Maize and Lupine Biomass as Raw Material for Production of Feed Silage

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Sustainable animal feed options must balance nutritional, environmental, and ethical considerations. Maize (*Zea mays*) and yellow lupine (*Lupinus luteus*) biomass offer promising substrates for producing silage, due to their high nutritional value and potential to support controlled fermentation. Maize is widely used in animal feed, due to its content of digestible carbohydrates and protein. Lupine is a viable alternative to soy, with advantages including high protein and low environmental impact due to its nitrogen-fixing abilities. This study investigated the possible use of selected lactic acid bacteria (LAB) strains—*L. buchneri 1, P. acidilactici 4, L. buchneri 2.1,* and *P. acidilactici 2.2*—to optimize fermentation of maize and lupine biomass for improved silage quality. Controlled fermentations using these strains resulted in silages with favorable parameters: pH values ranging from 3.8 to 4.4, dry matter content between 35 and 45%, and total acidity in the range of 6.0 to 8.0%. The best results were achieved in a 1:1 maize–lupine mixture, which showed stable LAB counts and optimal fermentation conditions. The results showed the potential of microbial inoculation in improving silage quality. However, the conclusions are based on lab-scale studies and do not address practical factors such as scalability, cost-effectiveness, or long-term storage stability.

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INTRODUCTION

The rapidly growing human population is driving increased demand for animal products and food of animal origin. Animal production presents significant sustainability challenges, including the health impacts of meat consumption, the environmental effects of industrial farming, and ethical and social concerns regarding the welfare and well-being of farm animals. Animal production has significant economic and nutritional benefits, as it provides valuable food. However, it negatively affects the environment and health through methane emissions and the use of antibiotics. Optimal animal nutrition, incorporating sustainable feed practices, is essential for promoting animal health and well-being while reducing production costs and greenhouse gas emissions, especially in the context of climate change.

One of the ways to reduce methanogenesis and effective breeding is to use fermented feed and fermented feed components, which support animal growth and improve the quality of animal products (Dumont *et al.* 2019; Guyomard *et al.* 2021). Maize (*Zea mays*) is a cereal crop widely cultivated for its versatility and high nutritional value. It serves as a staple food for humans and a vital feed source for livestock, providing a rich supply of carbohydrates, vitamins, and minerals. Globally, 1,137 million tons of maize are produced each year, almost 70% of which is used for feed purposes. The advantages of using maize as animal feed include its relatively high protein and fat content, as well as its high content of easily soluble carbohydrates. These characteristics make maize well-suited for fermentation processes. Ensiling is also four times cheaper than drying, which reduces production and storage costs (Erenstein *et al.* 2022; Płacheta *et al.* 2022). Research on the effectiveness of feeding animals with fodder fermented with appropriate strains of maize has shown that the addition of fermented maize significantly improves the digestibility of nutrients, lactation efficiency, and the use of absorbed nitrogen (Zhang *et al.* 2020).

Lupine (*Lupinus spp.*) is a legume crop that is valued for its physico-chemical properties, including high content of crude protein, carbohydrates, and fat. Lupine has a much higher protein content than maize, and it is a viable alternative to imported soy for feed production. Compared to soybean, lupine requires less demanding soil conditions, has a shorter vegetation period, and it benefits the environment by improving soil health through its ability to fix nitrogen from the air (Trugo *et al.* 2016; Płacheta *et al.* 2022). Global lupine production amounts to over 1 million tons annually. The types of lupine most frequently cultivated for fodder purposes include narrow-leafed lupine (*Lupinus angustifolius*) and yellow lupin (*Lupinus luteus* L.). However, due to its high content of anti-nutritional substances (alkaloids, phytates, and oligosaccharides), the consumption of yellow lupin may negatively affect the health of animals and humans. Fermentation using selected strains of bacteria reduces the content of potentially dangerous substances. Research has shown that feeding animals with feed containing fermented lupine has a positive effect on reducing methanogenesis in ruminants (Trugo *et al.* 2016). Unfortunately, the composition of lupine poses difficulties in the fermentation process.

The aim of this study was to select strains of lactic acid bacteria (LAB) for use in maize and lupine-based silage production. It examined how the bacteria affect the natural microflora of the raw materials and assessed their impact on the efficiency of biomass fermentation. It was hypothesized that the selected strains of lactic acid bacteria (LAB) could significantly improve the fermentation quality of silage, by achieving optimal physicochemical parameters. This could provide a sustainable feed option for ruminant livestock, particularly dairy cattle, which require high-quality, fiber-rich feed and are sensitive to silage composition and fermentation stability. This research is part of a larger project focused on developing a feed component with specific beneficial characteristics. In future work, the most effective bacterial strains will be used to develop a feed component with probiotic properties.

The first stage of the study involved spontaneous ensiling of various ratios of maize and lupine. Lactic acid bacteria (LAB) isolated from maize or lupine biomass, as well as from rye and wheat, were used for controlled fermentation. After a set period, the LAB count in the resulting silages were measured as well as their pH. The silages meeting the criteria (bacteria count of 10^7 to 10^9 and pH 3.8 to 4.4) were analyzed for dry matter, protein content, and total acidity. Strains fulfilling five further criteria were selected and analyzed for their metabolic abilities, proteomic similarity, and survival during simulated gastrointestinal passage. The strains that demonstrated the highest survival under these conditions were genetically identified. The final stage involved the controlled fermentation of maize, lupine, and a mixture of the two, using the bacterial strains that successfully passed all selection stages. The goal was to test whether the resulting products met established parameters to serve as a balanced feed component.

EXPERIMENTAL

Research Design

The purpose of the first stage of the research was to select bacterial strains with suitable technological properties for the production of silage for cattle. Spontaneous fermentation of maize and lupine was carried out, allowing the natural development of microorganisms present in the environment. This process was conducted under controlled conditions of temperature and humidity, which made it possible to obtain autochthonous microflora responsible for fermentation. The bacterial strains were isolated from the silages obtained as a result of spontaneous fermentation.

The next stage was controlled fermentation of maize and lupine. Controlled fermentation was carried out using the strains isolated in the previous step and other environmental isolates owned by the Department of Environmental Biotechnology at Lodz University of Technology. The aim at this stage was to assess the effectiveness of individual strains under controlled fermentation conditions. The key technological microbiological and physicochemical parameters of the silages subjected to spontaneous and controlled fermentation were then compared. The analysis included the number of lactic acid bacteria in the silages, dry matter content, protein content, pH, and total acidity. The results enabled assessment of the quality and stability of the obtained silages. The isolates that produced silage with parameters meeting the desired criteria (bacteria count of 10^7 to 10^9, pH of 3.8 to 4.4, dry matter 35 to 45%, and total acidity of 6.0 to 8.0%) were selected for further studies.

The selected strains were characterized for their metabolic activity using API 50 CHL (bioMérieux) and proteomic activity using the MALDI-TOF mass spectrometry method. Their ability to survive in simulated intestinal passage conditions was also assessed. The four strains that demonstrated the highest survival rates were investigated to explore their mutual antagonism and potential use as monocultures and mixed cultures. Genetic identification of the selected strains was performed by genomic DNA isolation using a GeneMATRIX Soil DNA Purification Kit (EURX, Gdańsk, Poland). The quality and concentrations of DNA were assessed spectrophotometrically. NGS libraries were prepared and sequenced on the DNBSEQ G400 platform (MGI, Shenzhen, China). Finally, silage was produced from maize and lupine biomass mixed in appropriate proportions, inoculated with the selected bacterial isolates (both individually and in combination), and evaluated based on the pre-established criteria.

Biological Material

Maize and lupine biomass

The biomass consisted of maize grains (*Zea mays*) with a dry matter content of 98%, moisture content of 2%, total protein content of 8.87 ± 0.117%, and crude fiber content of 2.51 ± 0.015% and yellow lupine grains (*Lupinus luteus*) with a dry matter content of 90%, moisture content of 10%, total protein content of 45.61 ± 3.018%, and crude fiber content 15.36 ± 0.023%. The maize grains and lupine grains were obtained from GRANUM Sp. J. (Poland).

Bacterial strains

The 55 bacterial strains used in this study were environmental isolates previously collected and characterized at the Department of Environmental Biotechnology at Lodz University of Technology. These strains were originally isolated from plant-based environments, specifically maize, lupine, wheat, and rye substrates, and include both grain meal- and silage-derived strains. Their selection was based on preliminary screening for traits associated with fermentation potential. This collection represents a novel and diverse set of candidate LAB, chosen based on their relevance to the raw materials studied and prior characterization data supporting their suitability for silage production. The following environmental strains originating from wheat and rye were used: KBS1, 3, KBS4, 5, 6 (POP5), 7, 8 (POP4), 9, 16, 21, 22, 25, 26, 28, 31, 35, 36, 39, 40, 41, 42, 44, 45, 46, 48, 49, 50, 51, 52, 53, 56, 60, 61, 62, 63, 64, 65, 66. The following strains were isolated from maize meal and lupine: K1-T, 1, K2-T, K2, K4-T, 4, K5-T, K5, KK2-T, 2.1, KK4-T, KK4, 2.2, L4-T, L4, L5-T, L5.

Research Methods

Culture media and growth conditions

MRS broth, selective for LAB, was used for the cultivation and isolation of bacteria, with the following composition (g/liter): enzymatic digest of casein 10.0; meat extract 8.0; yeast extract 4.0; D(+)glucose 20.0; di-potassiumhydrogen-phosphate 2.0; Tween ®80 1.0; di-ammonium hydrogen citrate 2.0; sodium acetate 5.0; magnesium sulfate heptahydrate 0.2; manganese sulfate monohydrate 0.04 (Merck, Darmstadt, Germany). The bacteria were cultured at a temperature of 30 ± 1 °C for 48 h under anaerobic conditions using an AnaeroGen Sachet (Oxoid™ AnaeroGen™ 2.5L Sachet, Thermo Scientific™). The effectiveness of anaerobiosis was monitored using anaerobic indicator strips (Biomerieux, Sigma-Aldrich), which enabled real-time visual verification of oxygen exclusion during the entire fermentation period.

Strain isolation

Bacterial strains were isolated from spontaneous maize and lupine silages using selective MRS medium (de Man, Rogosa and Sharpe), which provides optimal growth conditions for LAB. First, samples of silage were collected after spontaneous fermentation. Then, 1 g of the fermented material was mixed under sterile conditions with 99 mL of physiological saline (0.85% NaCl) to obtain a homogenate. The mixture was mixed thoroughly. Next, the homogenate was subjected to serial tenfold dilutions in physiological saline solution. These dilutions allowed for a gradual reduction in the concentration of microorganisms, which was crucial for isolating single colonies. From the prepared dilutions, 1 mL of the solution was taken, and deep seeding was performed. The samples were incubated under anaerobic conditions at 30 ± 1 °C for 48 h. Strains displaying colony morphology characteristic of LAB—round, cream, or white colonies with smooth edges—and positive results for Gram staining were subsequently cultured in liquid MRS medium for further analysis.

Inoculum preparation

The inoculum for controlled fermentation of maize and lupin biomass was prepared in several stages. First, the selected strains of LAB isolated from spontaneously fermented silages were multiplied. Each strain was cultured in liquid MRS medium (de Man, Rogosa and Sharpe) under anaerobic conditions at 30 ± 1°C for 24 h to reach the logarithmic growth phase. After completion of the culture, the bacterial cell suspension was centrifuged (4000 rpm for 10 min). The obtained cell pellet was washed with sterile physiological saline solution (0.85% NaCl). The pellet was suspended in fresh physiological saline solution to obtain a suspension with a specific concentration. The bacterial cell suspensions were initially standardized by measuring optical density (OD) at 600 nm. Bacteria were introduced into the plant biomass in an amount sufficient to obtain a final concentration of 10^4 CFU/mL in the fermentation medium.

Spontaneous fermentation for silage preparation

Spontaneous fermentation of maize–lupine biomass was initiated by adding tap water to adjust the moisture content to levels suitable for effective fermentation. The biomass consisted of crushed maize and lupine grains, which were mixed thoroughly with the water to achieve the desired moisture level. After thorough mixing, the fermentation material was placed in tight, vacuum-sealed bags, which created anaerobic conditions conducive to fermentation. Silages were prepared from maize and lupine biomass in the following maize to lupine ratios: 0 : 1 (test L1), 1 : 4 (test M1L4), 2 : 3 (test M2L3), 1 : 1 (test M1:L1), 3 : 2 (test M3L2), 4 : 1 (test M4L1), and 1 : 0 (test M1).

Controlled fermentation for silage preparation

The appropriate amount (5%) of inoculum was added to crushed maize and lupine biomass, in such a way as to ensure an even distribution of bacteria in the material. The dry mass of each type of biomass was first measured, to determine the volume of inoculum that should be introduced into the biomass to obtain final silage with the specified dry mass content. After thorough mixing, the fermentation material was placed in tight, vacuum-sealed bags, which created anaerobic conditions conducive to fermentation. Silages were prepared from maize and lupine biomass separately, and in the final stage from mixtures in the following maize to lupine ratios: 0:1 (test L1), 1:4 (test M1L4), 2:3 (test M2L3), 1:1 (test M1:L1), 3:2 (test M3L2), 4:1 (test M4L1), and 1:0 (test M1).

Metabolic abilities

Metabolic abilities were determined using a commercial API 50 CHL test (bioMérieux SSC Europe Sp. z o. o.). The API 50 CHL tests were performed according to the procedure recommended by the manufacturer. The samples were incubated at 30 °C for 48 h. The results were read after 24 h and 48 h of incubation.

MALDI-TOF mass spectrometry

For MALDI-TOF MS proteomic similarity, isolates were grown at 30 °C for 18 h on MRS medium plates and analyzed with the AXIMA-iD Plus Confidence MALDI-TOF MS System (Kratos Analytical Ltd and Shimadzu Corporation, Kyoto , Japan) and SARAMIS Premium software (ver. 4.11) (Spectral Archive and Microbial Identification System, bioMérieux, France), according to the methodology developed by Liszkowska *et al.* (2023).

Survival under conditions of intestinal passage

The survival rates of the isolated strains under conditions mimicking intestinal transit were determined. The tested strains were cultured in 25 mL of liquid MRS medium (Merck, Darmstadt, Germany), until the cells reached the late logarithmic growth phase or the early stationary growth phase—*i.e.*, approximately 109 cfu/mL. The biomass was separated from the substrate by centrifugation (9000 rpm, 15 min) and washed twice with PBS solution. The obtained biomass was suspended in 25 mL of crushed raw material (maize and lupine) and adjusted to pH 4.6 using 1M HCl. Next, 5 mL of sterile stock solution A was added to the cell suspension, and a seed sample was taken to determine the initial number of cells. Subsequently, 3 mL of stock solution A (pH=5.0) was added to the suspension. The composition (g/L) of stock solution A was as follows: sodium chloride 6.2, potassium chloride 2.2, calcium chloride 0.22, sodium bicarbonate 1.2. Lysozyme and pepsin were added to the solution A, to final concentrations of 0.01% lysozyme and 0.3% pepsin. Samples were taken for sowing. Conditions analogous to those prevailing in the stomach (*i.e.*, low pH of gastric juice) were obtained by adding 1 M HCl to the suspension. Three different pH values were determined: pH=2.0, pH=3.0, and pH=4.0. After acidification, the suspensions were incubated for 20 min at 37 °C, and samples were taken from each suspension for sowing. The remaining suspension was neutralized to pH=6.5, which corresponds to the pH prevailing in the duodenum, using 1 M $NaHCO_3$. Basic solution B (pH 8.0) was added to the tested suspension. The composition (g/L) of basic solution B was as follows: sodium chloride 5, potassium chloride 0.6, calcium chloride 0.3. Bile salts and pancreatin were added to the solution B, to final concentrations of 0.45% bile salts and 0.1% pancreatin. The suspensions were incubated for 120 min, and then samples were taken for sowing. Before each sowing, the samples were neutralized with 0.1 M NaOH. The survival of cells exposed to intestinal passage was calculated as the ratio of the number of bacteria per mL of the control sample to the number of bacteria in samples after simulation, expressed in log CFU/mL.

Genetic Identification

DNA isolation and sequencing

Genomic DNA was isolated using a GeneMATRIX Soil DNA Purification Kit (EURX, Gdańsk, Poland). The concentration and quality of DNA were assessed using a spectrophotometer. DNA integrity was analyzed using 0.7% agarose gel electrophoresis. NGS libraries were prepared using a DNA Library Prep Set (MGI, Shenzhen, China), according to "Whole Genome Sequencing Library Preparation (DNBSEQ) instruction" (Document NO.: BGI-NGS-HKJK-DNA-001, v. A0). The isolated genomic DNA was sequenced using a DNBSEQ G400 sequencing platform (MGI, Shenzhen, China). In total, 4,002,937 to 4,116,274 pairs of 150 bp reads were generated per library. NGS library preparation and sequencing were performed at BGI-TECH (Wuhan, China).

Genome and metagenome assembly and annotation

The reads generated for each library were assembled using SPAdes (v. 3.13.0; (Prjibelski *et al.* 2020)) with default options in the metagenomic mode. The quality of the assemblies was assessed using QUAST (v. 4.1; (Gurevich *et al.* 2013)). The sequence data were deposited in the NCBI database under the BioProject ID PRJNA1200942. Prediction of ribosomal RNA was performed using Barrnap (v. 0.9; (Seemann 2014)). Taxonomic assignment was performed by searching 16S rRNA sequences using the online version of nucleotide BLAST at NCBI and the 16S ribosomal RNA sequences (Bacteria and Archaea) database (access date December 2024). MetaQUAST (v. 5.0.2; (Mikheenko *et al.* 2016)) was used to further confirm the taxonomic assignment. Processing of annotation information was performed using custom-made scripts written in Perl v. 5.26.2 or Python v. 3.7.9. The calculations were performed using the BlueOcean computational cluster, which is part of TUL Computing and Information Services Centre infrastructure.

Physicochemical Analysis

Determination of dry matter content

Mass was measured on six weighing dishes. About 1 g of each sample was added to each dish. The samples were placed in an oven (muff oven 12L PRO, Adverti) with the temperature set at 130 °C for 3 h.

Determination of protein content

Protein content was determined using the Kjeldahl method, according to the method described by Dygas and Berłowska (2023). The silage sample was placed in a combustion flask with a 5 g Missouri tablet as a catalyst (Büchi, Flawil, Switzerland). Concentrated sulfuric acid was added and the sample was burned at 550 °C in a SpeedDigester K-425 (Büchi, Flawil, Switzerland). The burned sample was then neutralized with a 30% sodium hydroxide solution (m/m) in a KjelFlex K-360 (Büchi, Flawil, Switzerland). The steam was distilled and titrated to pH 4.5 using the attached SI Analytics TitroLine®5000 (Xylem, Washington, USA). The control sample consisted of dry maize and lupine grains.

Determination of crude fiber content

The crude fiber content was determined using a FOSS Fibertec™ 8000 (Hilleroed, Denmark), according to the method described by Dygas and Berłowska (2023).

Determination of total acidity and pH

The pH of all samples was determined by the potentiometric method using a pH-meter (METTLER TOLEDO Five Easy Plus FP20). The total acidity was measured by titration with sodium hydroxide. For this purpose, 1 g of the silage was added to a mortar and then, through mechanical processing, thoroughly ground and mixed with 99 mL of distilled water. The solution was titrated with a 0.1 M NaOH solution using an automatic titrator (TitroLine500, SI Analytics), calibrated to pH 9. The titration was carried out until the solution reached pH 9, indicated by the purple color change of phenolphthalein.

Microbiological Analysis

Determination of microbial abundance

In sterile conditions, 1 g of the silage was mixed with 99 mL of physiological saline (0.85% NaCl) to obtain a homogenate. The mixture was mixed thoroughly. Then, the homogenate was subjected to serial ten-fold dilutions in physiological saline solution. Deep seeding was performed. The samples were incubated in anaerobic conditions at 30 ± 1 °C for 48 h.

Determination of antagonistic abilities

The antagonistic abilities of the strains of lactic acid bacteria isolated the raw materials were tested using the column method, which allows for parallel growth of the examined strains. This method enables the measurement of growth inhibition zones, showing the inhibitory effects of specific test strains. Three posts with diameters of 10 mm were cut out from the MRS medium overgrown with lactic acid bacteria and incubated for 24 h at 30 °C and placed on grass plates (wort or broth medium with the indicator strain previously inoculated in the amount of 10^5 to 10^6 CFU/mL). The plates were incubated for 16 h at 30 °C, and then the clear zones of the turf around the posts were read.

Statistical analysis

Statistical analysis (ANOVA, Tukey test, $p<0.05$) was performed for each result usingStatisticav.14.0.1 (TIBCO Software Inc., Palo Alto, California, USA). All experimental procedures were performed in three independent replicates to ensure the reliability and reproducibility of the results across biological and technical levels.

RESULTS AND DISCUSSION

Spontaneous Fermentation of Raw Material Mixtures

The initial step in the screening of bacteria for use in the production of feed components from maize and lupine was the spontaneous fermentation of maize and lupine mixtures with the following weight ratios: 1:0, 4:1, 3:2, 1:1, 2:3, 1:4, and 0:1. Spontaneous fermentation was continued for 4, 6, or 8 weeks. The counts of LAB (cfu/g) and pH were assessed to identify samples meeting the quality criteria: LAB in the range of 10^7 to 10^9 and pH in the range of 3.8 to 4.4. The results show that LAB counts (Table 1) generally increased during fermentation, reaching the highest values in samples with higher amounts of maize. However, not all samples met both criteria simultaneously. The sample containing only lupin (0:1) showed the lowest LAB count, significantly below the acceptable range. Adding maize in a ratio of 1:4 (M1L4) increased the LAB count to a level meeting the criterion, but the pH remained too high. The LAB counts were the highest in the samples with higher proportions of maize, such as 2:3 (M2L3) and 1:1 (M1L1), which was the only sample to meet both criteria.

Table 1. Spontaneous Fermentation of Mixtures with Different Amounts of Maize and Lupine Biomass over 4, 6, and 8 Weeks

Types and ratio of biomass in the mixture		LAB (cfu/g)			pH		
Maize:Lupine	Sample name	Fermentation time (weeks)					
		4	6	8	4	6	8
0:1	**L1**	4.5×10^4	7.6×10^6	5.3×10^6	4.49 ± 0.01*	4.46 ± 0.00*	4.44 ± 0.00*
1:4	**M1L4**	5.6×10^8	5.4×10^8	1.0×10^9	4.54 ± 0.00*	4.56 ± 0.01*	4.54 ± 0.00*
2:3	**M2L3**	5.4×10^8	8.3×10^8	8.3×10^9	4.57 ± 0.02*	4.60 ± 0.00*	4.59 ± 0.00*
1:1	**M1L1**	3.4×10^9	6.7×10^9	4.3×10^9	4.33 ± 0.00*	4.22 ± 0.01*	4.20 ± 0.00*
3:2	**M3L2**	2.9×10^9	6.2×10^9	5.4×10^9	4.42 ± 0.00*	4.49 ± 0.02*	4.49 ± 0.00*
4:1	**M4L1**	4.7×10^8	8.2×10^9	4.7×10^9	4.53 ± 0.01*	4.58 ± 0.01*	4.58 ± 0.00*
1:0	**M1**	4.5×10^8	8.4×10^8	6.0×10^8	4.52 ± 0.00*	4.23 ± 0.00*	4.20 ± 0.00*

*$p<0.05$; Values marked in blue meet the adopted criteria for LAB counts in the range of 10^7–10^9. Values marked in yellow meet the adopted criteria for pH values in the range of 3.8–4.4. The best samples based on the LAB counts and pH values are marked in green.

As shown in Fig. 1, the protein content decreased with larger amounts of maize in the ensiled mixture, while the acidity of all samples (except the lupine sample after 4 weeks) was within the desired range of 6.0 to 8.0%. The samples with a predominance of maize (3:2 and 4:1) had high LAB counts, but the pH values were not within the required range. The biomass consisting of maize only (1:0) showed acceptable pH, but the LAB count was lower than for the other samples. The best results were achieved in the 1:1 mixture (M1L1), which showed both high lactic acid bacteria and an appropriate pH. This suggests that equivalent proportions of maize and lupin biomass may be optimal for fermentation under the studied conditions. Significant differences were observed (Tukey test, $p<0.05$), particularly in protein enrichment and acidification of the substrates, reflecting the effectiveness of the nutritional and preservation parameters of the silages.

The initial assessment of spontaneous fermentation highlighted the importance of maize–lupine ratios for achieving optimal lactic acid bacteria abundance and pH levels. Based on the results, specific bacterial strains could then be selected that further enhance the fermentation process and meet recommended standards for silage production.

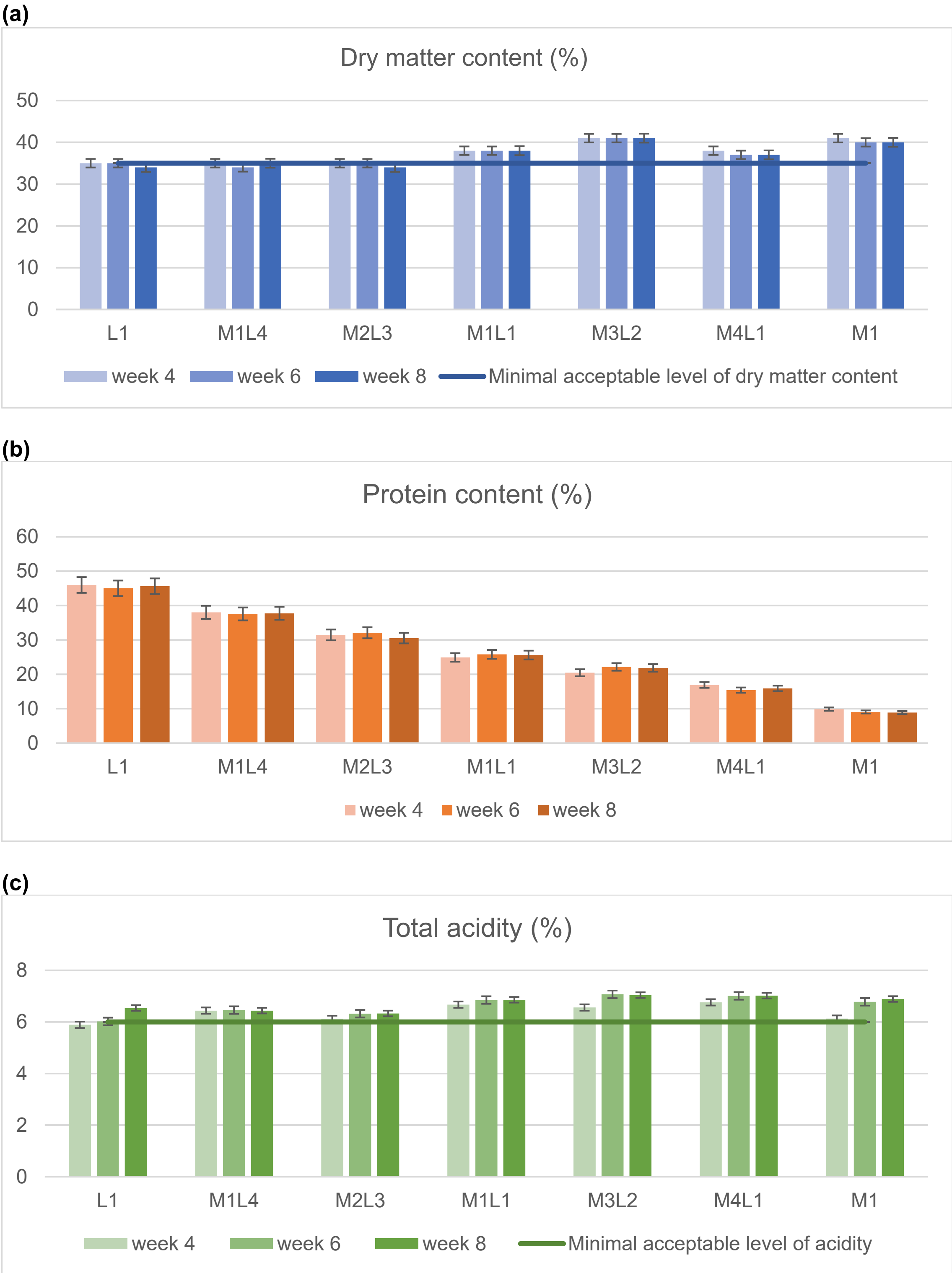


Fig. 1. Physicochemical characteristics (a) dry matter content, b) protein content and c) total acidity) of mixtures with different proportions of maize and lupine fermented spontaneously for 4, 6, or 8 weeks

Controlled Fermentation using a Selected Strain

The development of microorganisms during the fermentation process depends on various factors. These factors include the amount of soluble sugars in water and the dry matter content of fermenting materials, the temperature in the mass undergoing the ensiling process, the rate of production of organic acids and the decrease in pH, the presence of undesirable bacteria, the presence of oxygen, and the buffering capacity of the plants. When the pH is too high, some plants undergoing fermentation use up amino acids, especially basic amino acids. Biochemical transformations carried out by microorganisms may lead to deamination and decarboxylation of amino acids. Insufficiently well-ensiled plant biomass may contain toxic biogenic amines and undesirable organic acids. Achieving the appropriate pH is a crucial part of the process of feed production (Radkowski 2016). The pH of feed can have an impact on animal development. The pH value also regulates the passage of feed through the stomach. Low pH slows the passage of food, so digestive juices can act on the feed for longer. This ensures better digestion and higher feed assimilation. Additionally, feeding animals with appropriately acidic feed reduces the likelihood of animals developing intestinal problems (Zhang *et al.* 2023). Consequently, pH determination is a cost-effective and widely accessible technique commonly used in agrotechnics for feed quality assessment.

Of the 55 tested strains, 11 strains isolated from wheat and rye cereals met the criteria for effective ensiling of maize or lupine: 9, 21, 26, 31, 39, 44, 45, 46, 47, 48, and 50. All of the strains isolated from maize and lupine yielded satisfactory results (Table 2), with LAB content and pH values within the standard recommended ranges for the production of silage for animals. The ensiled biomass from these strains was analyzed to determine the content of dry matter, protein, and total acidity in the silages. Interestingly, a larger number of strains demonstrated better ensiling performance with lupine than with maize, despite the favorable physicochemical properties of maize, which typically support efficient fermentation (Fig. 2, Fig. 3).

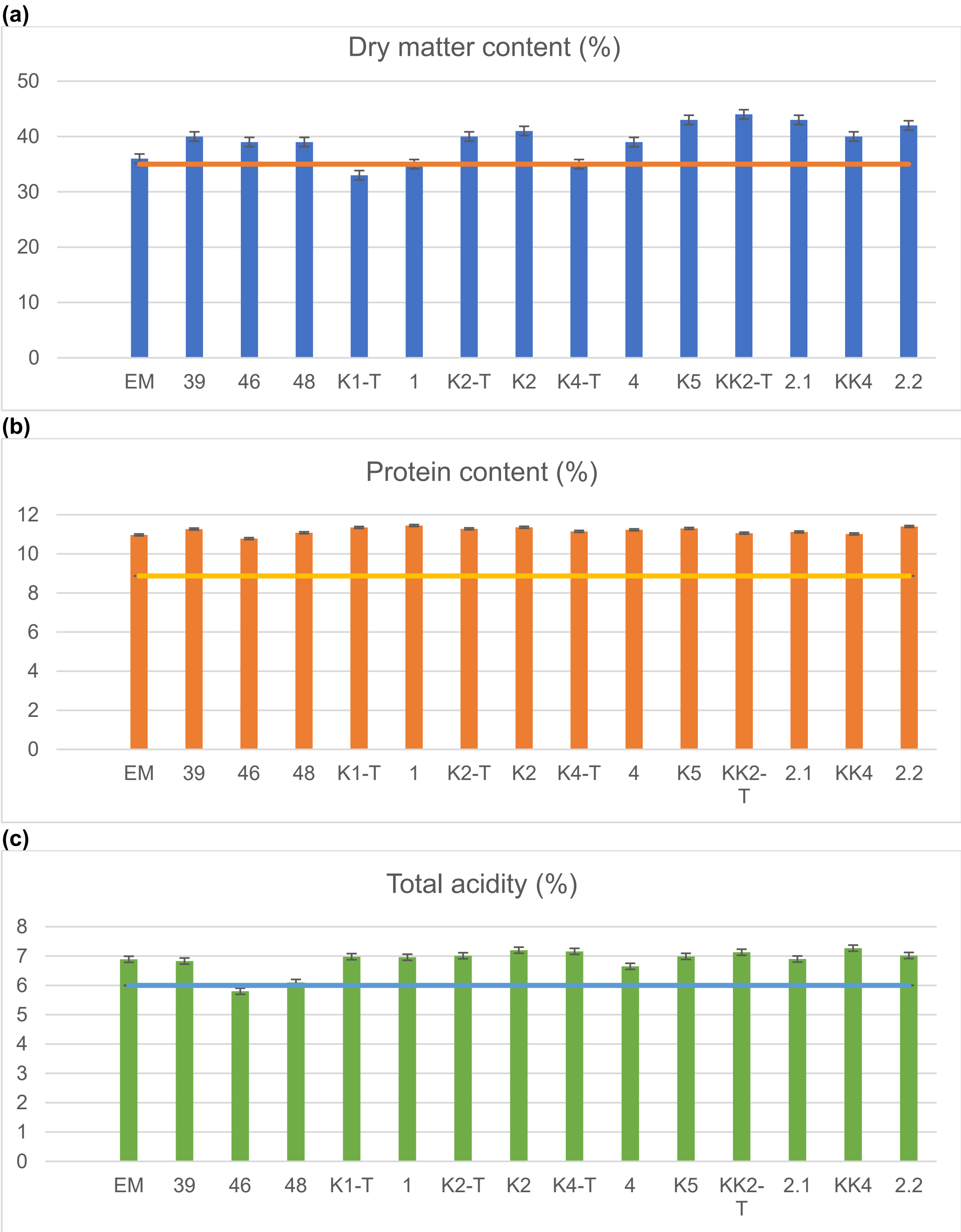
Table 2. Microbiological Growth and pH Values of Silages Obtained Using Selected Strains

Sample	Microorganism	Maize		Lupine	
		LAB (cfu/g)	pH	LAB (cfu/g)	pH
Dry biomass	Environmental microorganisms	1.8×10^8	-	5.3×10^4	-
Meal after spontaneous fermentation	Environmental microorganisms	6.0×10^8	4.20 ± 0.00*	5.3×10^6	4.44 ± 0.00*
Meal after controlled fermentation	strain KBS1	3.0×10^8	5.71 ± 0.00*	1.2×10^8	4.43 ± 0.00*
	strain 3	5.9×10^7	4.59 ± 0.00*	2.0×10^8	4.61 ± 0.00*
	strain KBS4	4.9×10^7	5.20 ± 0.00*	3.5×10^8	4.85 ± 0.00*
	strain 5	1.1×10^6	4.66 ± 0.00*	8.6×10^8	4.51 ± 0.00*
	strain 6 (POP5)	1.3×10^8	4.49 ± 0.00*	1.6×10^9	4.52 ± 0.00*
	strain 7	1.8×10^8	4.53 ± 0.00*	1.02×10^9	4.45 ± 0.00*
	strain 8 (POP4)	2.2×10^8	4.45 ± 0.00*	3.2×10^7	4.45 ± 0.00*
	strain 9	3.5×10^7	4.53 ± 0.01*	3.6×10^7	3.85 ± 0.00*
	strain 16	1.3×10^6	5.32 ± 0.00*	9.6×10^7	4.48 ± 0.00*
	strain 21	3.4×10^7	4.60 ± 0.01*	1.9×10^8	4.13 ± 0.00*
	strain 22	1.7×10^7	4.50 ± 0.01*	2.4×10^9	5.14 ± 0.00*
	strain 25	4.9×10^5	4.56 ± 0.00*	3.2×10^8	4.45 ± 0.00*
	strain 26	9.8×10^7	4.50 ± 0.00*	2.8×10^7	4.01 ± 0.00*
	strain 28	1.9×10^8	4.54 ± 0.00*	1.0×10^9	4.48 ± 0.00*
	strain 31	3.8×10^8	4.47 ± 0.00*	1.5×10^7	3.83 ± 0.00*

	strain 35	2.7×10^8	4.56 ± 0.00*	4.0×10^6	4.44 ± 0.00*
	strain 36	4.6×10^8	4.51 ± 0.00*	1.9×10^9	3.60 ± 0.00*
	strain 39	4.3×10^9	4.18 ± 0.00*	4.0×10^4	3.99 ± 0.00*
	strain 40	5.7×10^7	4.39 ± 0.00*	3.2×10^9	4.96 ± 0.00*
	strain 41	5.6×10^8	4.49 ± 0.00*	1.5×10^7	4.58 ± 0.00*
	strain 42	6.7×10^7	4.58 ± 0.00*	9.0×10^7	4.71 ± 0.00*
	strain 44	5.6×10^6	4.20 ± 0.00*	6.8×10^7	4.12 ± 0.00*
	strain 45	5.3×10^7	4.42 ± 0.00*	1.8×10^7	4.02 ± 0.00*
	strain 46	5.6×10^8	4.40 ± 0.00*	2.8×10^6	3.81 ± 0.00*
	strain 48	6.7×10^7	4.20 ± 0.00*	2.5×10^4	4.11 ± 0.00*
	strain 49	5.4×10^5	4.18 ± 0.01*	2.1×10^8	4.57 ± 0.00*
	strain 50	6.7×10^5	4.57 ± 0.00*	4.9×10^7	4.05 ± 0.00*
	strain 51	5.4×10^5	4.77 ± 0.02*	4.5×10^8	4.61 ± 0.00*
	strain 52	6.2×10^4	5.05 ± 0.02*	4.0×10^6	4.69 ± 0.00*
	strain 53	7.8×10^5	5.54 ± 0.00*	3.0×10^9	4.97 ± 0.01*
	strain 56	5.8×10^6	4.57 ± 0.00*	5.6×10^5	4.68 ± 0.02*
	strain 60	4.9×10^7	4.52 ± 0.00*	1.2×10^8	4.52 ± 0.00*
	strain 61	5.2×10^6	5.04 ± 0.00*	3.0×10^6	4.45 ± 0.00*
	strain 62	5.3×10^8	4.55 ± 0.00*	1.4×10^6	4.50 ± 0.00*
	strain 63	5.9×10^8	5.02 ± 0.00*	3.6×10^7	4.52 ± 0.00*
	strain 64	6.3×10^9	4.49 ± 0.00*	15×10^9	4.45 ± 0.00*
	strain 65	1.9×10^8	4.62 ± 0.00*	1.4×10^5	4.65 ± 0.00*
	strain 66	2.1×10^9	4.48 ± 0.00*	1.6×10^5	4.65 ± 0.00*
	strain K1-T	4.2×10^8	4.34 ± 0.00*	3.2×10^7	4.40 ± 0.00*
	strain 1	7.8×10^8	3.98 ± 0.00*	1.8×10^8	4,32 ± 0.00*
	strain K2-T	3.3×10^8	4.20 ± 0.00*	1.2×10^8	4.34 ± 0.00*
	strain K2	6.5×10^8	4.12 ± 0.01*	4.4×10^8	4.24 ± 0.00*
	strain K4-T	7.2×10^8	4.04 ± 0.01*	5.6×10^8	4.22 ± 0.00*
	strain 4	5.4×10^8	4.02 ± 0.00*	5.4×10^7	4.32 ± 0.00*
	strain K5-T	4.8×10^8	4.44 ± 0.00*	3.6×10^8	4.36 ± 0.00*
	strain K5	1.2×10^8	4.40 ± 0.00*	1.1×10^7	4.44 ± 0.00*
	strain KK2-T	1.3×10^8	4.32 ± 0.01*	6.9×10^8	4.35 ± 0.01*
	strain 2.1	4.8×10^8	3.94 ± 0.00*	6.6×10^8	4.02 ± 0.01*
	strain KK4-T	3.4×10^5	4.88 ± 0.00*	4.3×10^4	5.16 ± 0.01*
	strain KK4	6.8×10^8	4.06 ± 0.00*	4.6×10^8	4.23 ± 0.00*
	strain 2.2	4.2×10^8	4.12 ± 0.00*	3.8×10^9	4.12 ± 0.00*
	strain L4-T	3.2×10^4	5.02 ± 0.00*	2.7×10^4	5.87 ± 0.00*
	strain L4	1.8×10^5	4.68 ± 0.00*	4.0×10^5	5.04 ± 0.00*
	strain L5-T	1.2×10^7	4.60 ± 0.00*	1.2×10^9	4.12 ± 0.00*
	strain L5	2.6×10^7	4.54 ± 0.00*	1.4×10^9	4.01 ± 0.00*

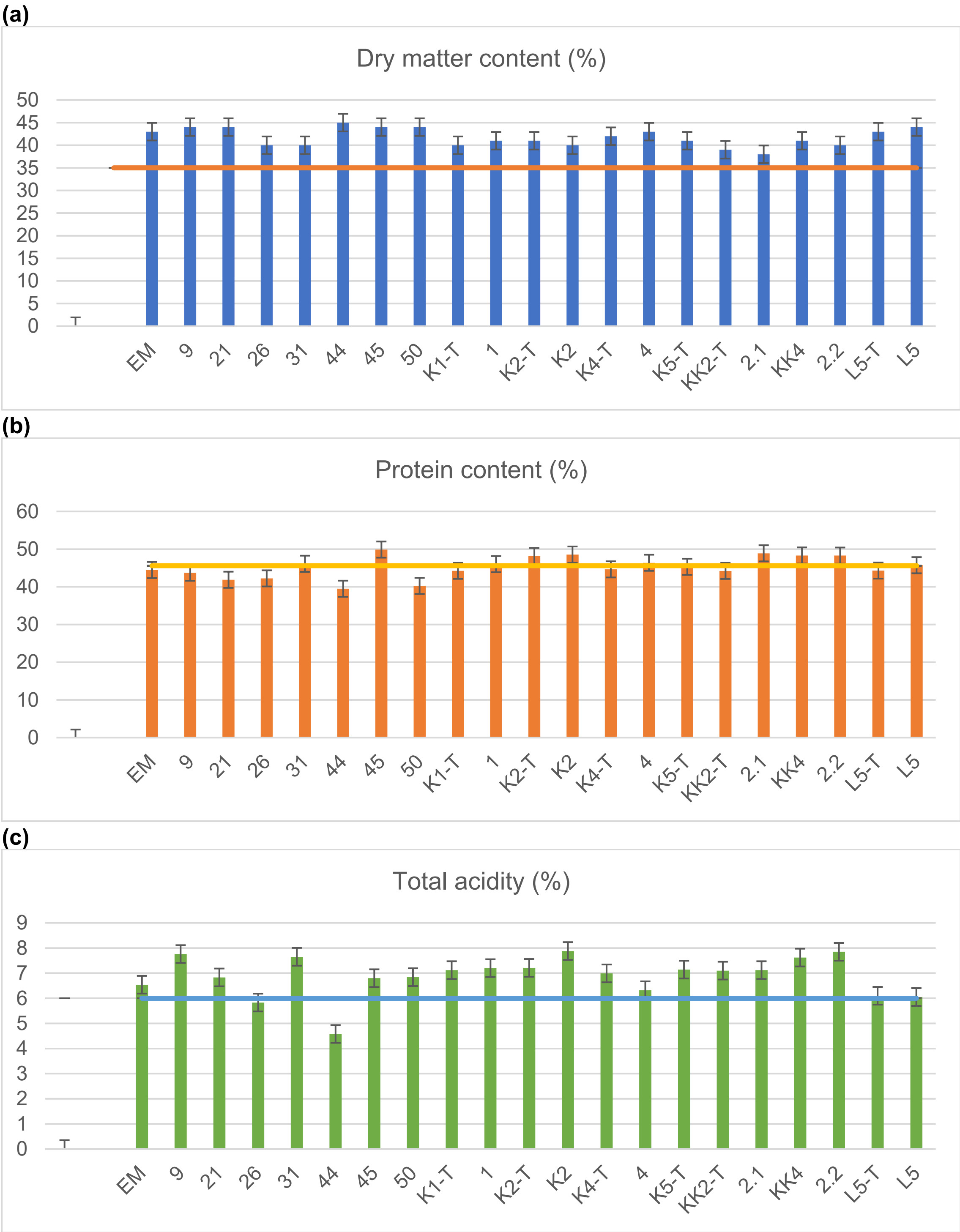
*$p<0.05$: Note: Values marked in blue meet the adopted criteria for LAB counts in the range of 10^7–10^9. Values marked in yellow meet the adopted criteria for pH values in the range of 3.8–4.4. The best samples based on the LAB counts and pH values are marked in green.

The nutritional value of silage depends primarily on the species of plants used and on the development stage of the plants when they are harvested. The stage of plant development also affects the chemical composition of the final product, including the content of protein, fibers, sugars, fats, vitamins, and minerals. How the silage is obtained is also important, as well as how it is stored—whether as wet silage, fermented silage, or dried silage. Each of these forms of silage can constitute an important component of the diet for farm animals and help provide them with the necessary nutrients (Yang *et al.* 2021). The form of animal silage is selected depending on the animal species, season, availability of raw materials, and farmer preferences. Both the quality and nutritional value of silage may change, depending on factors such as weather conditions, soil, fertilization, and harvesting and storage methods. Therefore, regular examination and testing of silage is important to ensure that animals receive adequate nutrition.



Note: EM - environmental microorganisms

Fig. 2. Physicochemical characteristics (a) dry matter content, b) protein content, and c) total acidity) of maize fermented with selected strains obtaining silage with the desired pH parameters and LAB counts



Note: EM - environmental microorganisms

Fig. 3. Physicochemical characteristics (a) dry matter content, b) protein content, and c) total acidity) of lupine fermented with selected strains obtaining silage with the desired pH parameters and LAB counts

The selected environmental strains isolated from wheat and rye cereals showed greater potential for ensiling lupine grain meal than for maize meal, as indicated by the number of silages meeting the pH value criteria and the fact that seven of the strains could be used for ensiling lupine, and only three for ensiling maize. Adding tap water to the grain in the ratio of 2:1, resulting in a dry matter content of 35 to 45%, had a positive effect on the process of ensiling the substrates. The ensiling results with this addition were within the desired range, from 6.10 ± 0.021 to 7.27 ± 0.028 in the case of maize silage and from 6.05 ± 0.690 to 7.85 ± 0.008 in the case of lupine samples. The ensiling results for spontaneous fermentation of substrates were also within the norm. However, controlled fermentation has advantages, as the process conditions may vary depending on the substrate used and how it is prepared (Li *et al.* 2023; Yi *et al.* 2023). The acidity of silages obtained in the spontaneous fermentation process is influenced by the number of LAB produced. In the present study, the LAB count in the case of maize was 6×10^8 and in the case of lupine 5.3×10^6, which resulted in differences in the pH value. The pH obtained for maize was 4.20 and the pH obtained for lupine was 4.44. These results do not meet the adopted feed standards. The results falling within the accepted range of pH, dry matter, and acidity are marked in colors in Table 2. Significant differences were observed (47 test, $p<0.05$), particularly in protein enrichment and acidification of the substrates, reflecting the effectiveness of specific strains in enhancing the nutritional and preservation parameters of the silages.

After determining the physicochemical parameters of the silages, such as pH, dry matter content, total acidity, and the LAB count, it was possible to conduct an analysis to select strains with the best fermentation properties. This is crucial because it allows for the selection of microorganisms that not only effectively reduce pH, but also stabilize the fermentation process and ensure silages with high nutritional value.

Metabolic Abilities and Proteomic Similarity of Selected Strains and Isolated Strains from Fermented Maize and Lupine

The next step was to assess the metabolic and proteomic capabilities of the selected strains, which allows for a deeper understanding of their mechanisms of action. Metabolic analysis was used to assess the ability of the strains to produce organic acids (*e.g.* lactic, acetic), which are crucial for silage stabilization and quality. Proteomic analysis was used to identify proteins associated with adaptation to specific fermentation conditions and assess the biological effectiveness of the strains.

The proteome similarity of the isolated strains compared to the control samples from the MALDI-TOF MS database showed that most strains were similar to the strains *L. brevis, L. buchneri*, and *P. acidilactici* (Table 1). All isolated strains were of plant origin (da Silva *et al.* 2021; Bao *et al.* 2023). The strains were also evaluated for their ability to ferment and grow on various substrates, such as simple sugars (*e.g.*, D-glucose, D-fructose), disaccharides (*e.g.*, cellobiose, maltose), and polysaccharides (*e.g.*, starch, glycogen). The strains showed varying ability to utilize these substrates, which reflects their adaptive metabolic potential. The most versatile strains were similar to *L. brevis* and fermented a wide range of substrates, including D-glucose, cellobiose, maltose, and trehalose. Other strains, similar to *L. buchneri* and *P. acidilactici*, also showed metabolic activity towards several basic substrates, although their ranges of ability were more limited compared to *L. brevis*. Strains similar to the *Pediococcus* genus, although they had limited metabolic capabilities, were characterized by high activity towards basic sugars such as D-glucose and sucrose, which may indicate their specialization for fermenting simple substrates. The incubation time (24 h or 48 h) significantly affected the fermentation abilities of the studied strains. In most cases, longer fermentation time (48 h) led to the fermentation of a larger number of substrates, suggesting gradual activation of the full metabolic potential of the bacteria.

Table 3. Metabolic Activity and Proteomic Similarity of Selected Strains Isolated from Wheat and Rye, Maize, and Lupine

	Time (h)	control	L-arabinose	Ribose	D-xylose	Galactose	D-Glucose	D-Fructose	D-Mannose	Mannitol	Methyl-D-glucoside	Glucosamine	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	D-sacharose	Sucrose	Trehalose	D-Raffinose	Starch	Glycogen	B-Gentiobiose	D-turanose	Gluconate	Strain	Proteomic Similarity (%)
31	24						+	+	+				+	+	+							+						+		*L. brevis*	99.4
	48						+	+	+	+			+	+	+							+						+			
39	24		+	+			+	+	+				+	+	+			+					+					+		*L. brevis*	99.9
	48		+	+		+	+	+	+				+	+	+			+					+					+	+		
45	24		+	+			+	+	+				+	+	+							+	+		+			+		*L. brevis*	89.5
	48		+	+			+	+	+				+	+	+							+	+		+			+			
48	24			+	+		+	+	+				+	+	+							+	+					+		*L. brevis*	99.9
	48			+	+		+	+	+				+	+	+							+	+					+			
K1-T	24						+	+	+			+					+	+												*P. acidilactici*	81.7
	48		+	+			+	+	+			+					+	+													
1	24			+		+	+	+	+			+					+													*L. buchneri*	75.5
	48			+		+	+	+	+			+			+	+	+								+						
K2-T	24		+				+	+	+			+					+	+												*P. acidilactici*	87.5
	48		+	+			+	+	+			+					+	+													
K2	24		+	+		+	+	+	+	+																				*L. buchneri*	77.4
	48		+	+		+	+	+	+	+																					
K4-T	24						+	+	+			+					+													*Pediococcus* sp.	92.7
	48		+	+			+	+	+			+					+	+													
4	24						+	+	+								+	+							+					*L. brevis*	76.3
	48						+	+	+								+	+							+						
K5-T	24						+	+	+			+					+	+												*P. acidilactici*	92.3
	48		+	+			+	+	+			+					+	+													
K5	24			+	+		+	+	+			+						+			+	+						+		*L. buchneri*	60.7
	48			+	+		+	+	+			+				+	+	+			+	+						+			
KK2-T	24			+			+	+	+			+					+	+												*P. acidilactici*	69.7
	48		+	+			+	+	+			+					+	+													
2.1	24						+	+	+								+	+							+					*P.acidilactici*	59.4
	48						+	+	+								+	+							+						
KK4	24		+	+		+	+	+	+	+						+	+	+		+	+	+			+			+		*P. acidilactici*	71.4
	48		+	+		+	+	+	+	+		+				+	+	+		+	+	+			+			+			
2.2	24					+	+	+	+			+						+				+								*L. pentosus/ L.plantarum*	90.4
	48					+	+	+	+			+						+				+									
L5-T	24						+	+	+									+												*Pediococcus* sp.	76.3
	48		+	+			+	+	+			+					+	+													
L5	24					+	+	+	+			+			+		+	+												*L. buchneri*	73.5
	48					+	+	+	+			+			+		+	+													

"+" – positive test result compared to control sample (growth or fermentation)

Their metabolic diversity allows for the use of the strains in various industrial applications, especially in the fermentation of cereals and legumes. The natural sugar composition of maize and lupin determines the selection of bacteria for fermentation processes (Coda *et al.* 2014; Kanengoni *et al.* 2015). Strains similar to *L. brevis* are versatile, with the ability to ferment many sugars found in both maize and lupin, including glucose, fructose, sucrose, L-arabinose, and D-xylose. This allows such strains to be used in a wide range of fermentation processes. Strains similar to *P. acidilactici* and *L. buchneri* also ferment glucose, fructose, and sucrose, making them effective in maize processing. Of particular note is the ability of some strains to ferment raffinose, which is a characteristic of legumes such as lupin. The ability of the bacteria to degrade galactose and hemicelluloses (*e.g.*, L-arabinose and D-xylose) highlights their usefulness in optimizing fermentation of lupin (Zhang and Vadlani 2015; Qiu *et al.* 2017).

Survival of Environmental Strains Isolated from Maize and Lupine in Conditions Simulating Ruminant Intestinal Transit

The survival of the environmental strains isolated from maize and lupine was examined in conditions simulating intestinal transit. Although the strains had already been selected based on their physicochemical properties, metabolic capabilities, and proteomic similarity, it was important to assess how they would cope with the difficult conditions prevailing in the intestines, such as low pH, the presence of bile and digestive enzymes, and variable temperatures. Understanding whether strains are able to survive and grow in these conditions is especially important for probiotics, because one of the requirements for probiotic microorganisms is the ability to survive in the digestive tract, where they exert their positive health effects (Naissinger da Silva *et al.* 2021). Testing survival under such conditions can also contribute to a better understanding of the adaptive mechanisms that allow microorganisms to adapt to changing conditions. It furthermore provides a more complete picture of their potential, especially with regard to their physicochemical and metabolic properties, which were assessed during selection. Although these properties are important, the conditions prevailing in the digestive tract can induce changes in the functioning of microorganisms, making it crucial to assess their actual ability to survive in other organisms. The isolated strains of bacteria were suspended in solutions of crushed raw materials (maize or lupine). Survival assessment was carried out in conditions reflecting the conditions of intestinal transit. The survival of the strains was monitored over six consecutive stages. Each strain demonstrated the ability to survive in the conditions prevailing in the simulated gastrointestinal tract. Strains with a survival rate of 70% or higher at pH=2 were selected for further studies, because this is the value prevailing in the stomach of monogastric animals and is a critical factor in determining the sensitivity of bacteria to changes in the gastrointestinal environment (Table 4, Table A1). When examining the survival of bacterial strains isolated from maize and lupine, it was shown that the strains marked with the following symbols had the highest survival, falling within the accepted survival range of above 70%: 1 (82%), 4 (80%), 2.1 (81%), and 2.2 (70%). In Table 4 (Table A1), values indicate statistically significant differences in the survival of bacterial strains isolated from maize and lupine at various stages of the simulated digestive system (Tukey test, $p<0.05$). Significant changes were observed primarily after exposure to low pH conditions and bile salts, reflecting the varying tolerance of the strains to gastrointestinal stress. Strains 1, 4, 2.1, and 2.2 demonstrated superior survival rates, maintaining above 70% viability after completing stage VI at pH 2.0, suggesting enhanced resilience compared to the other tested isolates.

Table 4. Survival of Bacterial Strains Isolated from Maize and Lupine in the Simulated Digestive System

Stage	**I**	**II**	**III**	**IV**			**V**			**VI**		
Factors Acting on Selected Strain	**Solution A**	**pepsin and lysozyme solution**	**pepsin and lysozyme solution (after 20 minutes of incubation)**	**low pH (after 30 minutes of incubation)**			**Solution with salts bile and pancreatin**			**Solution with salts bile and pancreatin (after 120 minutes of incubation)**		
				pH 2.0	**pH 3.0**	**pH 4.0**	**pH 2.0**	**pH 3.0**	**pH 4.0**	**pH 2.0**	**pH 3.0**	**pH 4.0**
Strain	**Log number of bacteria [CFU/ml] (DS)****											
1	8.67 ± 0.06	8.96 ± 0.06	9.17 ± 0.04*	7.94 ± 0.02*	8.90 ± 0.02*	8.68 ± 0.02*	7.09 ± 0.11	8.88 ± 0.07	8.81 ± 0.01*	7.15 ± 0.04*	8.91 ± 0.01*	8.90 ± 0.07
Survival after stage VI of the experiment at pH=2 compared to stage I: 82%												
4	878 ± 0.06	8.66 ± 0.06	8.87 ± 0.04*	7.54 ± 0.02*	8.60 ± 0.02*	8.68 ± 0.03*	6.89 ± 0.11	8.12 ± 0.07	8.81 ± 0.06	7.05 ± 0.04*	8.81 ± 0.03*	8.70 ± 0.06
Survival after stage VI of the experiment at pH=2 compared to stage I: 80%												
2.1	8.82 ± 0.50	8.86 ± 0.04*	8.56 ± 0.04*	6.94 ± 0.02*	8.90 ± 0.02*	8.68 ± 0.02*	7.09 ± 0.11	8.88 ± 0.07	8.81 ± 0.01*	7.15 ± 0.04*	8.91 ± 0.01*	8.90 ± 0.07
Survival after stage VI of the experiment at pH=2 compared to stage I: 81%												
2.2	8.98 ± 0.07	8.63 ± 0.27	8.80 ± 0.05	8.63 ± 0.01*	8.49 ± 0.03*	9.00 ± 0.02*	6.32 ± 0.21	8.73 ± 0.02*	8.93 ± 0.14	6.27 ± 0.06	8.35 ± 0.01*	8.94 ± 0.09
Survival after stage VI of the experiment at pH=2 compared to stage I: 70%												

*p<0.05

Genetic Identification of Selected Strains

The strains selected based on all previously described criteria were submitted to genetic identification. Advanced genetic methods including DNA isolation, sequencing, and bioinformatic analysis were used to study the environmental strains. Genetic analysis of environmental strains isolated from maize (1, 4, and 2.1) and lupine (2.2) revealed diverse results in taxonomic identification, as well as differences in parameters such as genome coverage and the percentage of mapped reads (Table 5). The microscopic observations made allowed to determine the morphology of the bacteria, which contributed to the identification of the bacteria.

Table 5. Genetic Identification of Selected Strains

Strain	Taxonomy - metaQuast	Taxonomy – 16S rRNA vs BLAST NCBI	Genome fraction (%)	Mapped reads (%)
1	*L. buchneri* 1	*L. buchneri* 1	94.9	94.8
4	*P. acidilactici* 4	*P. acidilactici* 4	81.3	45.4
2.1	*L. buchneri* 2.1	*L. buchneri* 2.1	91.6	96.4
2.2	*P. acidilactici* 2.2	*P. acidilactici* 2.2	80.9	11.6

Strains 1 and 2.1 were identified as *Lactobacillus buchneri* by both metaQuast and 16S rRNA analysis (using BLAST NCBI). These strains were characterized by high genome coverage (94.9% for 1 and 91.6% for 2.1) and a high percentage of mapped reads (94.8% and 96.4%, respectively). These results indicate high sequencing quality and strong similarity to the reference *L. buchneri* genome.

Strain 4, identified as *Pediococcus acidilactici* by both metaQuast and 16S rRNA analysis, displayed features that set it apart from the *L. buchneri* strains 1 and 2.1. It showed lower genome coverage (81.3%) and a markedly lower percentage of mapped reads (45.4%), suggesting that large portions of its genome differ from the reference sequences. This discrepancy may be due to the presence of unique genetic elements, such as plasmids or chromosomal rearrangements, which are absent in the reference genome. These differences could also reflect adaptive changes in the 4 genome, possibly shaped by its environmental origin from maize, which may include exposure to specific metabolites, varying pH levels, or microbial competition.

Similarly, strain 2.2 was assigned to *P. acidilactici* by both identification methods. It exhibited genome coverage of 80.9% and only 11.6% of mapped reads. Despite its taxonomic classification, the low percentage of mapped reads indicates substantial genetic divergence from the reference strain. 2.2 was isolated from lupin, a leguminous plant with a distinctive microbiome and specific environmental conditions, which may have driven the evolution of strain-specific genes in 2.2. These adaptations might include metabolic capabilities or stress responses tailored to the lupin environment.

L. buchneri and *P. acidilactici* are LAB widely used as inoculants in the ensiling of maize. *L. buchneri* is valued for its ability to produce 1,2-propanediol and propionic acid, which suppress fungal and yeast growth, enhancing aerobic stability. *P. acidilactici* is known for its tolerance to high osmotic pressure, making it suitable for materials with high dry matter content. Use of these strains in silage improves lactic fermentation, reduces pH, inhibits spoilage organisms, and enhances feed stability and quality (da Silva *et al.* 2018; Romero *et al.* 2021; Kleinschmit and Kung 2006).

Although *P. acidilactici* strain 2.2 has low mapped reads compared to the reference, its identification suggests it may still play a role in the lupin microbiome, potentially participating in fermentation or plant protection. Moreover, *P. acidilactici* has been linked to probiotic effects, including protection against *Salmonella enterica* in animal models (Abatemarco *et al.* 2018; Krooneman *et al.* 2002), indicating possible broader functional capabilities beyond its plant-associated habitat.

Antagonism of Bacteria Isolated from Fermented Maize and Lupine

The bacteria isolated from maize and lupine that showed the highest survival during intestinal passage showed no antagonistic effects during tests on plates, where their mutual interactions were observed (Table A2). No zones of growth inhibition were observed on the plates. This lack of antagonistic behavior indicated the possibility of creating a feed component inoculated with a mixture of strains.

Fermentation of Maize and Lupine Biomass Using Selected LAB Strain

To obtain a balanced feed component, silage was prepared from a mixture of maize and lupine inoculated with selected strains. After selecting the LAB strains, they were used to ferment maize and lupine biomass and a maize-lupine mixture. The LAB count, silage pH, dry matter content, protein content, and total acidity were determined again (Table 6). The results within the accepted ranges for pH, dry matter, and total acidity are in bold. After using *L. buchneri* 1, *P. acidilactici* 4, *L. buchneri* 2.1 and *P. acidilactici* 2.2 strains for fermentation, silages were obtained in which the pH, dry matter content, and acidity were within the assumed ranges (pH 3.8 to 4.4; dry matter 35 to 45%; and total acidity 6.0 to 8.0%) in each sample. The amounts of LAB ranged from 6.4×10^{7} for the fermented lupine sample using the *L. buchneri* bacterial strain to 5.2×10^{9} for the fermented maize–lupine mixture using all four selected strains in a 1:1:1:1 ratio. The highest cell count of 4.4×10^{9} CFU/mL and the highest protein content of 46.50% were observed in the mixture of strains 1, 4, 2.1, and 22. In the case of the mixture of maize and lupine (1:1), the cell count ranged from 6.4×10^{7} to 5.2×10^{9} CFU/mL and the pH ranged from 3.8 to 4.4, which are the optimal conditions for fermentation. The colors in Table 6 indicate the optimal results for acidity, protein, dry matter, and pH, as well as the number of CFU/mL, which confirms the high quality of fermentation. The results indicate that it is possible to create a feed component from maize and lupine using the selected LAB strains (Filya 2006; Li *et al.* 2022; Kim *et al.* 2021). The results show that various strains, particularly when combined, significantly impacted the LAB counts, pH, dry matter content, protein content, crude fiber content, and total acidity (Tukey test, $p<0.05$).

Although the present study demonstrates the effectiveness of selected LAB strains (*L. buchneri* 1, *P. acidilactici* 4, *L. buchneri* 2.1, and *P. acidilactici* 2.2) in improving the fermentation quality of maize and lupine biomass, economic feasibility remains an important consideration for potential large-scale application. Factors such as the cost of strain cultivation, inoculum preparation, and storage logistics must be taken into account. While the use of the selected strains contributed to optimal pH, dry matter content, total acidity, and microbial load, which are crucial for feed quality and stability, these benefits must be weighed against the additional production and operational expenses. Furthermore, effective storage methods, such as vacuum-sealed silage bags or silo tanks, are essential to maintain anaerobic conditions and preserve microbial viability over time. These methods may incur additional costs, but could also reduce feed losses and spoilage, potentially offsetting initial investments. Future research should include a cost–benefit analysis and long-term storage trials to assess the commercial viability and scalability of using the selected LAB strains in silage production. On an industrial scale, the inoculum could be cultivated on 5°Blg wort due to its lower cost, which further justifies the use of the selected LAB strains adapted to low-sugar environments.

Table 6. Fermentation of Maize and Lupine Biomass using Selected Isolated Strains of Lactic Acid Bacteria

Type of biomass	Strain used for fermentation	LAB (cfu/mL)	pH	Dry matter content (%)	Protein content (%)	Crude fiber content (%)	Total acidity (%)
Maize	Non-fermented	1.8×10^8	-	98 ± 0.00*	8.87 ± 0.117%	2,51 ± 0.015*	-
	L. buchneri 1	7.8×10^8	3.98 ± 0.00*	34 ± 0.00*	10.52 ± 0.041*	1.89 ± 0.032*	6.67 ± 0.120
	P. acidilactici 4	5.4×10^8	4.02 ± 0.00*	33 ± 0.00*	10.38 ± 0.050	1.85 ± 0.065	6.56 ± 0.041*
	L. buchneri 2.1	4.8×10^8	3.94 ± 0.00*	35 ± 0.00*	11.02 ± 0.071	1.78 ± 0.048*	6.72 ± 0.142
	P. acidilactici 2.2	4.2×10^8	4.12 ± 0.00*	34 ± 0.00*	10.06 ± 0.120	1.86 ± 0.065	6.30 ± 0.034*
	A mixture of 1, 4, 2.1 and 2.2 strains in equal quantities	5.4×10^9	3.90 ± 0.00*	35 ± 0.00*	10.54 ± 0.062	1.69 ± 0.235	6.86 ± 0.210
Lupine	Non-fermented	5.3×10^4	-	90 ± 0.00*	45, 61 ± 3.018%	15.36 ± 0.023*	-
	L. buchneri 1	1.8×10^8	4.32 ± 0.00*	34 ± 0.00*	38.34 ± 0.133	10.65 ± 0.068	6.12 ± 0.100
	P. acidilactici 4	5.4×10^7	4.32 ± 0.00*	33 ± 0.00*	37.54 ± 0.044*	11.28 ± 0.258	6.20 ± 0.051
	L. buchneri 2.1	6.6×10^8	4.02 ± 0.00*	36 ± 0.00*	35 ± 1.120	11.86 ± 0.064	6.44 ± 0.041*
	P. acidilactici 2.2	3.8×10^9	4.12 ± 0.00*	34 ± 0.00*	38 ± 0.050	9.68 ± 0.078	6.15 ± 0.121
	A mixture of 1, 4, 2.1 and 2.2 strains in equal quantities	4.4×10^9	3.89 ± 0.00*	37 ± 0.00*	46.50 ± 1.120	9.98 ± 0.022*	7.12 ± 0.032*
Maize and lupine in a ratio of 1:1	Non-fermented	2.7×10^6	-	94 ± 0.00*	27.03 ± 0.027*	9.23 ± 0.031*	-
	L. buchneri 1	6.4×10^7	4.15 ± 0.00*	34 ± 0.00*	24.68 ± 0.132	9.20 ± 0.025	6.38 ± 0.230
	P. acidilactici 4	6.3×10^8	4.23 ± 0.00*	39 ± 0.00*	22.45 ± 0.432	8.96 ± 0.020	6.20 ± 1.124
	L. buchneri 2.1	1.9×10^8	4.34 ± 0.00*	33 ± 0.00*	25.88 ± 0.343	8.25 ± 0.210	6.02 ± 0.244
	P. acidilactici 2.2	8.8×10^7	4.02 ± 0.00*	35 ± 0.00*	27.56 ± 0.043	8.36 ± 0.312	6.50 ± 0.051
	A mixture of 1, 4, 2.1 and 2.2 strains in equal quantities	5.2×10^9	4.20 ± 0.00*	39 ± 0.00*	27.12 ± 0.104	8.54 ± 0.542	6.05 ± 0.140

*$p<0.05$

Orange indicates acidity within the established range of 6.0–8.0%. Grey indicates dry matter content within the established range of 35–45%. Yellow indicates pH values within the established range of 3.8–4.4. Blue indicates CFU/mL higher than in the control samples. Green indicates strains used to produce silage from the substrates that met all the criteria listed in the table.

CONCLUSIONS

1. The lactic acid bacteria (LAB) strains isolated from maize and lupine biomass demonstrated better suitability for fermenting maize and lupine material compared to strains previously isolated from cereal-based sources such as wheat and rye sourdoughs, in terms of achieving desirable silage parameters. The effective strains included *L. buchneri* 1, *P. acidilactici* 4, *L. buchneri* 2.1, and *P. acidilactici* 2.2.
2. The absence of antagonistic interactions among the selected LAB strains allowed for their combined use in a single inoculant formulation. This approach led to improved and consistent silage characteristics—including pH, dry matter content, and microbial load—when applied to maize, lupine, or a 1:1 mixture of both substrates.
3. Spontaneous fermentation of maize and lupine mixtures (in varying proportions) also produced silage with acceptable pH, acidity, and dry matter values after 4 to 8 weeks. The 1:1 maize–lupine mixture yielded the most favorable results, particularly in terms of LAB count stability and pH control, suggesting its promise for future fermentation studies.
4. While these findings suggest potential benefits of using selected LAB strains for improving silage quality, the conclusions are limited to laboratory-scale fermentation under controlled conditions. Further research, including in vivo feeding trials and economic analysis, is necessary to fully assess the practical viability and sustainability of this approach in livestock production systems.

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APPENDIX

Table A1. Survival of Bacterial Strains Isolated from Maize and Lupine in the Simulated Digestive System

Stage	I	II	III	IV			V			VI		
Factors Acting on Selected	Solution A	pepsin and lysozyme solution	pepsin and lysozyme solution (after 20 minutes of incubation)	low pH (after 30 minutes of incubation)			Solution with salts bile and pancreatin			Solution with salts bile and pancreatin (after 120 minutes of incubation)		
				pH 2.0	pH 3.0	pH 4.0	pH 2.0	pH 3.0	pH 4.0	pH 2.0	pH 3.0	pH 4.0
Strain	Log number of bacteria [CFU/ml] (DS)**											
31 K	8,85 ± 0,20	8,80 ± 0,13	8,86 ± 0,12	8,76 ± 0,05			8,70 ± 0,08			8,84 ± 0,08		
31	8,80 ± 0,07	8,67 ± 0,04*	8,78 ± 0,03*	5,10 ± 0,05	5,12 ± 0,01*	5,28 ± 0,06	4,81 ± 0,06	5,17 ± 0,03*	5,78 ± 0,13	4,90 ± 0,04*	5,13± 0,05	5,43 ± 0,04*
Survival after stage VI of the experiment at pH=2 compared to stage I								56%				
39 K	8,78 ± 0,04*	8,72 ± 0,03*	8,70 ± 0,04*	8,71 ± 0,19			8,67 ± 0,07			8,70 ± 0,07		
39	8,70 ± 0,06	8,67 ± 0,03*	8,68 ± 0,05	4,78 ± 0,09	5,03 ± 0,08	5,30 ± 0,06	4,48 ± 0,13	5,15 ± 0,04*	6,02 ± 0,03*	4,56 ± 0,01*	5,02 ± 0,14	5,80 ± 0,16
Survival after stage VI of the experiment at pH=2 compared to stage I								52%				
45 K	8,50 ± 0,05	8,51 ± 0,03*	8,36 ± 0,04*	8,40 ± 0,13			8,50 ± 0,01*			8,64 ± 0,16		
45	8,34 ± 0,04*	8,38 ± 0,08	8,28 ± 0,12	4,67 ± 0,02*	5,04 ± 0,05	5,80 ± 0,03*	4,40 ± 0,04*	5,30 ± 0,06	6,14 ± 0,08	4,60 ± 0,04*	5,00 ± 0,08	5,90 ± 0,13
Survival after stage VI of the experiment at pH=2 compared to stage I								55%				
48K	7,90 ± 0,11	7,93 ± 0,08	7,80 ± 0,17	8,00 ± 0,10			8,04 ± 0,15			7,78 ± 0,02*		
48	7,70 ± 0,17	7,56 ± 0,08	7,60 ± 0,11	4,50 ± 0,09	5,12 ± 0,03*	6,30 ± 0,01*	4,58 ± 0,04*	5,25 ± 0,02*	6,12 ± 0,03*	4,44 ± 0,06	4,98 ± 0,09	6,00 ± 0,06
Survival after stage VI of the experiment at pH=2 compared to stage I								58%				
K1-T K	8,87 ± 0,08	8,80 ± 0,04*	8,91 ± 0,03*	8,80 ± 0,09			8,90 ± 0,07			8,84 ± 0,02*		

K1-T	8,80 ± 0,09	8,83 ± 0,03*	8,32 ± 0,03*	5,54 ± 0,15	5,30 ± 0,05	5,56 ± 0,04*	4,89 ± 0,11	5,56 ± 0,07	6,68 ± 0,05	5,02 ± 0,04*	6,12 ± 0,03*	6,86 ± 0,03*
Survival after stage VI of the experiment at pH=2 compared to stage I									57%			
1 K	8,89 ± 0,13	8,80 ± 0,05	8,78 ± 0,08	8,80 ± 0,11			8,72 ± 0,03*			8,86 ± 0,03*		
1	**8, 67 ± 0,06**	**8,96 ± 0,06**	**9,17 ± 0,04***	**7,94 ± 0,02***	**8,90 ± 0,02***	**8,68 ± 0,02***	**7,09 ± 0,11**	**8,88 ± 0,07**	**8,81 ± 0,01***	**7,15 ± 0,04***	**8,91 ± 0,01***	**8,90 ± 0,07**
Survival after stage VI of the experiment at pH=2 compared to stage I									**82%**			
K2-T K	8,80 ± 0,03*	8,78 ± 0,05	8,82 ± 0,01*	8,82 ± 0,02*			8,80 ± 0,03*			8,79 ± 0,08		
K2-T	8,14 ± 0,04*	7,89 ± 0,03*	8,22 ± 0,07	5,07 ± 0,05	5,32 ± 0,05	5,30 ± 0,04*	4,06 ± 0,03*	4,60 ± 0,07	6,78 ± 0,07	4,53 ± 0,04*	5,12 ± 0,08	6,76 ± 0,05
Survival after stage VI of the experiment at pH=2 compared to stage I									56%			
K2 K	8,88 ± 0,03*	8,78 ± 0,05	8,82 ± 0,01*	8,82 ± 0,02*			8,80 ± 0,03*			8,79 ± 0,08		
K2	8,68 ± 0,02*	8,59 ± 0,03*	8,32 ± 0,03*	5,07 ± 0,05	5,32 ± 0,07	6,12 ± 0,04*	4,16 ± 0,03*	5,20 ± 0,03*	6,58 ± 0,03*	4,28 ± 0,04*	6,02 ± 0,03*	6,86 ± 0,03*
Survival after stage VI of the experiment at pH=2 compared to stage I									49%			
K4-T K	8,56 ± 0,05	8,56 ± 0,05	8,42 ± 0,06	8,62 ± 0,05			8,65 ± 0,02*			8,75 ± 0,07		
K4-T	7,78 ± 0,05	7,69 ± 0,03*	7,22 ± 0,04*	4,47 ± 0,03*	5,12 ± 0,05	5,43 ± 0,04*	3,46 ± 0,03*	5,61 ± 0,03*	6,28 ± 0,04*	3,72 ± 0,04*	6,12 ± 0,03*	6,06 ± 0,06
Survival after stage VI of the experiment at pH=2 compared to stage I									47%			
4 K	8,98 ± 0,04*	8,88 ± 0,05	8,92 ± 0,03*	8,82 ± 0,04*			8,92 ± 0,02*			8,89 ± 0,05		
4	**8,78 ± 0,06**	**8,66 ± 0,06**	**8,87 ± 0,04***	**7,54 ± 0,02***	**8,60 ± 0,02***	**8,68 ± 0,03***	**6,89 ± 0,11**	**8,12 ± 0,07**	**8,81 ± 0,06**	**7,05 ± 0,04***	**8,81 ± 0,03***	**8,70 ± 0,06**
Survival after stage VI of the experiment at pH=2 compared to stage I									**80%**			
K5-T K	8,56 ± 0,08	8,68 ± 0,07	8,62 ± 0,04*	8,62 ± 0,02*			8,80 ± 0,06			8,59 ± 0,08		
K5-T	7,98 ± 0,04*	7,79 ± 0,05	7,32 ± 0,04*	4,07 ± 0,07	5,52 ± 0,02*	5,48 ± 0,04*	3,98 ± 0,03*	4,36 ± 0,04*	5,78 ± 0,07	4,56 ± 0,04*	5,18 ± 0,07	6,66 ± 0,05
Survival after stage VI of the experiment at pH=2 compared to stage I									57%			
K5 K	8,46 ± 0,12	8,48 ± 0,05	8,52 ± 0,04*	8,50 ± 0,02*			8,44 ± 0,05			8,42 ± 0,04*		
K5	8,10 ± 0,40	7,89 ± 0,02*	8,02 ± 0,02*	5,17 ± 0,05	5,42 ± 0,06	5,50 ± 0,05	5,01 ± 0,02*	5,62 ± 0,02*	6,18 ± 0,04*	5,42 ± 0,04*	6,02 ± 0,03*	6,76 ± 0,04*

Survival after stage VI of the experiment at pH=2 compared to stage I										67%		
KK2-T K	8,64 ± 0,11	8,58 ± 0,03*	8,62 ± 0,07	8,42 ± 0,02*			8,40 ± 0,04*			8,49 ± 0,06		
KK2-T	8,34 ± 0,04*	8,09 ± 0,03*	7,89 ± 0,03*	5,77 ± 0,05	5,38 ± 0,05	5,50 ± 0,04	5,06 ± 0,03*	5,64 ± 0,04*	6,18 ± 0,04*	5,19 ± 0,09	5,56 ± 0,04*	6,72 ± 0,02*
Survival after stage VI of the experiment at pH=2 compared to stage I										62%		
2.1 K	8,82 ± 0,30	8,88 ± 0,04*	8,82 ± 0,06	8,72 ± 0,05			8,76 ± 0,04*			8,79 ± 0,05		
2.1	**8,82 ± 0,50**	**8,86 ± 0,04***	**8,56 ± 0,04***	**6,94 ± 0,02***	**8,90 ± 0,02***	**8,68 ± 0,02***	**7,09 ± 0,11**	**8,88 ± 0,07**	**8,81 ± 0,01***	**7,15 ± 0,04***	**8,91 ± 0,01***	**8,90 ± 0,07**
Survival after stage VI of the experiment at pH=2 compared to stage I										**81%**		
KK4 K	8,70 ± 0,06	8,73 ± 0,03*	8,72 ± 0,04*	8,65 ± 0,02*			8,63 ± 0,03*			8,73 ± 0,03*		
KK4	8,40 ± 0,60	7,82 ± 0,03*	7,22 ± 0,07	4,67 ± 0,05	5,38 ± 0,05	5,48 ± 0,04*	5,16 ± 0,03*	5,60 ± 0,04*	6,18 ± 0,04*	5,02 ± 0,02*	6,12 ± 0,08	6,66 ± 0,03*
Survival after stage VI of the experiment at pH=2 compared to stage I										60%		
2.2 K	9,06 ± 0,01*	9,02 ± 0,02*	9,03 ± 0,01*	9,12 ± 0,05			9,01 ± 0,03*			9,14 ± 0,03*		
2.2	**8,98 ± 0,07**	**8,63 ± 0,27**	**8,80 ± 0,05**	**8,63 ± 0,01***	**8,49 ± 0,03***	**9,00 ± 0,02***	**6,32 ± 0,21**	**8,73 ± 0,02***	**8,93 ± 0,14**	**6,27 ± 0,06**	**8,35 ± 0,01***	**8,94 ± 0,09**
Survival after stage VI of the experiment at pH=2 compared to stage I										**70%**		
L5-T K	8,98 ± 0,03*	8,72 ± 0,05	8,78 ± 0,05	8,70 ± 0,02*			8,71 ± 0,06			8,87 ± 0,04*		
L5-T	8,83 ± 0,05	8,40 ± 0,13	8,36 ± 0,03*	5,31 ± 0,01*	8,24 ± 0,01*	8,68 ± 0,08	5,31 ± 0,01*	8,14 ± 0,01*	8,78 ± 0,08	5,31 ± 0,01*	8,34 ± 0,01*	8,88 ± 0,08
Survival after stage VI of the experiment at pH=2 compared to stage I										58%		
L5 K	8,78 ± 0,07	8,57 ± 0,20	8,64 ± 0,08	8,70 ± 0,06			8,68 ± 0,06			8,79 ± 0,02*		
L5	8,67 ± 0,06	8,54 ± 0,05	8,50 ± 0,04*	5,13 ± 0,01*	8,10 ± 0,04*	8,46 ± 0,04*	4,45 ± 0,01*	7,43 ± 0,10	8,61 ± 0,04*	5,15 ± 0,02*	8,04 ± 0,03*	8,76 ± 0,05
Survival after stage VI of the experiment at pH=2 compared to stage I										59%		

*p<0,05
**K – control samples performed for each strain
Strains selected for further analysis are marked in green, assuming the strain survival rate of 70% or more as the criterion.

Table A2. Assessment of Antagonistic Interactions Among Bacterial Strains

Strain		1	4	2.1	2.2
		Growth inhibition zone [mm]			
1	Growth inhibition zone [mm]	-	0	0	0
4		0	-	0	0
2.1		0	0	-	0
2.2		0	0	0	-