiaceae, Clostridiaceae, Cryptococcaceae, Enterobacteriaceae, Lachnospiraceae, Lactobacillaceae, Moraxellaceae, Micrococaceae, Paenibacillaceae, Promicromonosporaceae, Pseudomonadaceae, Rhizobiaceae, Saccharomycetaceae, Shewanellaceae, Sphingomonadaceae, and Staphylococcaceae, and 25 genera included *Acinetobacter, Arthrobacter, Alkalihalobacillus, Priestia, Bacillus, Blastomonas, Cellulosimicrobium, Citrobacter, Clostridium, Lacrimispora, Cryptococcus, Kazachstania, Lactobacillus, Levilactobacillus, Lentilactobacillus, Companilactobacillus, Secundilactobacillus, Lacticaseibacillus, Paenibacillus, Pseudomonas, Ralstonia, Rhizobium, Shewanella, Sphingomonas*, and *Staphylococcus* that were isolated from all samples of corn silage (Table 4). The most isolated family from all corn silage was Lactobacillaceae. The most isolated species was *Lentilactobacillus buchneri* (17%) (Figure 1). Bacteria, yeast, and fungi with detoxification abilities were isolated from different sources, and LAB are the preferred candidates for eliminating mycotoxins in silages because they play a critical role in the ensiling fermentation. *Lactiplantibacillus plantarum* and *Lentilactobacillus buchneri* are known to enhance lactic acid (LA) fermentation and acetic acid (ACA) production to improve fermentation quality and inhibit aerobic spoilage [23].

Table 3. The number of isolated species of bacteria and yeasts from corn silage.

Species of Bacteria/Sample	CONT			ALAB			NAUR			Total
	1	2	3	1	2	3	1	2	3	
Acinetobacter radioresistens			3							3
Arthrobacter crystallopoietes	4									4
Alkalihalobacillus gibsonii				2	3					5
Priestia megaterium				3	4	4				11
Bacillus pumilus				4			5			9
Bacillus safensis	3		3						3	9
Bacillus spp.	2					3			4	9
Blastomonas natatoria			2							2
Cellulosimicrobium cellulans	3									3
Citrobacter freundii		4	5							9
Clostridium cadaveris	2	3								5
Lacrimispora sphenoides							3			3
Cryptococcus neoformans	3									3
Kazachstania exigua	7	5	6	4	5	6		5		38
Kazachstania humilis	5						4		6	15
Lactobacillus amylovorus									5	5
Levilactobacillus brevis	5	5								10
Lentilactobacillus buchneri	5	6	8	10	9	6	9	10	7	70
Lactobacillus delbrueckii subsp. *delbruecki*			5							5
Lactobacillus delbrueckii subsp. *bulgaricus*			6							6
Companilactobacillus farciminis			3	4			5	3	6	21
Lactobacillus kalixensis			4							4
Lactobacillus kefiri	3						4		5	12
Secundilactobacillus malefermentas				5						5
Lentilactobacillus parabuchneri	5							5		10
Lacticaseibacillus paracasei subsp. *paracasei*				5	8	10	7	8		38
Lactiplantibacillus pentosus	4									4
Lactiplantibacillus plantarum									6	6
Lactobacillus sakei			5			4				9
Paenibacillus amylolyticus	5	4	3	4	6	3				25
Paenibacillus illinoisensis			5							5
Pseudomonas agarici				3					3	6
Pseudomonas lutea						3				3
Pseudomonas putida						4				4
Ralstonia pickettii	5	3	2			4				14
Rhizobium radiobacter						2				2
Shewanella fidelis	2									2
Sphingomonas parapaucimobilis							2	3		5
Sphingomonas wittichii			3							3
Sphingomonas yabuuchiae							3			3
Sphingomonas yabuuchiae		3								3
Staphylococcus epidermidis						5				5
Staphylococcus lugdunensis				3						3
Total isolates	63	33	63	47	35	54	42	34	45	416

CONT—control; ALAB—additive on the base of lactic acid bacteria; NAUR—nutritional additive: urea.

Table 4. Isolated species, genera, and families from corn silage samples.

Species	Genera	Family	%
Acinetobacter radioresistens	*Acinetobacter*	Moraxellaceae	0.7
Arthrobacter crystallopoietes	*Arthrobacter*	Micrococcaceae	1.0
Alkalihalobacillus gibsonii	*Alkalihalobacillus*	Bacillaceae	1.2
Priestia megaterium	*Priestia*	Bacillaceae	2.7
Bacillus pumilus	*Bacillus*	Bacillaceae	2.2
Bacillus safensis	*Bacillus*	Bacillaceae	2.2
Bacillus spp.	*Bacillus*	Bacillaceae	2.2
Blastomonas natatoria	*Blastomonas*	Sphingomonadaceae	0.4
Cellulosimicrobium cellulans	*Cellulosimicrobium*	Promicromonosporaceae	0.7
Citrobacter freundii	*Citrobacter*	Enterobacteriaceae	2.2
Clostridium cadaveris	*Clostridium*	Clostridiaceae	1.2
Lacrimispora sphenoides	*Lacrimispora*	Lachnospiraceae	0.7
Cryptococcus neoformans	*Cryptococcus*	Cryptococcaceae	0.7
Kazachstania exigua	*Kazachstania*	Saccharomycetaceae	10.0
Kazachstania humilis	*Kazachstania*	Saccharomycetaceae	3.7
Lactobacillus amylovorus	*Lactobacillus*	Lactobacillaceae	1.2
Levilactobacillus brevis	*Levilactobacillus*	Lactobacillaceae	2.4
Lentilactobacillus buchneri	*Lentilactobacillus*	Lactobacillaceae	17.0
Lactobacillus delbrueckii subsp. *delbruecki*	*Lactobacillus*	Lactobacillaceae	1.2
Lactobacillus delbrueckii subsp. *bulgaricus*	*Lactobacillus*	Lactobacillaceae	0.4
Companilactobacillus farciminis	*Companilactobacillus*	Lactobacillaceae	5.1
Lactobacillus kalixensis	*Lactobacillus*	Lactobacillaceae	1.0
Lactobacillus kefiri	*Lactobacillus*	Lactobacillaceae	2.9
Secundilactobacillus malefermentas	*Secundilactobacillus*	Lactobacillaceae	1.2
Lentilactobacillus parabuchneri	*Lentilactobacillus*	Lactobacillaceae	2.4
Lacticaseibacillus paracasei subsp. *paracasei*	*Lacticaseibacillus*	Lactobacillaceae	9.1
Lactiplantibacillus pentosus	*Lactiplantibacillus*	Lactobacillaceae	1.0
Lactiplantibacillus plantarum	*Lactiplantibacillus*	Lactobacillaceae	1.4
Lactobacillus sakei	*Lactobacillus*	Lactobacillaceae	2.2
Paenibacillus amylolyticus	*Paenibacillus*	Paenibacillaceae	6.0
Paenibacillus illinoisensis	*Paenibacillus*	Paenibacillaceae	1.2
Pseudomonas agarici	*Pseudomonas*	Pseudomonadaceae	1.4
Pseudomonas lutea	*Pseudomonas*	Pseudomonadaceae	0.7
Pseudomonas putida	*Pseudomonas*	Pseudomonadaceae	1.0
Ralstonia pickettii	*Ralstonia*	Burkholderiaceae	3.4
Rhizobium radiobacter	*Rhizobium*	Rhizobiaceae	0.4
Shewanella fidelis	*Shewanella*	Shewanellaceae	0.4
Sphingomonas parapaucimobilis	*Sphingomonas*	Sphingomonadaceae	1.2
Sphingomonas wittichii	*Sphingomonas*	Sphingomonadaceae	0.7
Sphingomonas yabuuchiae	*Sphingomonas*	Sphingomonadaceae	0.7
Sphingomonas yabuuchiae	*Sphingomonas*	Sphingomonadaceae	0.7
Staphylococcus epidermidis	*Staphylococcus*	Staphylococcaceae	1.2
Staphylococcus lugdunensis	*Staphylococcus*	Staphylococcaceae	0.7

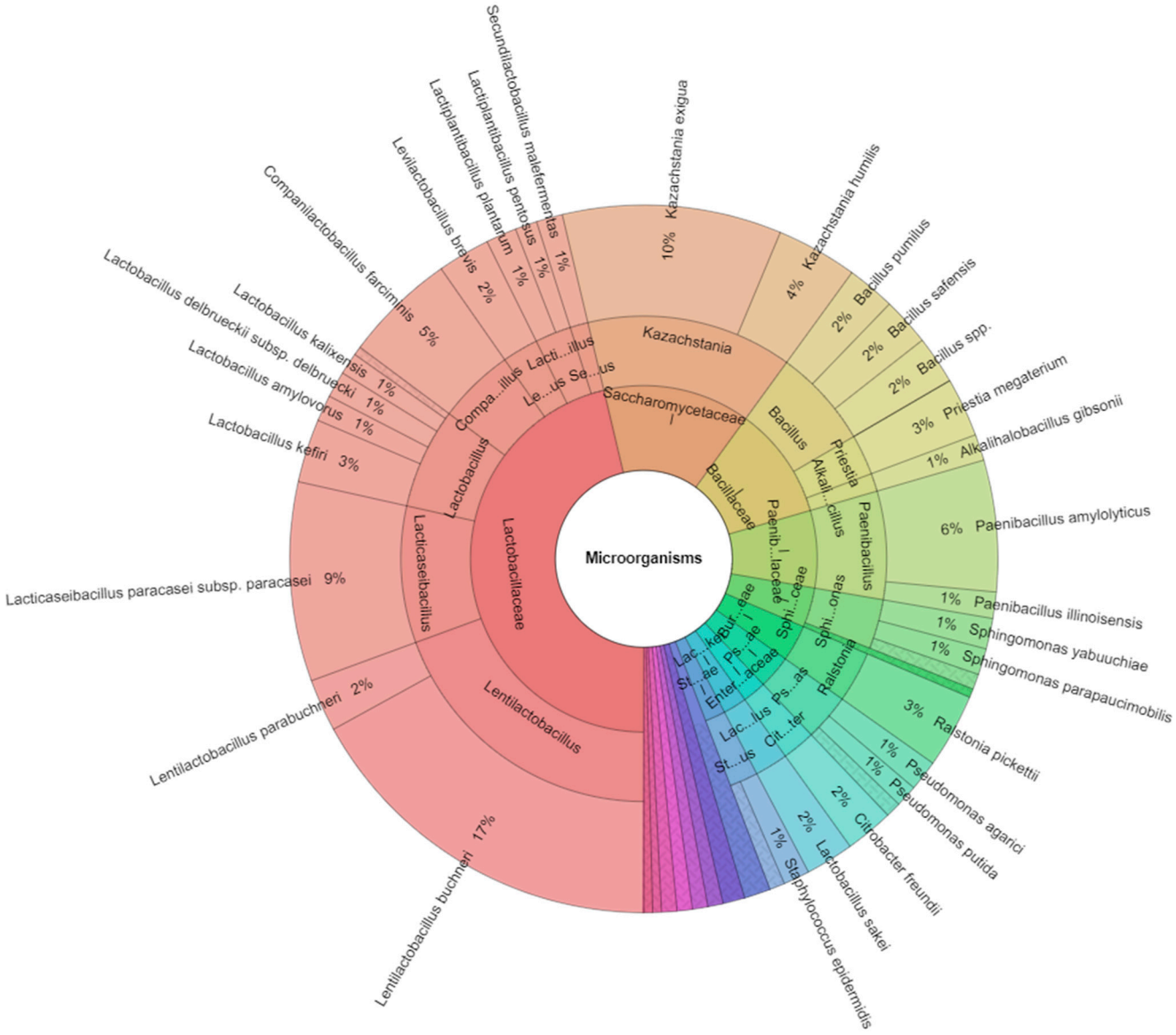

Figure 1. Krona chart of isolated species of bacteria and yeasts from all samples of corn silage.

In the study of Wang et al. [30] they examined the effects of lactic acid bacteria (LAB) *Lactiplantibacillus plantarum* subsp. ZA3 and *Artemisia argyi* (AA) on the fermentation characteristics, microbial community, and mycotoxins. The results showed that corn silage has microbial communities, *Acetobacter* and *Enterobacter*, which were inhibited in all AA groups, while a higher abundance of lactobacilli was maintained; moreover, *Candida*, *Pichia,* and *Kazachstania* abundances were decreased in both groups. In our study, different results were found, the most isolated species were from the family Lactobacillaceae and yeast *Kazachastania exigua* (10%). Our results did not confirm previous research. We can assume that the increased number of LAB increases the production of lactic acid, which affects the growth of yeast. The relatively lower pH values in inoculated silages, combining activities of acidification and antagonistic activity towards other bacteria, promotes the reduction in bacterial and fungi diversities, and ultimately improves feed quality. This observation indicated that the fungi community can change when the environment changes from anaerobic to aerobic. It is possible that acid-tolerant bacteria still dominate the bacterial community in the early period of aerobic exposure, and the variation in microbial community.

In our study, microscopic fungi in each group of samples were isolated and they were of the genera *Alternaria*, *Aspergillus*, *Fusarium*, *Mucor*, *Rhizopus,* and *Penicillium.* The

most isolated genera 25% resp. 25% in control samples were *Aspergillus* and *Penicillium*. Similar results were found in samples treated with the addition of LAB with an incidence of *Aspergillus* of 12.5% and *Penicillium* of 18%, and in samples with nutritional additive urea with an incidence of *Aspergillus* of 15% and *Penicillium* of 20%.

In the study of Krustev and Khristov [31], eight species of microscopic fungi were demonstrated in the sampled corn silage: *Mucor*, *Penicillium*, *Aspergillus*, *Alternaria*, and *Trichoderma*, similar as in our study. It was established that the number of species in the surface layer of the ensilaged mass was the highest.

Isolation of novel LAB strains for application in silage has been a common practice over the years but it is still an activity with current importance around the globe [32–34], due to the interest in collecting diverse strains for future applications not only as silage inoculants but also in other plant-based food for animal and human uses [35].

Lentilactobacillus buchneri is presently the gold standard to promote aerobic stability in corn silage [36,37]. Acetic acid is one of the main organic acids produced by heterofermentative LAB and it has the capacity of promoting aerobic stability when silos are opened [38].

All isolated species in control samples were 27 species and the most isolated species was from the family Lactobacillaceae (9 species, Figure 2). The most isolated species from control samples were *Lentilactobacillus buchneri* (12%) and *Kazachstania exigua* (11%), following with *Paenibacillus amylolyticus* (8%), *Citrobacter freundii*, and *Ralstonia picketii* (6%). *L. buchneri* Ls141 and 463 were used as external reference strains. *L. buchneri* Ls141 had been isolated from corn silage in a previous study [39]. *Candida ethanolica*, *Saccharomyces bulderi*, *Pichia anomala*, *Kazachstania unispora*, and *Saccharomyces cerevisiae* were the predominant yeasts. *Pichia anomala*, *Issatchenkia orientalis*, *S. cerevisiae*, and *Pichia fermentans* were the prevalent species in high moisture corn [36].

All isolated species in corn samples with the addition of LAB were 19 species and the most isolated species were from the families Lactibacillaceae (5 species) and Bacillaceae (4 species, Figure 3). The most isolated species from corn samples with the addition of LAB were *Lentilactobacillus buchneri* (18%) and *Lacticaseibacillus paracasei* subsp. *paracasei* (16%), following with *Kazachstania exiqua* (10%), *Paenibacillus amylolyticus* (9%), and *Priestia megaterium* (8%). Driehuis et al. [40] observed that strains of *Lentilactobacillus buchneri* (*Lactobacillus buchneri*) [41] were able to degrade lactic acid into acetic acid and 1,2-propanediol [42], which could then be metabolized into propionic acid [43]. Since both acetate and propionate are strong yeast inhibitors [44,45], these modifications positively improve the aerobic stability of silage. More recently, co-inoculation with *L. buchneri* NCIMB 40788 and *Lentilactobacillus hilgardii* CNCM-I-4785 (*Lactobacillus hilgardii*) was reported to increase the stability of different silages [46,47]. While microbial dynamics during fermentation were recently characterized in corn silage inoculated with these two microorganisms [48], little research has been undertaken to characterize microbial succession and mycotoxin production in inoculated vs. uninoculated silages during the feed-out phase [49,50]. *Kazachstania exiqua* was isolated from corn silage in the study of Santos et al. [36].

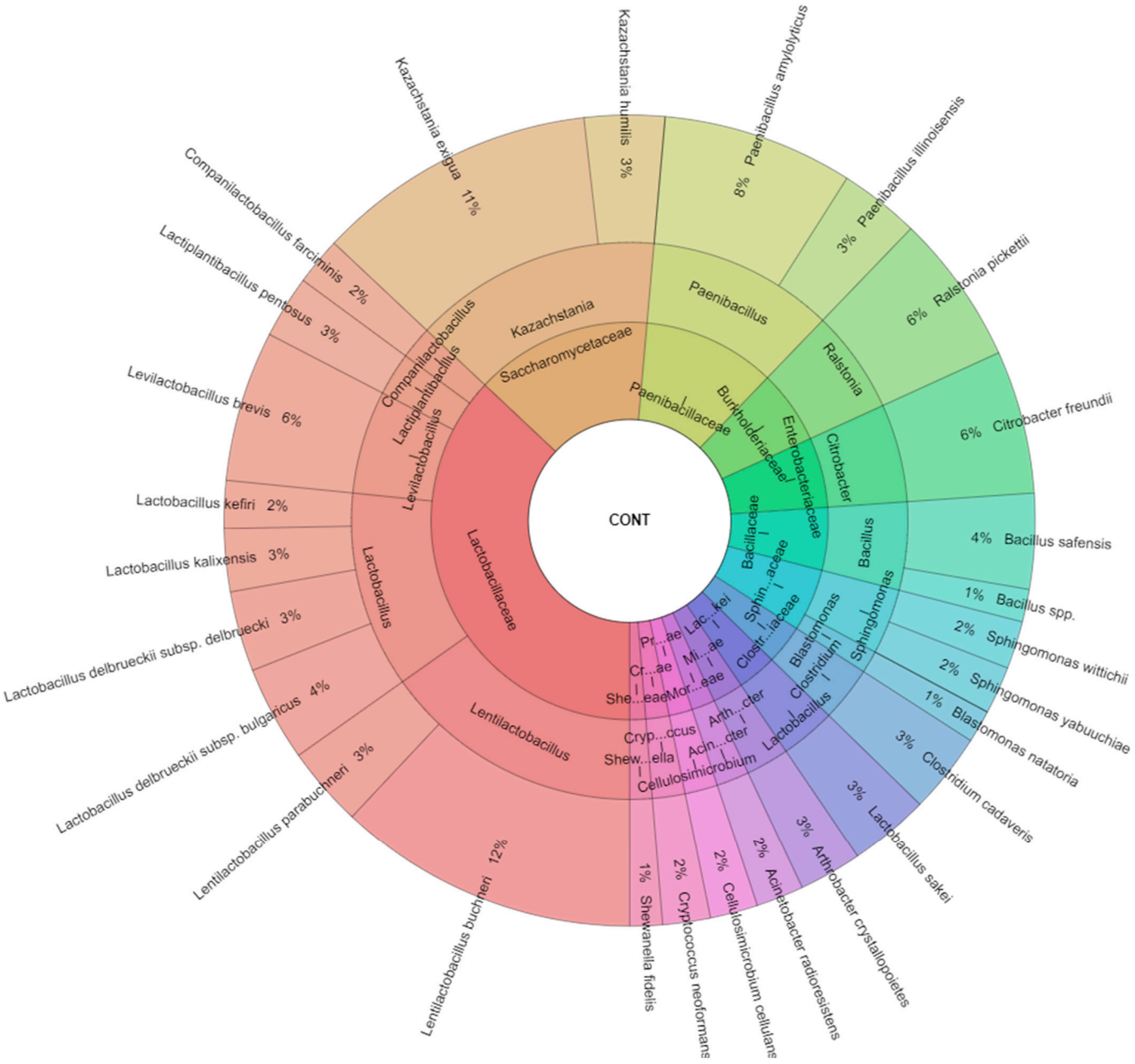

Figure 2. Krona chart of isolated species of bacteria and yeasts from control samples of corn silage.

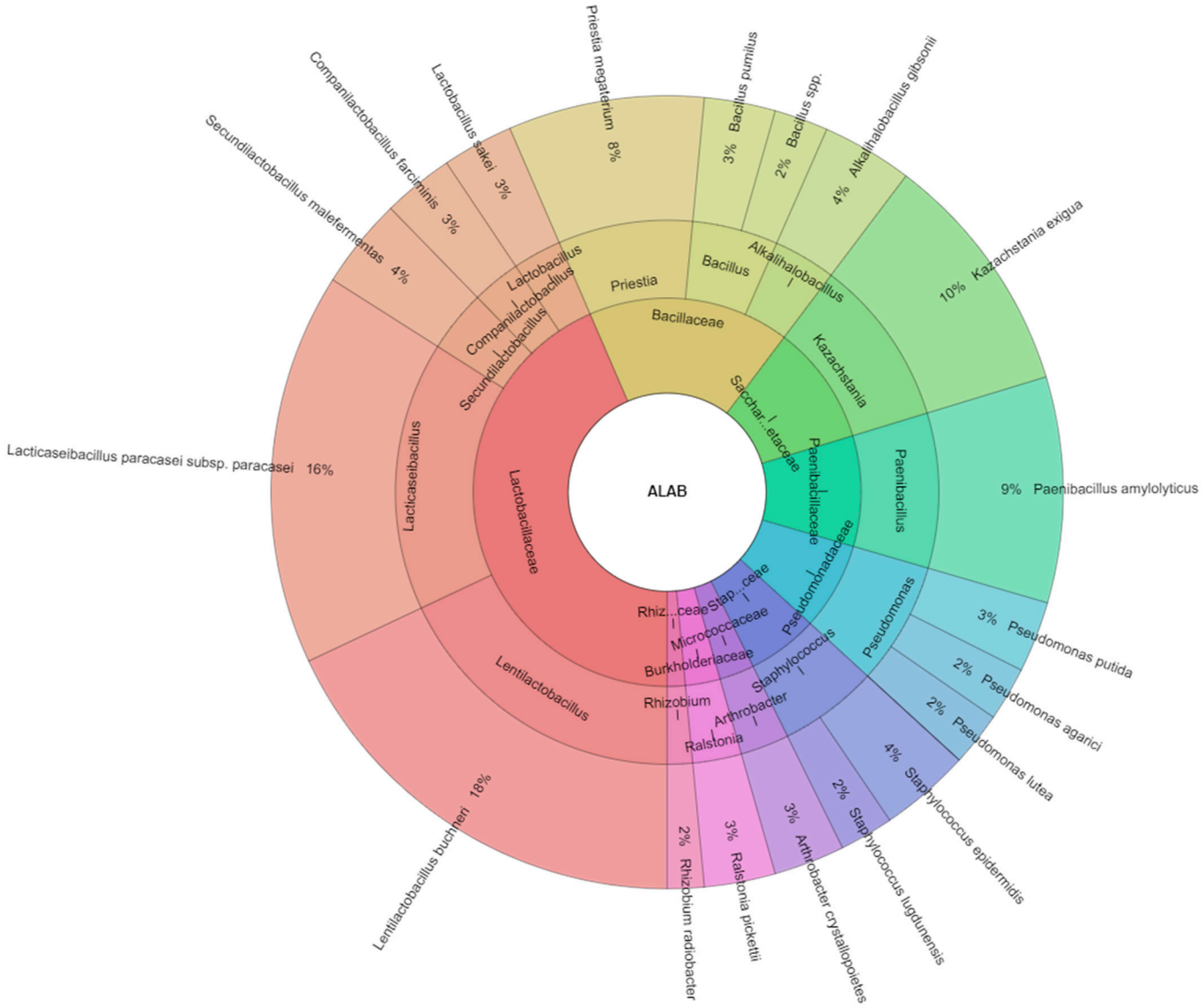

Figure 3. Krona chart of isolated species of bacteria and yeasts from samples of corn silage with additive on the base of lactic acid bacteria.

Ruminal microorganisms are capable of transforming nitrogen from NPN compounds into protein of high nutritional value. However, if the release of ammonia promoted by NPN exceeds the use capacity by ruminal microbiota, there will be an excretion of this excess with a consequent loss of energy. If the ammonia concentration extrapolates the excretion capacity, the intoxication of the animal may occur [51]. Nitrogen from NPN compounds can be converted by ruminal microbes into protein with a high nutritional value [52]. However, there will be the expulsion of this surplus and a resulting loss of energy if the release of ammonia encouraged by NPN exceeds the capacity for utilization by ruminal bacteria [53].

All isolated species from samples with nutritional additive urea were 16 species and most isolated species were from the Lactibacillaceae family similar to 7 species (Figure 4). The most isolated species from the treated samples with nutritional additive urea were *Lentilactobacillus buchneri* (22%) and *Lacticaseibacillus paracasei* subsp. *paracasei* (12%), following with *Companilactobacillus farciminis* (11%) and *Lactobacillus kefiri* (8%). Pang et al. [54] reported that most of the bacterial community in silage belonged to the phylum Firmicutes and the genera *Lactobacillus, Pedicoccus*, and *Weissella*. These results showed that the

dominant phyla in the measured samples were Proteobacteria and Firmicutes. This result was different from that of other researchers [54,55], who found that most bacteria involved in lactic acid fermentation of silage belonged to the genera *Lactobacillus*, *Pedicoccus*, *Weissella*, and *Leuconostoc.* Metagenomic analysis revealed that urea addition in the sheep diet significantly increased the relative abundance of genera involved in nitrogen metabolism especially. Increasing nitrogen sources by urea addition may be beneficial to microbial protein production. This could improve microbial utilization of additional N sources during ruminal fermentation. Therefore, the synchronization between ruminal ammonia nitrogen release and carbohydrate availability resulted in greater microbial protein synthesis [56]. The bacterial composition was also altered by lysine supplementation to support energy metabolism, in which the microbial diversity was unchanged [57].

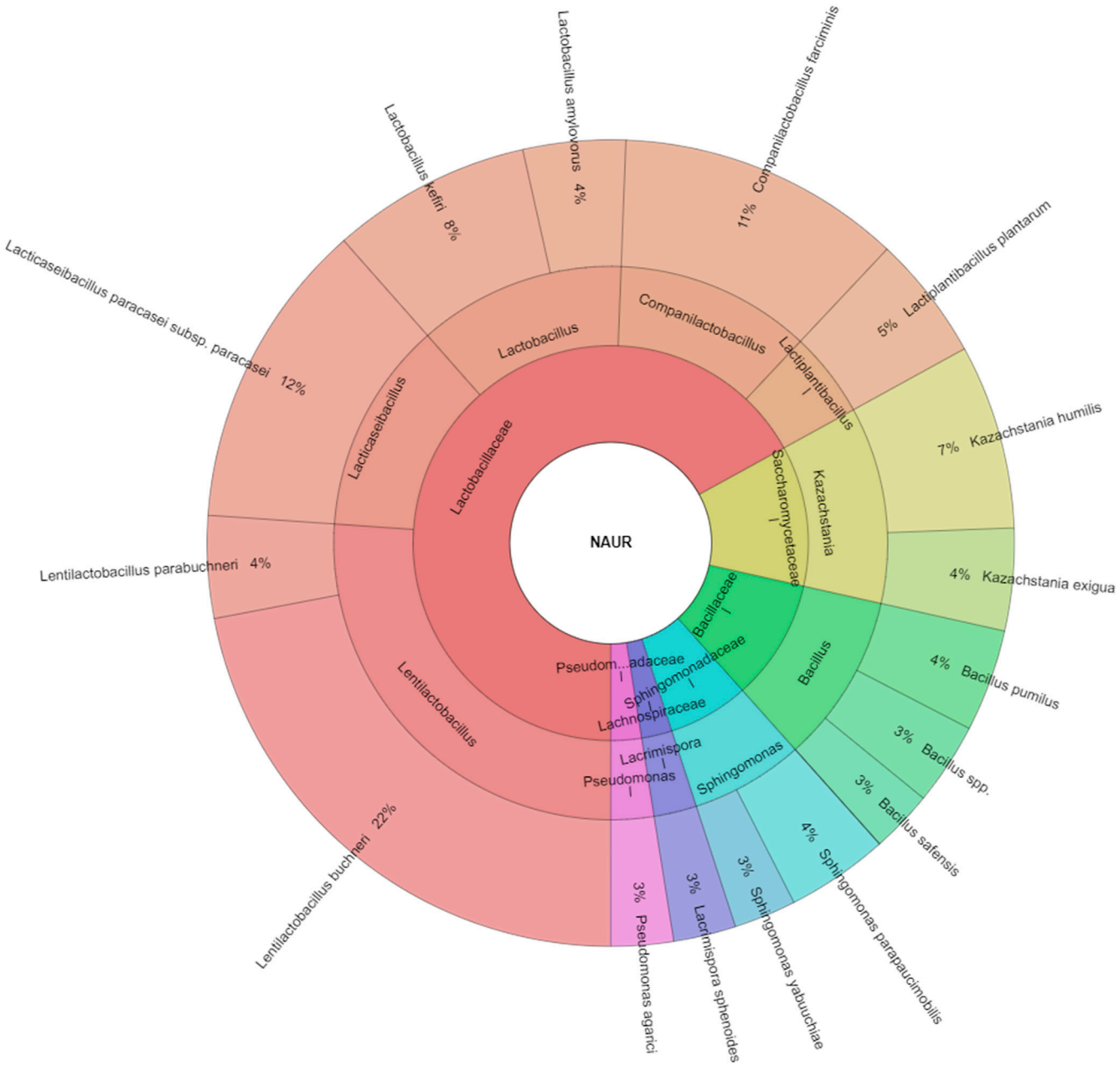

Figure 4. Krona chart of isolated species of bacteria and yeasts from samples of corn silage with nutritional additive: urea.

3.2. Mycotoxin Composition of Corn Silage

Several mycotoxin species were identified in corn silage samples. Tables 5 and 6 provide an overview on detected mycotoxin concentrations as well as on the level of significance. In this study, there were no statistically significant differences between variants of each mycotoxin species. Penagos-Tabares et al. [58] confirmed the occurrence of the same mycotoxins as detected in this experiment. When mycotoxins are present, there are few ways to avoid unwanted problems and therefore prevention is essential. Some silage additives can reduce growth of fungi and hence mycotoxin formation [14,59]. Whitlow and Hagler [59] found that these can be additives such as ammonia, propionic acid, sorbic acid, and bacterial or enzymatic additives. Dong et al. [60] examined the interactions between the harvest stage and the dose of inoculant in corn silages and found that inoculant lowered ($p > 0.05$) concentrations of deoxynivalenol at the milk stage. Furthermore, the inoculant significantly decreased or increased deoxynivalenol content with its different dosing at the dough stage [60]. Aflatoxins and ochratoxins were not identified neither in control variant nor in experimental variants. In contrast, Kalúzová et al. [16] determined these types of mycotoxins and decreased them with urea and inoculant addition. The effect of silage additives was manifested in reducing zearalenone content in experimental variants (Table 5). Corn silage with urea achieved a significantly ($p < 0.05$) higher concentration of zearalenone but its concentration after inoculant addition was even higher [16]. An increased concentration of zearalenone was also reported by Drouin et al. [61] when inoculant was added to the corn silage. The application of inoculant to corn silage resulted in increased fumonisin production, and Bakri [62] registered the same effect in his experiment. Gallo et al. [63] also detected fumonisins and found a greater level of fumonisin B1 ($p > 0.05$) and fumonisin B2 ($p < 0.05$) in inoculated corn. Corn silage NAUR resulted in a more than two times higher mean value of fumonisin B1 compared to CONT. Kalúzová et al. [16] found a similar result with this nutritional additive but the differences were not significant. Teller et al. [64] studied the effect of various additives on mycotoxin concentration in corn silage. A higher content of deoxynivalenol and fumonisin B1 was observed after inoculant treatment. As for zearalenone, the microbial additive lowered its content in silage samples [64]. The highest value of all mycotoxins reached nivalenol in both CONT and ALAB variants. In comparison with the control, a higher content of nivalenol was observed in variants with inoculant addition. Wang et al. [65] found a significant tendency in differences in nivalenol content depending on storage temperature and type/use of lactic acid bacteria. Eckard et al. [66] detected nivalenol in 8 of 19 samples of corn silage between a range of 190 and 760 µg/kg. A lowering trend ($p > 0.05$) of this mycotoxin in inoculated corn silage was noticed by Bakri [62]. Variant NAUR was higher in nivalenol content than CONT, but lower compared to variant ALAB. Although type A trichothecenes and ergot alkaloids are present in cereal crops, they were not detected by the HPLC-MS/MS method in this experiment. Type A trichothecenes include, for instance, mycotoxins such as T-2 toxin and HT-2 toxin, which are quite often found in silage. Some authors [16,65,67] confirmed a change in T-2 toxin concentration by using urea and various microbial additives in corn silages. Contamination by ergot alkaloids is mostly seen in forages such as tall fescue, sorghum, and ryegrass, but their presence in other forages is not refuted [68,69]. Zhang et al. [69] noted that ergot alkaloids are rarely present in corn silage in China, but still, they occurred in one of their samples of corn silage at a concentration of 15.3 µg/kg.

Table 5. The content of regulated mycotoxins in corn silage variants in μg/kg.

Mycotoxin Species	Variant	Mean	S.D.	Minimum	Maximum	*p* Value
Deoxynivalenol	CONT	163.46	80.23	117.03	256.10	0.404
	ALAB	260.85	250.09	116.33	549.62	
	NAUR	373.33	158.00	215.00	531.00	
	Total	265.88	178.21	116.33	549.62	/
Aflatoxins	CONT	<0.3/1 *	/	/	/	/
	ALAB	<0.3/1 *	/	/	/	
	NAUR	<0.3/1 *	/	/	/	
	Total	<0.3/1 *	/	/	/	
Ochratoxins	CONT	<0.5/2 *	/	/	/	/
	ALAB	<0.5/2 *	/	/	/	
	NAUR	<0.5/2 *	/	/	/	
	Total	<0.5/2 *	/	/	/	
Zearalenone	CONT	56.61	43.09	31.52	106.36	0.626
	ALAB	36.47	6.89	28.57	41.23	
	NAUR	41.03	8.55	36.00	50.90	
	Total	44.71	24.04	28.57	106.36	/
Fumonisin B1	CONT	94.35	42.47	50.75	135.59	0.076
	ALAB	96.71	54.95	57.02	159.42	
	NAUR	208.33	67.16	144.00	278.00	
	Total	133.13	74.27	50.75	278.00	/
Nivalenol	CONT	203.26	108.71	140.44	328.79	0.277
	ALAB	592.26	454.57	139.52	1048.63	
	NAUR	331.33	48.21	277.00	369.00	
	Total	375.62	290.98	139.52	1048.63	/
Type A trichothecenes	CONT	<3/10 *	/	/	/	/
	ALAB	<3/10 *	/	/	/	
	NAUR	<3/10 *	/	/	/	
	Total	<3/10 *	/	/	/	
Ergot alkaloids	CONT	<2/6 *	/	/	/	/
	ALAB	<2/6 *	/	/	/	
	NAUR	<2/6 *	/	/	/	
	Total	<2/6 *	/	/	/	

S.D.—standard deviation; CONT—control; ALAB—additive on the base of lactic acid bacteria; NAUR—nutritional additive: urea; * < LOD/LOQ—limit of detection/limit of quantification.

Besides free *Fusarium* mycotoxins (deoxynivalenol, fumonisin B1, nivalenol, and zearalenone), the presence of some other *Fusarium* contaminants known as emerging mycotoxins (beauvericin, enniatins, and moniliformin) were also found. Beauvericin was detected only in corn silage with the addition of urea. An average concentration of 47 μg/kg of beauvericin by Zachariasova et al. [70] was determined in corn silage samples. Reisinger et al. [71] found 120 samples of corn silage positive on beauvericin, and this mycotoxin belonged to five most frequently detected mycotoxins in their study. In corn silage samples, Sørensen et al. [72] did not detect the presence of enniatin A and A1 but confirmed the presence of enniatin B and B1. In contrast to Sørensen et al. [72], the mean value of enniatin B was 28.01 μg/kg lower and that of enniatin B1 was 9.50 μg/kg lower in our corn silage samples without additive. On the other hand, neither enniatin A nor B1 were confirmed in the corn silage from Rasmussen et al. [73], but the mean value of enniatin B (44.00 μg/kg) was lower compared to our study of silage without additive. Similarly, Storm et al. [74] only reported the occurrence of enniatin B with an average value higher (53.00 μg/kg) than that reported in Table 6 for the silage without additive. In whole-plant corn silage, enniatins did not appear in any of the samples or were detected at very low concentrations [75]. However, Shimshoni et al. [76] detected the occurrence of enniatin A (0.3 μg/kg), enniatin A1 (0.8 μg/kg), enniatin B (0.2 μg/kg), as well as enniatin B1 (0.9 μg/kg). Identical to the previous author, McElhinney et al. [77] also found the presence of these mycotoxins, but in grass silage. Moreover, the presence of enniatin

B was confirmed by Wambacq et al. [78] in 82 corn silage samples with concentrations up to 5000 µg/kg. As in the present study, these values were not statistically significant in studies, which all the authors mentioned. Moniliformin was present in all samples; however, lower concentrations were obtained in both treated variants. Zhang et al. [69] reported relatively low concentrations of moniliformin, and the highest concentration of 116 µg/kg was found. On the contrary, Gräfenhan et al. [79] did not detect moniliformin in red clover silage (control and treated variant), but after addition of soil, moniliformin concentration jumped up to 222 µg/kg. As Kalúzová et al. [14] mentioned, the effect of urea on mycotoxin concentrations has not been widely monitored so far and the effect on chemical composition and fermentation parameters was more closely monitored in this case. Some studies confirmed a positive suppression effect of silage additives on mycotoxin concentrations [23,61–63]. However, other studies reveal the increasing mycotoxin content after the application of silage additives [17,65]. Contradictory results are caused probably by many factors such as temperature, water activity, and pH value [80–82], which are affecting the environment of microscopic fungi producing the mycotoxins in stressful conditions; thus other experiments are necessary in this research field.

Table 6. The content of emerging mycotoxins in corn silage variants in µg/kg.

Mycotoxin Species	Variant	Mean	S.D.	Minimum	Maximum	*p*-Value
Beauvericin	CONT	<2/6 *	/	/	/	/
	ALAB	<2/6 *	/	/	/	
	NAUR	496.67	175.51	355.00	693.00	
	Total	496.67	175.51	355.00	693.00	
Enniatin A1	CONT	16.36	14.78	5.72	33.24	0.428
	ALAB	6.67	1.99	5.30	8.95	
	NAUR	13.17	0.99	12.50	14.30	
	Total	12.07	8.61	5.30	33.24	/
Enniatin B	CONT	44.99	48.93	16.49	101.49	0.368
	ALAB	14.55	5.05	9.21	19.25	
	NAUR	13.53	0.78	12.90	14.40	
	Total	24.36	29.06	9.21	101.49	/
Enniatin B1	CONT	30.50	37.58	4.50	73.59	0.463
	ALAB	7.11	2.42	4.37	8.95	
	NAUR	16.57	1.46	15.40	18.20	
	Total	18.06	21.42	4.37	73.59	/
Moniliformin	CONT	42.30	12.32	28.56	52.34	0.323
	ALAB	24.73	18.64	13.95	46.25	
	NAUR	35.13	3.55	31.30	38.30	
	Total	34.06	13.65	13.95	52.34	/

CONT—control; ALAB—additive on the base of lactic acid bacteria; NAUR—nutritional additive: urea; * <LOD/LOQ—limit of detection/limit of quantification

3.3. Relationship between the Mycotoxin Concentrations and Microscopic Fungi and Yeasts, Harmful Effects, and Mycotoxin Limits in Feeds

The main correlation characteristics between the mold and yeast populations and detected mycotoxins in corn silages were not statistically significant; however, the coefficient of determination between the May and FUMB1 (r^2) was relatively high (0.79%) (Table 7). This was also confirmed by Barug et al. [83], where a direct relationship between the microscopic fungi and yeast and certain mycotoxins in silage was not observed. Similarly, no correlations were found between fungal DNA and mycotoxin concentrations [84]. According to Schenck et al. [85], a correlation between the presence of *Fusarium* toxins (NIV, DON, 3-ACDON, HT-2, T-2, BEAU, and ENNB) and the presence of *Fusarium culmorum*, *F. equiseti*, *F. graminearum*, or *F. poae* could not be proved. However, there were negative significant correlations between the nivalenol and enniatin A1 ($p < 0.01$) and moniliformin ($p < 0.05$). On the other side, the positive correlation between the enniatin A1 and moniliformin ($p < 0.05$) was observed. *Aspergillus*, *Fusarium*, and *Penicillium* species comprise

a well-known group of microscopic filamentous fungi that are infamous for their ability to make many potent mycotoxins. Mycotoxins play a significant role in the defensive strategies of mycotoxigenic fungi. The fungal species more frequently identified in this work have been previously reported in silage [86]. The presence of mycotoxins, which are produced by *Aspergillus* and *Penicilium* species, was not evaluated in corn silages in this study.

Table 7. Mold and yeast population and mycotoxin Pearson's correlation relationship.

	DON	ZEA	FUMB1	NIV	ENNA1	ENNB	ENNB1	MON
MaY	0.567	0.179	0.876	−0.629	0.629	−0.066	0.367	0.558
DON		−0.709	0.894	0.283	−0.283	−0.859	−0.558	−0.368
ZEA			−0.318	−0.877	0.877	0.970	0.981	0.916
FUM				−0.176	0.176	−0.540	−0.128	0.088
NIV					−1.000 **	−0.734	−0.954	−0.996 *
ENNA1						0.734	0.954	0.996 *
ENNB							0.904	0.791
ENNB1								0.977

MaY—molds and yeasts; DON—deoxynivalenol; ZEA—zearalenone; FUMB1—fumonisin B1; NIV—nivalenol; ENNA1—enniatin A1; ENNB—enniatin B; ENNB1—enniatin B1; MON—moniliformin; * $p < 0.05$; ** $p < 0.01$.

Aflatoxins are mainly produced by toxigenic strains of *Aspergillus* molds. In ruminants, reduced milk production in dairy cows, decreased milk quality and safety due to carry-over of toxins from contaminated feed, liver malfunctions, decreased feed efficiency and rate of gain in beef cows, and compromised immune and ruminal functions were observed [86]. The maximum content of aflatoxin B1 for complete foodstuffs for cattle, sheep, and goats is 0.02 mg/kg with the exception of complete foodstuffs for dairy animals (0.005 mg/kg) and for calves and lambs (0.01 mg/kg). The maximum content of aflatoxin B1 for complementary foodstuffs for cattle, sheep, and goats is 0.02 mg/kg (except complementary foodstuffs for dairy animals, calves, and lambs: 0.005 mg/kg) [87].

Ochratoxins are produced by several *Penicilium* and *Aspergillus* species, have hepatotoxic and nephrotoxic effects, causing poor feed conversion and limiting weight gains in ruminants [88]. The guidance limit of ochratoxin A for feed materials (cereals and cereal products) is 0.25 mg/kg, and for complementary and complete foodstuffs are limited only for poultry and pigs [89].

In analyzed corn silages, only fusarium mycotoxins were found. Deoxynivalenol causes feed refusal and lower weight gains, diarrhea, lower milk production, hepatotoxicity (in young preruminants), and immune alterations [86,88,90,91]. The regulatory level (guidance value) of deoxynivalenol for complementary and complete foodstuffs for ruminants is 5 mg/kg. For feed materials, guidance values are 8 mg/kg (for cereals and cereal products) and 12 mg/kg (for maize by-products) [89].

Clinical signs in ruminants caused by fumonisins are decreased feed intake, milk production, and mild liver diseases [86,92]. The results of Roberts et al. [93] revealed that exposure to deoxynivalenol and fumonisins was detrimental to the welfare of finishing steers and may compromise their ability to withstand other stressors such as disease, heat stress, or other toxins. The regulatory level (guidance value) of fumonisins (B1 + B2) for complementary and complete foodstuffs for adult ruminants (>4 months) is 50 mg/kg, and for feed materials (maize and maize products) is 60 mg/kg [89].

Higher zearalenone contamination is linked with a risk of vaginal and rectal prolapses, infertility, hyperestrogenism, swelling of mammary glands, and milk production reduction in dairy cows [88,94]. The regulatory level (guidance value) of zearalenone for complementary and complete foodstuffs for calves, dairy cattle, sheep (including lambs), and goats (including kids) is 0.5 mg/kg. For feed materials, guidance values are 2 mg/kg (for cereals and cereal products) and 3 mg/kg (for maize by-products) [89].

Nivalenol belongs to the B group of trichothecene mycotoxins along with deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, and fusarenon-X [95]. Generally, the toxicity of deoxynivalenol and nivalenol is similar [88,96] and effects of nivalenol include immunotoxicity and hematotoxicity. With the exception of forage maize (and maize silage), levels of nivalenol in forages are generally low. For lactating dairy cows and beef cattle, the estimated lower-bound and upper-bound exposures to nivalenol are between 0.077 and 0.69 μg/kg of live body weight per day, except for maize-silage-based diets (1.9 and 4.6 μg/kg body weight per day) [95]. The concentrations of regulated mycotoxins in our experiment did not exceed the maximum permitted, guidance, and daily tolerable intake according to the limits [87,89,95,97].

Animal exposure to enniatins and beauvericin is primarily from feed intake of cereal grains and cereal by-products [97]. The primary toxic action of beauvericin and enniatins is related to their ability to form ion channels and transport NH_4^+ or K^+ ions across the cell membrane, resulting in disturbance of the ion homeostasis and eventually cell death [98,99].

Enniatins are mutagenic and embryotoxic for animals [100,101]. For the sum of enniatins, the calculated lower-bound and upper-bound (UB) chronic exposures for ruminants ranged from 3.30 to 8.26 μg/kg body weight per day and estimated acute UB exposure is 32.6 μg/kg body weight per day, for ruminants [97].

Moniliformin is mainly detected in cereal grains and cereal-based feed [102]. The main pathological change observed in sheep was the degeneration of the proximal tubules of the kidneys after moniliformin intake [103]. No toxicity data suitable for hazard characterization of moniliformin were identified for ruminants, farmed rabbits, horses, farmed fish, dogs, and cats. Therefore, the EFSA Panel on Contaminants in the Food Chain (CONTAM) [104] considered of 0.20 mg moniliformin/kg body weight identified for pigs as an indicative reference point [102].

4. Conclusions

The number of microbiota varied with the control to nutritional additives. The most isolated group of bacteria was lactic acid bacteria in all groups of corn silages. The most isolated species of LAB were similar as bacterial additives added to silages. The application of additives did not affect the number of lactic acid bacteria, in both groups with additives. The total numbers of microorganisms in both groups with additives were affected; however, the number of microscopic filamentous fungi only in the group with the addition of lactic acid bacteria compared to the control group was lower. In both groups with the additive treatment, there was a lower diversity of isolated species of microorganisms, and a higher proportion of main species of lactic acid bacteria compared to the control group. The following *Fusarium* mycotoxins have been recorded in corn silages: deoxynivalenol, zearalenone, fumonisin B1, nivalenol (regulated mycotoxins), beauvericin, enniatin A1, B, B1, and moniliformin (emerging mycotoxins). Nivalenol reached the highest total mean value of regulated mycotoxins in silage samples. The highest prevalence of deoxynivalenol and beauvericin in silage with urea addition was observed. While beauvericin was found only in silage with urea, other emerging mycotoxins (enniatin A1, B, B1, and moniliformin) were present in all variants. However, the mycotoxin content after the addition of silage additives was not statistically significant, hence, their effect in corn silage was not confirmed. On the other side, it can be stated that monitored mycotoxin did not affect the hygienic quality and safety of analyzed corn silage. More studies for investigation of the effect of silage additives on mycotoxin concentration in silages are needed.

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