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Lentilactobacillus buchneri Preactivation Affects the Mitigation of Methane Emission in Corn Silage Treated with or without Urea

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Abstract: The aim of this study was to investigate the effect of different forms of *Lentilactobacillus buchneri* on the in vitro methane production, fermentation characteristics, nutritional quality, and aerobic stability of corn silage treated with or without urea. The following treatments were applied prior to ensiling: (1) no urea treatment and LB; (2) no urea treatment+freeze dried LB; (3) no urea treatment+preactivated LB; (4) with urea treatment+no LB; (5) with urea treatment+freeze dried LB; (6) with urea treatment+preactivated. LB was applied at a rate of 3×10^8 cfu/kg on a fresh basis, while urea was applied at a rate of 1% on the basis of dry matter. Data measured at different time points were analyzed according to a completely randomized design, with a $2 \times 3 \times 5$ factorial arrangement of treatments, while the others were analyzed with a 2×3 factorial arrangement. Preactivated LB was more effective than freeze-dried LB in reducing silage pH, ammonia nitrogen, cell-wall components, yeast count, and carbon dioxide production, as well as increasing lactic acid and residual water-soluble carbohydrate and aerobic stability ($p < 0.0001$). A significant reduction in the methane ratio was observed after 24 h and 48 h incubation with preactivated forms of LB ($p < 0.001$). The results indicated that preactivated LB combined with urea improved fermentation characteristics, nutritional quality, and aerobic stability and reduced the methane ratio of corn silages.

Keywords: gas production; *Lentilactobacillus buchneri*; methane; silage; urea

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1. Introduction

The role of mitigating methane (CH_4) emissions in global climate change has received increasing attention in a number of disciplines in recent years. Extensive research has shown that almost 16% of human-induced greenhouse gas (GHG) emissions are derived from the livestock sector, with enteric CH_4 being the single largest source [1–3]. In addition to its negative contribution to global GHG emissions, CH_4 may also show a 2 to 12% loss of feed energy ingested depending on breed of the animal, the composition of feed, and intake of feed [4,5]. So far, different strategies have been devised to reduce CH_4 emissions from ruminants, such as genetic selection of lower CH_4-producing animals, manipulation of the rumen environment using ionophores, electron receptors, or plant-derived essential oils, and dietary manipulation by increasing forage quality and diversity [2,6,7].

Corn (*Zea mays* L.) is mainly produced to obtain silage globally due to its high yield potential, high specific energy content, palatability, and ease of ensiling [8]. Furthermore, it has been proposed that ensiling is used to produce high-quality forages and mitigate GHG emissions by diluting the total energy intake for daily requirements [9]. The higher concentration of corn starch could increase propionate formation by decreasing pH and the

number of protozoa, eliminating the inhibition of H_2 in rumen fermentation, thus reducing ruminal methanogenesis [10–12].

On the one hand, the potential probiotic effects of obligate heterofermentative *Lentilactobacillus buchneri* (LB), which are primarily used to augment the aerobic stability of the silage, on animal performance and ruminal fermentation have been revealed by several studies [13–15]. LB is a Gram-positive bacterium that produces lactic acid (LA) and acetic acid (AA). Although homofermentative bacteria are lactic acid bacteria (LAB) that produce only LA, heterofermentative bacteria make several end products, such as acetic acid (AA), formic acid (FA) and ethanol [16]. Further, it is believed that LB interferes with the connection between cell wall carbohydrate and lignin by producing ferulic acid esterase (FAE), resulting in a higher level of cell wall degradation and improved fiber digestion [17]. Previous research has also established that the inoculant preactivation has proven to be more effective in enhancing silage fermentation due to the ongoing fermentation phase [18].

On the other hand, silage supplemented with non-protein nitrogen sources, such as urea ($CO(NH_2)_2$, CAS number 57-13-6), has been shown to increase rumen microbe activity, resulting in an increase microbial N levels and ammonia in rumen, as well as an increased structural carbohydrate degradation, leading to the increased production of volatile fatty acids (VFAs) [19,20]. Furthermore, urea is capable of increasing the production of crude protein (CP) by microorganisms and, therefore, increasing the supply of metabolizable proteins host animals [21]. There is, however, conflicting evidence regarding the effects of urea on CH_4 in the literature. For example, Kara [22] found that CH_4 production was positively associated with the microbial population and ammonia-N (NH_3-N) concentration, while Bharanidharan et al. [23] observed that rumen metabolites and NH_3-N had a negative correlation with CH_4 production.

The effect of preactivated LB and urea on the fermentation profile, aerobic stability, and nutrient composition in corn silage has already been studied by dos Santos et al. [18]. There is, however, a lack of information on how various forms of LB affect CH_4 production of CH_4 when combined with a non-protein nitrogen source. Therefore, we hypothesized that (i) LB and urea would increase fermentable substrates, resulting in a reduction in enteric CH_4 emissions due to the reduced time that materials take to reach methanogens; (ii) combining preactivated LB with a non-protein nitrogen source would improve the fermentation characteristics and nutritional quality of corn silages. Hence, the current work addresses these knowledge gaps by investigating the effect of different forms of LB (non-, freeze-dried, and preactivated) on the in vitro CH_4 ratio, fermentation, and nutritional quality of corn silage treated with or without urea.

2. Materials and Methods

2.1. Experimental Design and Treatments

A completely randomized design with a factorial arrangement of 2 × 3 × 5 with two urea treatment (U), three forms of LB (F), and five fermentation periods (P), with four replicates was used. The second crop Pioneer variety 32K61 corn was harvested in the milk stage and cut with a forage harvester (Tinaz S04, Balikesir, Turkey) to achieve a theoretical length of 20–30 mm from a local farm in November 2021. Then, a representative 120 kg of chopped second crop whole corn was transferred to the Animal Feed and Nutrition Laboratory of Tekirdag Namik Kemal University, Tekirdağ, Turkey, where the silage was prepared and further analyzes were performed.

Fresh corn had 237.0 g/kg DM, 78.1 g/kg DM of CP, 537.6 g/kg neutral detergent fiber (NDF), 281.3 g/kg acid detergent fiber (ADF), 117.9 g/kg DM of water-soluble carbohydrate (WSC), 5.63 of pH, 8.75 log cfu/g of lactic acid bacteria (LAB) and 8.76 log cfu/g of yeast and without mold before ensiling (Figure 1).

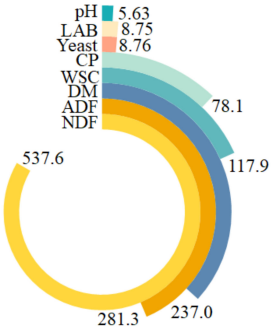

Figure 1. Fresh corn and its chemical and microbial composition; ADF: acid detergent fiber [g/kg DM] (DM = dry matter [g/kg]), ADL: Acid detergent lignin [g/kg DM], CP: crude protein [g/kg DM]; LAB: lactic acid bacteria [log cfu/g], NDF: neutral detergent fiber [g/kg DM], WSC: water soluble carbohyrate [g/kg DM], yeast [log cfu/g].

In the laboratory, a portion of the chopped corn was spread in a thin layer on a polyamide cover and treated with 1.0% fertilizer grade urea (UC, urea treated corn silage) by hand spraying on the basis of dry matter (DM) [18]. The other part, the remaining chopped corn, was also spread in a thin layer on a polyamide cover, and the same volume of distilled water was sprayed (C, corn silage). Then, approximately 1 kg of UC or C was spread in a thin layer on a 1 m^2 polyamide cover and subjected to one of the following treatments before ensiling: without LB (nLB), freeze-dried LB (LB) or preactivated LB (pLB). LB (*L. buchneri* NCIMB 40,788-CNCM I-4323; Lalsil AS, Lallemand Inc., Canada) and pLB were applied on a fresh basis at 3×10^8 cfu/kg as recommended by EFSA [24]. For each treatment, LB and pLB were dissolved in 20 mL of sterile distilled water, then applied similarly to urea treatment by hand sprayer, mixed well with sterile gloves in each replicate, and finally packed in polyethylene bags (Petkim, Izmir, Turkey; dimensions 250 × 400 mm; 41 cm^3/m^2 film per d O_2 and 160 cm^3/m^2 film per d CO_2 permeability measured at 23 °C and 0% relative humidity) and sealed by vacuum sealer (CVP-260PD, manufactured by CAS, Seoul, Korea). The nLP treatment was the same without any LB.

LB preactivation was performed using reconstituted skim milk (RSM) and table sugar before 24 h of treatment, according to dos Santos et al. [18]. In brief, 10 g of RSM and 2 g of sucrose (as an energy source for microbial growth) were dissolved in 90 mL of distilled water, sterilized at 120 °C for 15 min. The inoculations were carried out according to the manufacturer's specifications and then incubated in a biochemical oxygen demand oven for 24 h at 25 °C. A total of 120 vacuum bags (2 urea treatment × 3 forms of LB × 5 fermentation period × 4 replicates) were prepared and stored for 75 d at an ambient temperature of 25–30 °C.

2.2. Chemical and Microbiological Analyses

After 75 d of ensiling, the opened bags were subsampled for chemical analysis, microbial count, and determination of their aerobic stability. For the determination of the chemical composition, the subsampled silages and fresh corn were dried in an air-forced oven at 60 ± 2 °C for 72 h and then ground to pass a 1 mm sieve and stored at −20 °C until analysis could be performed. The DM of the samples was determined drying at 105 ± 2 °C in an air-forced oven for 24 h. The CP content in each sample was determined using the AOAC assay (method 976.15; AOAC; Esen et al., [25]). The ash content was measured by incinerating the silage samples in a muffle furnace at 550 °C for 3 h. The content of NDF (neutral detergent fiber) and ADF (acid detergent fiber) of the silages was measured according to the procedure used by Van Soest et al. [26]. The WSC (water-soluble carbohydrate) content of the silages was determined using a 0.2% anthrone reagent [25]. The pH, ammonia nitrogen (NH_3-N), and organic acid content of the silage samples was determined after extracting a representative 20 g of fresh silage in 180 mL of distilled water at room temperature for 1 h. A digital pH meter (inoLab ph 730, WTW, Weilheim,

Germany) and a microdistillation apparatus were deployed for the determination of pH and NH_3-N analysis [27]. After deproteinization of the silage extract with a mixture of metaphosphoric acid and formic acid (3:1, v:v), the organic acid content of the silages, i.e., acetic acid (AA), propionic acid (PA), butyric acid (BA), was determined using a gas chromatograph (Shimadzu GC-201, Kyoto, Japan) with a capillary column (Restek, Bellefonte, PA, USA; 30 m, id: 0.25 mm, f.t.: 0.25 µm), and with a flame ionization detector (FID) in a temperature range of 45–230 °C [28]. The lactic acid (LA) content of the silages was determined according to the procedure of Koc and Coskuntuna [29]. Fresh corn and silage measurements were made in triplicate and calculated on a fresh and wet silage basis. 20 g of silage samples were transferred to 180 mL of sterile saline solution; For LAB and yeast counts, serial dilutions were made. LAB counts were performed using MRS agar (De Man, Rogosa, and Sharpe agar; Merck KGaA, Darmstadt, Germany) in a 30 °C anaerobic jar for 72 h, while yeasts were counted on PDA agar (Potato Dextrose Agar; Merck KGaA, Darmstadt, Germany) incubated at 25 °C for 5 d [30].

2.3. Determination of Aerobic Stability

Two approaches were followed to determine the aerobic stability of corn silage: data loggers and PE (polyethylene) bottles, which are gastight and corrosion resistant. Room temperature and the inner temperature of each silage were recorded by thermocouples (HOBO Pendant Temperature/Light 64K Data Logger, Onset Computer Corporation, Bourne, MA, USA) during the 7 d of aerobic exposure, in intervals of 2 h. During aerobic exposure, the room temperature ranged between 23.11 ± 1.25 °C (Mean ± SD), and it was considered that aerobic deterioration was expected to begin once the temperature of the corn silage was 2 °C higher than the ambient temperature. Changes in pH, CO_2 production, and yeast count after 7 d of aerobic exposure were also used to assess the aerobic stability of corn silages [31].

2.4. Determination of in vitro Gas Production, Methane Content, Metabolizable Energy, Organic Matter Digestibility, and Protein Degradability

Gas production (GP) profiles of corn silage have been extensively studied in vitro. In this study, calibrated 100 mL glass syringes were used in triplicate for 96 h in a 39 °C water bath. In this investigation, no animals were used directly; rumen liquor was obtained from 3 different freshly slaughtered 2-year-old Holstein cattle, pooled, and then filtered through four layers of cheesecloth into a pre-warmed thermos at 39 °C and transferred to the laboratory within 20 min. Approximately 200 mg of dry corn silage samples were incubated in 100 mL glass syringes in twelve replicates each occasion. On each day of fermentation (24, 48, 72, and 96 h), three syringes in each treatment were randomly selected to determine the CH_4 content. The glass syringes were pre-warmed at 39 °C before injecting a 30 mL mixture of rumen liquor-buffer mixture free of particles (10/20, *v/v*; including no trypticase) under continuous injection of CO_2 following the procedure of Menke and Steingass [32]. The total gas volume of corn silage was determined at 3, 6, 12, 24, 48, 72, and 96 h and corrected with blank bottles and standard alfalfa hay. The CH_4 content (%) of the total gas produced after each specified time was measured using a CH_4 analyzer (S-AGM Plus 1010, Sensors Europe GmbH, Erkrath, Germany) according to the procedure described by Goel et al. [33].

The cumulative CP data of the corn silages were fitted to the exponential model of Ørskov and McDonald [34] by using the Solver function in the software Microsoft Excel software (Equation (1)).

$$Y = a + b(1 - e^{-ct}) \quad (1)$$

In Equation (1), Y is the volume [mL] of gas produced at time t; a, the volume [mL] of gas produced from the immediately soluble fraction of corn silages; b, the volume produced by gas [mL] from the insoluble fraction of corn silages; c, the gas production rate constant [mL/h]; t, the time of incubation [h].

The metabolizable energy (ME, [MJ/kg DM]) and organic matter digestibility (OMD, [% DM]) contents of corn silage were calculated using the following Equations (2) and (3) of Menke et al. [35].

$$\text{ME (MJ/kgDM)} = 2.20 + 0.136\ \text{GP} + 0.057\ \text{CP} \quad (2)$$

$$\text{OMD (\% DM)} = 14.88 + 0.889\ \text{GP} + 0.45\ \text{CP} + 0.0651\ \text{Ash} \quad (3)$$

In Equation (2), GP is the net production of gas 24 h [mL/200 mg]; CP, crude protein.

The enzymatic degradation method is one of the more practical ways of determining protein degradability. Therefore, crude protein degradation (ICVPD) of corn silage in vitro was determined by enzymatic hydrolysis for 24 h by protease enzyme (extracted from *Streptomyces griseus*) by using the method of Aufrere and Cartailler method [36].

2.5. Statistical Analysis

For statistical analysis of chemical composition (DM, CP, Ash), fermentation indices (pH, WSC, LA, NH_3-N), and microbial count (Yeast and LAB), a 2 × 3 × 5 factorial arrangement was used with the SAS-JMP software MIXED procedure (version 13.2; SAS Institute Inc., Cary, NC, USA), considering U (UAC, AC), F (nLB, LB, pLB), P (1, 3, 7, 14, 75 days) and their interactions (U × F, U × P, F × P, and U × F × P) as the fixed effects and experimental error as the random effect. A 2 × 3 factorial arrangement was used to analyze other parameters using U, P and their interactions. Using Duncan's new multiple range tests, the means were compared at $p < 0.05$, which is accepted as a statistically significant difference. The correlogram was drawn for visualization in the 'corrplot' package of the software, to assess the strength and direction of association between the parameters studied [37].

3. Results and Discussion

3.1. Effect of Urea-Treatment and Preactivation of L. buchneri on Chemical Compositions of Corn Silage

The DM content of corn silage was significantly affected by the fermentation period ($p < 0.0001$) and the form of LB ($p = 0.0286$), whereas urea was not ($p > 0.05$; Figure 2a). C-nLB had the highest DM content on day 1 (249.4 g/kg), while CLB had the lowest DM content on day 14 (199.4 g/kg). The DM content of the silages of the pLB and LB groups was reduced by 1.91% and 2.75%, respectively, compared to that of the nLB group. Conversely, no significant interaction was observed between urea treatment, LB form, and fermentation period. Consistent with these findings, da Silva et al. [38] reported that *L. hilgardii*, *L. buchneri,* and their combination interacted with the storage period and had a significant effect on the DM content of corn silages.

Corn silage treated with urea had a higher CP content (87.2 vs. 77.8 g/kg DM, $p < 0.0001$). This result can be attributed to the fact that the higher N fraction of urea increases the CP content of corn silage [19,20]. This view is also supported by dos Santos et al. [18], who write that the increase in nitrogen fractions could be used more effectively to promote the synthesis of microbial proteins in the rumen. Similar findings have also been reported for in vivo experiments by Sinclair et al. [39] and Gómez-Vázquez et al. [40]. However, compared to nLB, LB inoculation caused a significant decrease in CP ($p < 0.0001$), however, this ratio is lower in pLB (8.68%) than in LB (12.29%). A decreasing trend in CP content was also observed during fermentation (85.5 to 79.8 g/kg DM). The interactions between the urea treatment, LB form, and fermentation period all significantly affected the CP content in corn silage [$p < 0.0001$, with the exception of the urea × fermentation period interaction ($p = 0.0449$] (Figure 2b). A significant increase in the number that utilizes urea as a source of nitrogen may explain this result. These results are in line with those obtained by Canbolat [41].

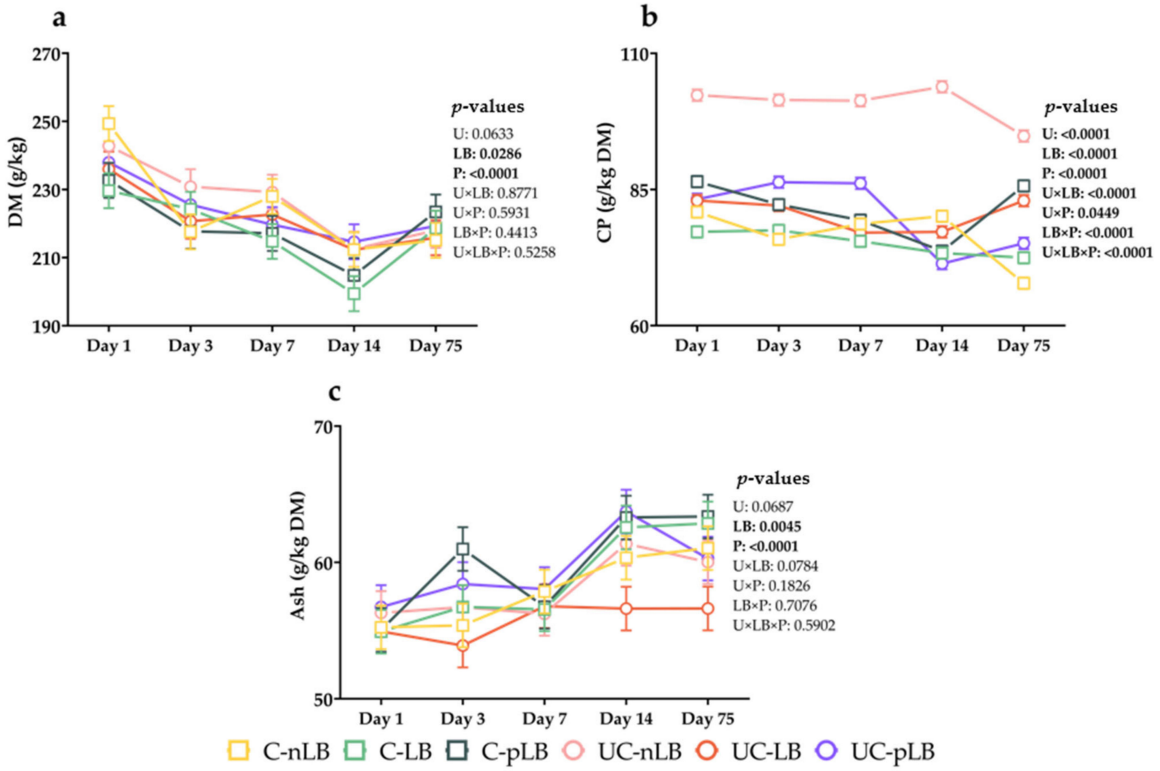

Figure 2. Effect of the ensiling length, the treatment with urea, and preactivation of *L. buchneri* on the chemical composition of the corn silages. Changes in dry matter [DM, (**a**)], crude protein [CP, (**b**)], and ash (**c**) content. C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*. Factors that are statistically significant are highlighted in bold ($p < 0.05$).

As spoilage occurs, organic matter disappears, but the volume of ash remains constant, so measuring ash provides an indirect method of estimating losses [42]. In the current study, the forms of LB ($p = 0.0045$) as well as fermentation time ($p < 0.0001$) significantly affected the ash content of corn silage, unlike the urea treatment ($p > 0.05$; Figure 2c). The preactivation of LB resulted in an increase in the ash content of corn silage (59.7 vs. 58.0 kg/kg DM), while the ash content of corn silage inoculated with freeze-dried LB decreased (57.22 g/kg DM). The ash content in the corn silage increased on day 14, but it did not change significantly until the fermentation process ended. These results are differ to those of Ariola et al. [43], who found a lower ash content in their study, which examined the effect of inoculation with *L. hilgardii* with or without *L. buchneri* on the fermentation, chemical composition, and aerobic stability of sorghum and corn silage after 30 and 90 days.

Several studies have indicated that digestible cell wall fractions from plant biomass can be hydrolyzed with the extension of ensiling during hydrolytic activities, such as acidolysis, microorganisms, and enzymes [25,44]. In the current study, Urea-treated corn silages showed a significant decrease in NDF content after 75 d of fermentation by 2.52% ($p = 0.0010$; Figure 3a). A reduction of 4.79% and 15.79% in the NDF content was also observed in LB and pLB silages compared to uninoculated silages (nLB). As a result of the interaction between urea treatment and LB forms, the NDF content of corn silage was significantly affected ($p < 0.0001$). However, contrary to expectations, preactivated LB was more effective in reducing the NDF content of corn silage in C-pLB than in UC-pLB (22.01 vs. 13.80%). Neither urea-treated corn silage nor non-urea-treated corn silage showed

any significant differences ($p > 0.05$; Figure 3b). However, LB inoculations in different forms and their interactions with urea significantly affected corn silage ($p < 0.0001$). Corn silages inoculated with LB significantly reduced their ADF content by 6.86%, while those inoculated with pLB reduced their ADF content by 2.21%. The highest reduction in ADF was observed in UC-LB (15.38%) and C-pLB (13.36%). A possible explanation for this could be that the synergetic effect of FAE-producing LB and urea would increase the fiber degradation rate and enhance the available WSC for LAB. These results are in agreement with those of previous studies [17,19]. Buettner et al. [45] stated that the high affinity of ammonia with water promotes the expansion of cell walls, causing the disruption of tissue components in treated silage. Therefore, the reduction of cell walls in urea treated silages can be a result of the hydrophilicity of urea and the destruction of cell walls.

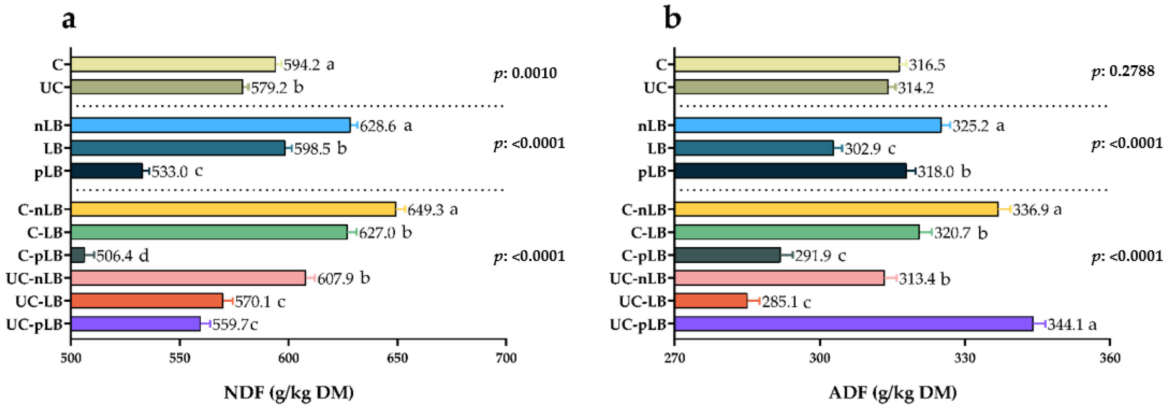

Figure 3. Effect of urea-treatment and preactivation of *L. buchneri* on the cell wall component of corn silages. Changes in the content of neutral detergent fiber [NDF, (**a**)], and aciddetergent fiber [ADF, (**b**)] of corn silages. A dotted line separates the urea treatment, the *L. buchneri* forms, and their interaction. C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*, DM: dry matter, U: urea treatment, LB: *L. buchneri* forms, U×LB: interaction between urea treatment and *L. buchneri* inoculation. Factors that are statistically significant are highlighted in bold ($p < 0.05$). Values with different letters (a–c) in each graph are statistically different ($p < 0.05$).

3.2. Effect of Urea-Treatment and Preactivation of L. buchneri on Fermentation Characteristics of Corn Silage

As expected, urea-treated silage had a higher pH due to the buffering effect of urea (3.88 vs. 3.84; $p < 0.0001$). The preactivation of LB was more effective than the freeze-dried form of LB and unioculated corn silage (3.84 vs. 3.87 and 3.87, respectively; $p < 0.0001$). Although the pH value on day 14 was the lowest (3.68), it increased statistically at the end of the fermentation period (3.72). Furthermore, all interactions were significant except for the urea × LB form ($p < 0.0001$; Figure 4a). It seems possible that adding urea would assist in silage preservation without any production BA, and the higher WSC content in the urea-treated groups confirms this assumption, which is consistent with the results of Islam et al. [46].

It has been demonstrated previously that the amount of WSC in silage, along with the activity of natural LAB, determines the amount of LA that accumulates during early stages of ensiling, as well as the rate at which pH decreases. In contrast to urea [41], heterofermentative LAB has been associated with an increase in WSC in silages [47]. In the current study, a significant increase in the WSC content was observed in urea-treated corn silages compared to untreated corn silages (40.67 vs. 22.57 g/kg DM). The pLB

(39.34 g/kg DM) was more effective in increasing the WSC content of corn silages than the LB (32.04 g/kg DM) and the nLB (23.48 g/kg DM) ($p < 0.0001$). With increasing fermentation period, a linear and marked reduction in WSC was also observed ($p < 0.0001$). As compared to C-nLB (18.14 g/kg DM), UC-pLB contained the highest content of WSC (52.09 g/kg DM), followed by UC-LB (41.11 g/kg DM) and UC-nLB (28.82 g/kg DM) ($p < 0.0001$). Additionally, all other interactions between urea treatment, LB form, and fermentation period were significant ($p < 0.0001$; Figure 4b).These results are in line with those obtained by Wang et al. [48] and Arriola et al. [43].

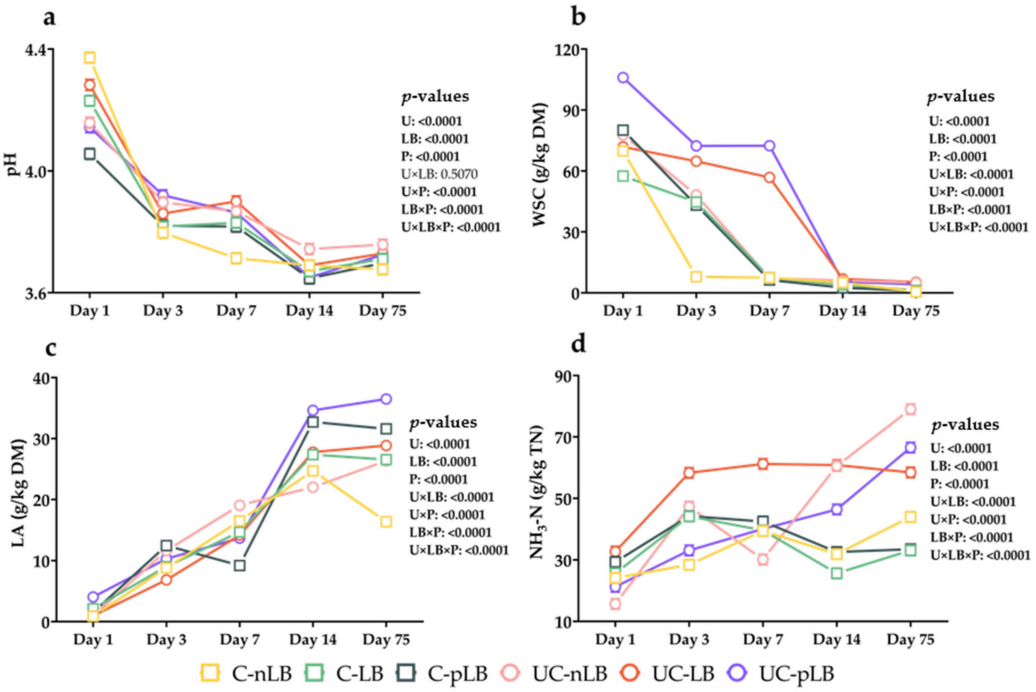

Figure 4. Effect of the ensiling length, the treatment with urea, and preactivation of *L. buchneri* on fermentation indices. Changes in pH (**a**), water-soluble carbohydrate [WSC, (**b**)], lactic acid [LA, (**c**)], and ammonia-nitrogen [NH_3-N, (**d**)] content of corn silage. C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*. Factors that are statistically significant are highlighted in bold ($p < 0.05$).

The LA content of the corn silage was significantly affected by urea treatment, LB forms, fermentation period, and all their interactions ($p < 0.0001$; Figure 4c). Corn silage treated with urea had a 9.92% increase in LA content. These results are in agreement with those obtained by Canbolat [41]. When corn silage was inoculated with LB, the LA content increased by 7.47%, while with pLB, it increased by 23.48%. UC-pLB exhibited the highest LA content (19.83 g/kg DM), while C-nLB exhibited the lowest (13.46 g/kg DM). Similar findings were also reported by Wang et al. [48] and Rabelo et al. [49].

In both plants and microbes, proteases hydrolyze proteins into NPN intermediate products, such as peptides and free amino acids, which are then further degraded to form ammonia, amines, and amides. Since NPN is less effective than true protein when consumed by ruminants, it results in a reduced quality of nutrients in silage [25]. In the current study, urea treatment significantly increased the NH_3-N content of corn silages, as expected (34.53 vs. 47.44 g/kg TN; $p < 0.0001$). LB significantly increased the amount of NH_3-N in corn silage (43.97 g/kg TN) compared to pLB (40.05 g/kg TN) and nLB (38.95 g/kg TN). The lowest and highest NH_3-N concentrations were determined on day 1 (24.79 g/kg TN)

and day 75 (52.43 g/kg TN), respectively. Furthermore, all interactions between urea treatment, LB form, and fermentation period were significant ($p < 0.0001$; Figure 4d). It is noteworthy that preactivation of LB reduced the increased NH_3-N ratio in urea-treated groups. Possibly, this is related to the preactivation process, which accelerates LA production in the preactivated groups and suppresses the buffer effect of urea treatment. This assumption is supported by the increase in LA concentrations in the preactivated group.

The AA content and the LA/AA ratio of corn silages were significantly affected by urea treatment, the forms of LB and their interactions, as shown in Table 1 ($p < 0.0001$). As a result of preactivation of LB, corn silage had significantly less AA content than C-nLB silages (4.46 vs. 7.69 g/kg DM); therefore, this group exhibited the highest LA/AA ratio (8.18). The reduced NDF content of these groups suggests that both the inoculated LB and the epiphytic LAB content of corn used the hemicellulose decomposition product during ensiling to produce LA through unknown mechanisms. Note that similar findings were reported by Muck et al. [50], Restelatto et al. [51], and Wu et al. [52]. Although none of the urea treatments or LB forms was significant on the PA content of corn silages ($p > 0.05$), their interaction was significant ($p < 0.0001$). Contrary to Witziq et al. [53], urea supplementation significantly affected the PA content of corn silage in the current study; however, the lowest PA (0.34 g/kg DM) was observed in the pLB group. Aside from this, no BA was detected in any of the treated silage groups.

Table 1. Effect of urea treatment and *L. buchneri* forms on fermentation indices of corn silage.

Item	C			UC				*p*-Values		
	nLB	LB	pLB	nLB	LB	pLB	SEM	U	LB	U×LB
AA (g/kg DM)	7.69 ^{d}	9.14 ^{c}	6.13 ^{e}	10.96 ^{b}	13.12 ^{a}	4.46 ^{f}	0.01	<0.0001	<0.0001	<0.0001
PA (g/kg DM)	0.48 ^{a}	0.41 ^{bc}	0.34 ^{d}	0.38 ^{cd}	0.42 ^{bc}	0.44 ^{ab}	0.01	0.6903	0.0058	<0.0001
BA (g/kg DM)	ND	ND	ND	ND	ND	ND	-	-	-	-
LA/AA ratio	2.13 ^{d}	2.90 ^{c}	5.15 ^{b}	2.40 ^{cd}	2.20 ^{d}	8.18 ^{a}	0.11	<0.0001	<0.0001	<0.0001

C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*, AA: acetic acid, PA: propionic acid, BA: butyric acid, SEM: standard error of mean, ND: Not detected, U: urea treatment, LB: *L. buchneri* forms, U×LB: interaction between urea treatment and *L. buchneri* inoculation. Values with different letters (a–f) in each row are statistically different ($p < 0.05$).

The primary cause of spoilage, as observed in several reports [54,55], is yeasts, which result in high pH levels. Yeast counts in corn silage treated with urea and untreated corn silage did not differ significantly ($p > 0.05$; Figure 5a). However, LB inoculated corn silages either preactivated (5.90 log cfu/g) or freeze dried (5.93 log cfu/g) showed a significant increase in LAB number over the nLB group (5.72 log cfu/g) ($p = 0.0008$). Moreover, it was observed that the number of yeast increased as fermentation time was extended ($p < 0.0001$). It was found that there were significant bilateral interactions between urea treatment, the LB form, and fermentation period (varied between $p < 0.05$ and $p < 0.0001$). According to this study, the results were higher than those obtained by Ariola et al. [43] and Xu et al. [56] using corn and *L. buchneri*; but the results were lower in Xu et al. [57]'s study.

LAB counts, however, were significantly higher in corn silage treated with urea than in corn silage that was not treated with urea (6.74 vs. 6.67 log cfu/g; $p < 0.0001$). Furthermore, it was found that the LAB counts of the corn silage were positively affected by freeze-dried (6.86 log cfu/g) or preactivated LB (6.84 log cfu/g) inoculation compared to unioculated corn silage (6.42 log cfu/g; $p < 0.0001$). The highest and lowest LAB counts were observed on day 75 (7.37 log cfu/g) and day 3 (6.44 log cfu/g), respectively, and the differences between fermentation period was significant. A significant interaction between urea treatment, LB form, and fermentation period was also observed in corn silage LAB counts

($p < 0.0001$; Figure 5b).These results are in line with those obtained by Xu et al. [56] and Arriola et al. [43].

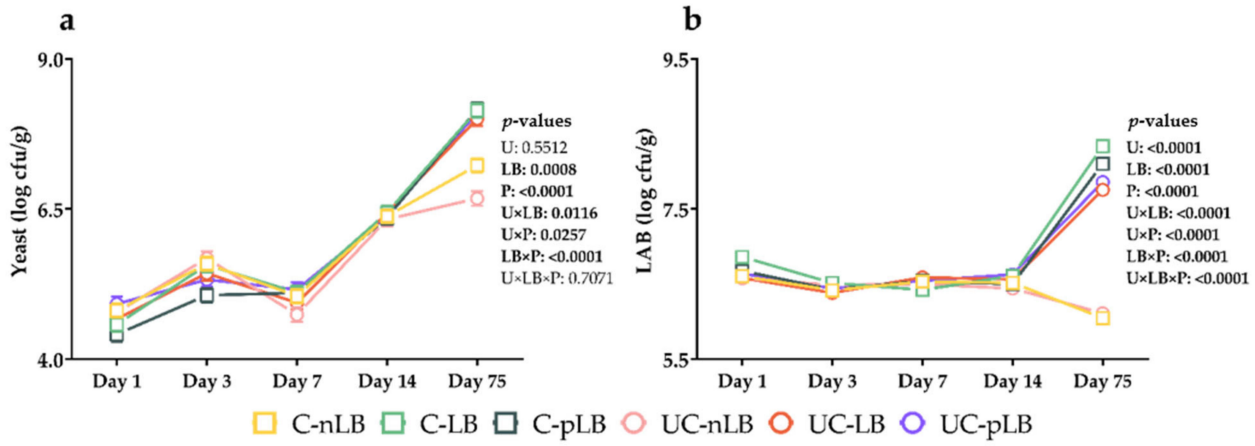

Figure 5. Effect of the ensiling length, the treatment with urea, and preactivation of *L. buchneri* on microbial count. Changes in yeast (**a**), and lactic acid bacteria [LAB, (**b**)] content of corn silage. C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*. Factors that are statistically significant are highlighted in bold ($p < 0.05$).

3.3. Effect of Urea Treatment and L. buchneri Forms on Aerobic Stability of Corn Silage

The DM content of corn silage was not affected by urea treatment, LB forms, or their interactions, as shown in Table 2 ($p > 0.05$). LB treatment had been shown to significantly affect the pH of corn silage ($p < 0.0001$), whereas urea treatment did not; however, its interaction was also found to be significant ($p = 0.0002$). Although urea treatment and LB form statistically reduced the number of yeasts after 7 d of aerobic exposure, they were unable to maintain the desired level of yeast growth; therefore, a higher CO_2 production was observed in all silages (except the UC-LB and UC-pLB groups). Even so, based on the results obtained with the data logger, the aerobic stability period for all corn silages was over 168 h, except C-nLB (132 h).

The low CO_2 content of the UC-pLB group was possibly related to its high LA content, making the pH more stable and inhibiting yeast activity longer. On the other hand, the synergistic effect of AA and urea reduced the CO_2 content in the UC-LB group but still made them unstable due to higher pH. Previous research has indicated that LB is the most common heterofermentative LAB used to (a) improve aerobic stability and (b) to avoid the growth of spoiling microorganisms after silo opening due to its well-known AA producing ability [50,58–60]. The restraining activity of urea on spoilage microorganisms, including yeast and mold, was also stated by Fang et al. [19]. Furthermore Koç et al. [27] stated that such factors could affect the aerobic stability of silage, such as the initial epiphytic LAB population and its abundance, additive types, organic acid, and WSC content of ensiled material. These results mirror previous studies examining LB and urea on silages' aerobic stability. The highest values of the aerobic stability were recorded for UC-pLB and UC-LB. In general, the effect of treatments on improving aerobic stability results from the following: increased AA production by bacteria decrease in remaining WSC lower accumulation of ammonia nitrogen.

Table 2. Effect of urea treatment and *L. buchneri* forms on the aerobic stability of corn silages.

Item	C			UC				*p*-Values		
	nLB	LB	pLB	nLB	LB	pLB	SEM	U	LB	U×LB
DM (g/kg)	220.1	221.7	224.7	222.0	220.9	223.4	3.27	0.9748	0.6044	0.8682
pH	5.90 ^{b}	5.77 ^{bc}	5.03 ^{d}	5.54 ^{c}	6.31 ^{a}	5.08 ^{d}	0.07	0.2346	<0.0001	0.0002
Yeast (log cfu/g)	9.81 ^{a}	9.46 ^{b}	9.13 ^{d}	9.41 ^{bc}	9.34 ^{c}	9.07 ^{d}	0.02	<0.0001	<0.0001	<0.0001
CO_2 (mL)	125.0 ^{a}	117.2 ^{b}	86.4 ^{c}	120.1 ^{ab}	51.8 ^{d}	41.0 ^{e}	1.38	<0.0001	<0.0001	<0.0001
Aerobic stability (h)	132 ^{b}	>168 ^{a}	>168 ^{a}	>168 ^{a}	>168 ^{a}	>168 ^{a}	0.47	<0.0001	<0.0001	<0.0001

C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*, DM: dry matter, CO_2: carbon dioxide, SEM: standard error of mean, U: urea treatment, LB: *L. buchneri* forms, U×LB: interaction between urea treatment and *L. buchneri* inoculation. Values with different letters (a–e) in each row are statistically different ($p < 0.05$).

3.4. Effect of Urea-Treatment and Preactivation of L. buchneri on Total Production of Gas and Methane from Corn Silage

No significant differences were found between urea treatment and the form of LB for total GP and estimated parameters ($p > 0.05$; Table 3). On the contrary, the interaction between urea treatment and LB form had a significant effect on total GP at 3 h ($p = 0.0237$) and the "a" parameter ($p = 0.0280$). It is possible that the difference in GP at the 3 h might be related to the difference in the AA:PA ratio between treatment groups. Specifically, Esen et al. [25] highlighted the importance of hydrogen in the formation of CH_4, which is influenced by the ratio of AA:PA. Similar findings were also reported by Guo et al. [61].

The estimated and total GP volume of corn silage varied between 61.25–66.98 and 61.92–68.05 mL/200 mg of DM, respectively, after the incubation period of 96 h and the differences between treated groups were not significant. In addition, there was no significant difference between OMD and ME of corn silage, as they ranged from 58.31 to 61.99% and 8.83–9.24 MJ/kg DM, respectively. The observed increase in GP could be attributed to readily degradable transient compounds, such as organic acids, water-soluble carbohydrates, fats and proteins increasing the degradation rate of forages on the first day of fermentation in accordance with Negri et al. [62]. Another possible explanation could be the hydrolysis of 60–65% of cellulose catalyzed by a complex and diverse microbial community of bacteria, protozoa, archaea and fungi within 48 h [63]. The enzymatic breakdown of the remaining parts continues at a slower rate in the following fermentation period.

Figure 6 illustrates that urea treatment and its interaction with the forms of LB did not significantly affect the CH_4 ratio at 24, 48, 72, and 96 h; however, preactivated LB significantly reduced the CH_4 ratio at 24 and 48 h. It was found that preactivated forms of LB significantly reduced the CH_4 ratio after 24 h and 48 h incubation by 4.88% ($p = 0.0039$) and 4.63% ($p = 0.0060$). The reduced methane ratio might be attributed to an increase in fermentable substrates under the influence of preactivated LB, resulting in a faster passage through the rumen, thereby decreasing the time spent in the presence of methanogens.

Table 3. Effect of urea treatment and *L. buchneri* forms on the gas production profile and digestibility parameters of corn silages.

Item	C			UC				*p*-Values		
	nLB	LB	pLB	nLB	LB	pLB	SEM	U	LB	U×LB
3 h, mL	16.42 ^{a}	13.79 ^{b}	16.51 ^{a}	15.14 ^{ab}	17.15 ^{a}	15.19 ^{ab}	0.84	0.7145	0.8898	0.0237
6 h, mL	24.97	23.20	25.10	23.90	27.58	24.64	1.19	0.3498	0.7293	0.0801
12 h, mL	35.03	32.28	34.71	32.98	36.66	34.43	1.73	0.6377	0.9417	0.2007
24 h, mL	48.44	44.72	48.19	44.76	47.77	45.91	2.31	0.6166	0.9414	0.3405

Table 3. *Cont.*

Item	C			UC				*p*-Values		
	nLB	LB	pLB	nLB	LB	pLB	SEM	U	LB	U×LB
48 h, mL	58.50	54.81	57.79	54.52	57.52	55.02	2.57	0.5325	0.9908	0.4086
72 h, mL	64.03	60.19	61.84	59.23	63.57	59.75	2.85	0.6249	0.9239	0.3737
96 h, mL	68.05	62.88	64.37	61.92	66.60	62.78	2.87	0.5811	0.8722	0.2682
c	0.045	0.047	0.051	0.048	0.045	0.052	0.0029	0.8815	0.1744	0.7584
a, mL	10.62 ^{ab}	8.38 ^{b}	9.78 ^{b}	9.70 ^{b}	12.78 ^{a}	9.43 ^{b}	0.93	0.1960	0.5941	0.0280
b, mL	56.36	53.84	53.84	51.55	53.31	52.04	2.64	0.2912	0.9274	0.7129
a + b, mL	66.98	62.22	63.62	61.25	66.09	61.47	2.86	0.5771	0.8164	0.2746
ME, MJ/kg DM	9.17	8.70	9.24	8.83	9.17	8.87	0.32	0.7580	0.9284	0.3459
OMD, % DM	61.40	58.31	61.99	59.33	61.44	59.46	2.05	0.7772	0.9180	0.3429
IVCPD, % CP	88.68 ^{c}	91.16 ^{bc}	93.89 ^{ab}	93.84 ^{ab}	92.39 ^{ab}	94.67 ^{a}	0.66	0.0008	0.0013	0.0110

C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*, a: gas production from the immediately soluble fraction; b: gas production from the insoluble fraction; c: the gas production rate constant for the insoluble fraction (b); a + b: potential gas production; ME: metabolizable energy; OMD: organic matter digestibility; IVCPD: in vitro crude protein degradability, SEM: standard error of mean, U: urea treatment, LB: *L. buchneri* forms, U×LB: interaction between urea treatment and *L. buchneri* inoculation. Values with different letters (a–c) in each row are statistically different ($p < 0.05$).

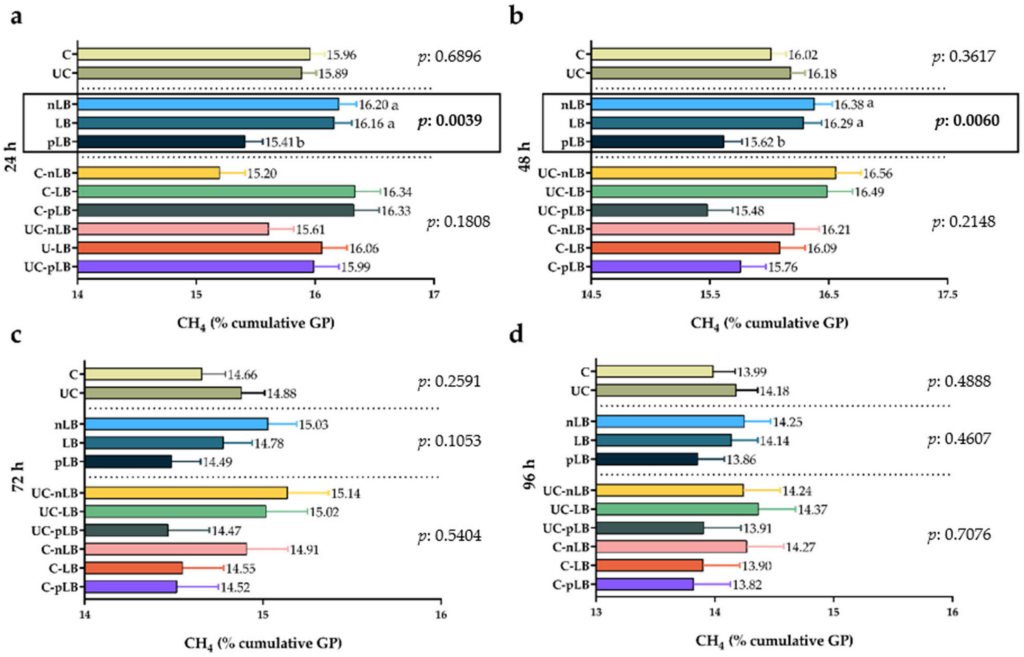

Figure 6. Effect of urea treatment and *L. buchneri* forms on the in vitro methane ratio of corn silage. Changes in the methane (CH_4) ratio of corn silage after 24 h (**a**), 48 h (**b**), 72 h (**c**), and 96 h (**d**). A dotted line separates the urea treatment, the *L. buchneri* forms, and their interaction. C: corn silages not treated with urea, UC: corn silages treated with 1.0% urea on dry matter basis, C-nLB: corn silages not treated with urea and uninoculated with *L. buchneri*, C-LB: corn silages not treated with urea and inoculated with *L. buchneri*, C-pLB: corn silages not treated with urea and inoculated with preactivated *L. buchneri*, UC-nLB: corn silages treated with urea and uninoculated with *L. buchneri*, UC-LB: corn silages treated with urea and inoculated with *L. buchneri*, UC-pLB: corn silages treated with urea and inoculated with preactivated *L. buchneri*, CH_4: methane, GP: gas production. Factors that are statistically significant are highlighted in bold ($p < 0.05$). Values with different letters (a–c) in each graph are statistically different ($p < 0.05$).

3.5. Factors Affecting In Vitro Gas Production and Methane Ratio in Corn Silages

A Pearson correlation analysis was performed to assess the strength of the parameters studied in the GP and CH_4 ratio (Figure 7). A negative correlation was observed between GP and pH ($p < 0.05$), while LA, LA/AA, and IVCPD negatively affected the CH_4 ratio ($p < 0.05$). A moderate and high positive correlation was also observed between CH_4-Yeast (AS) ($p < 0.01$) and CH_4-NDF ($p < 0.001$). The positive relationship between CH_4-NDF is an unexpected outcome and might be attributed to the FAE-producing ability of LAB, which decomposes hemicellulose during anaerobic fermentation. This is inconsistent with the data obtained by Ansah et al. [64] but similar to Singh et al. [65], who reported positive but not significant results. Singh et al. [65] also reported a positive and significant relationship between ADF and total GP, which aligns with our results.

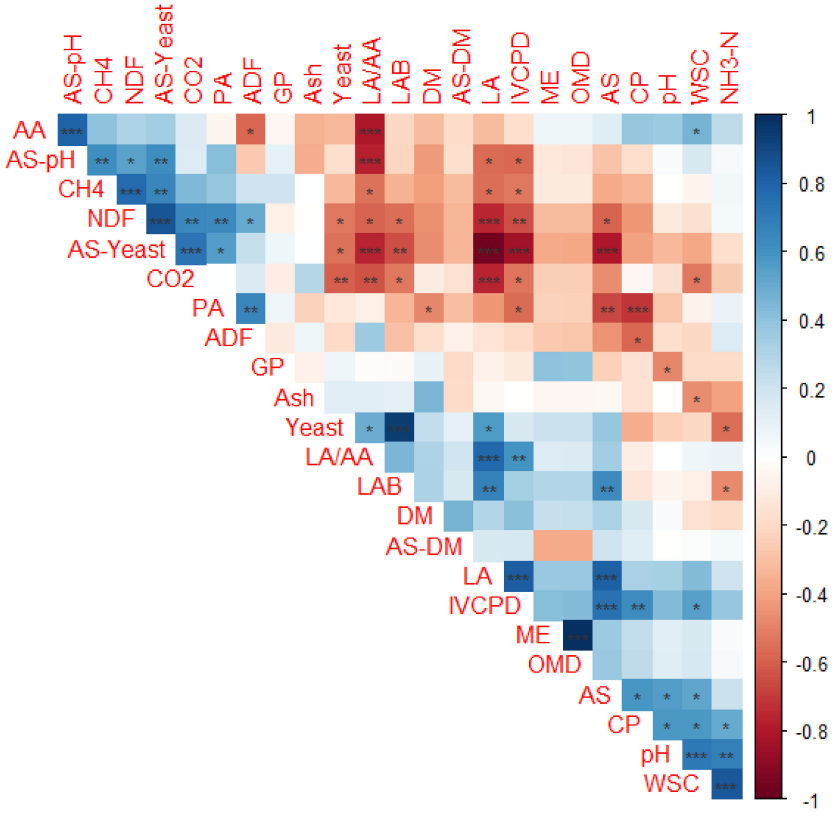

Figure 7. Pearson's correlation coefficients for fermentation characteristics, microbial and chemical compositions, cell wall components, aerobic stability, gas production in vitro and methane values of corn silage. The blue and red squares indicate positive and negative correlations, respectively, and the empty cases refer to non-significant correlations. AA: acetic acid, AS-pH: pH after 7 days of aerobic exposure, CH_4: methane, AS-Yeast: yeast count after 7 days of aerobic exposure, CO_2: carbon dioxide, PA: propionic acid, ADF: acid detergent fiber, GP: gas production, LA/AA: lactic acid/acetic acid ratio, LAB: lactic acid bacteria, DM: dry matter, AS-DM: dry matter content after 7 days of aerobic exposure, LA: lactic acid, IVCPD: in vitro crude protein degradability, ME: metabolizable energy, OMD: organic matter digestibility, AS: aerobic stability, CP: crude protein, WSC: water-soluble carbohydrates, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

4. Conclusions

Overall, these results indicate that preactivation of LB significantly reduces CH_4 emissions without affecting total GP in corn silage treated with or without urea. Furthermore, LB preactivation combined with urea treatment has been shown to produce significant improvements in cell wall degradation, CP, residual WSC and LA content, and aerobic stability of corn silage. More research is required to investigate the mechanisms involved

in the reduction of methane in the use of bacteria. This can be accomplished by performing other experiments, such as labelling with radioactive elements.

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