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Effects of *Bacillus coagulans* and *Lactobacillus plantarum* on the fermentation quality, aerobic stability and microbial community of triticale silage

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Abstract

Background Due to its hollow stem, ensiling triticale presents a challenge as it may cause an overabundance of oxygen during the fermentation process. This study investigated the effects of *Bacillus coagulans* (BC) and *Lactobacillus plantarum* (LP) on the fermentation characteristics, microbial community, and aerobic stability of ensiled triticale. Fresh triticale was wilted at a dry matter content of 350 g/kg. The experiment was arranged in a 2 × 2 factorial design, with both BC and commercial LP added at 0 or 1×10^6 cfu/g of fresh weight (FW) of chopped triticale.

Results After 60 days of ensiling, the pH, water-soluble carbohydrates (WSC), neutral detergent fiber (NDF), and ammonia nitrogen (NH_3-N) of inoculated groups were lower than those of the control group ($P < 0.05$), especially in the LP + BC treatment ($P < 0.05$). The lactic acid (LA) concentration, lactic acid/acetic acid (LA/AA), and aerobic stability were also higher ($P < 0.05$) in the LP + BC treatment than in other treatments. The bacterial diversity was reduced, and the richness was increased by the application of LP and BC individually ($P < 0.05$). Compared with the control silage, LP-treated silage had higher *Lactobacillus* ($P < 0.05$), while BC-treated silage had higher *Bacillus* and *Pediococcus* ($P < 0.05$). The LP + BC-treated silage had higher *Lactobacillus*, *Bacillus*, *Enterococcus*, and *Serratia* ($P < 0.05$). *Bacillus* was negatively correlated with NDF ($P < 0.05$) and AA ($P < 0.05$). *Lactobacillus* was positively correlated with LA ($P < 0.05$) and LA/AA but negatively with pH and NH_3-N ($P < 0.05$).

Conclusions The combination of BC and LP may lead to improved ensiled triticale fermentation quality and aerobic stability by inducing alterations in the composition of bacterial communities, which is crucial for the efficient utilization of triticale resources.

Keywords Triticale, Microbial diversity, *Bacillus coagulans*, *Lactobacillus plantarum*, Aerobic stability

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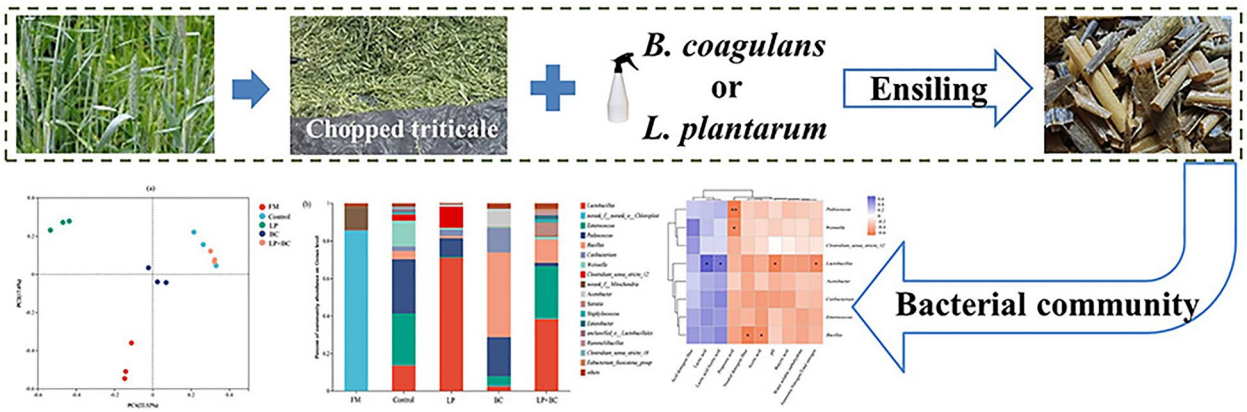
Graphical Abstract



Background

Triticale, an artificial allopolyploid hybrid, has been cultivated to combine yield efficiency and grain quality, resulting in high biomass and protein [1, 2]. Currently, it covers 3 million hectares (ha), with an average annual production of nearly 15 million tons in 2020. This resource could be beneficial in alleviating the shortage of feed for livestock [3, 4], as well as accelerating crop rotation and enhancing the utilization of fallow ground in winter [5].

Ensiling is an effective way to preserve wet crops by creating anaerobic conditions and acidification through the activity of lactic acid bacteria (LAB) [6, 7]. However, ensiling triticale can be challenging due to its hollow stem, which can lead to aerobic degradation during the process [8]. *Lactobacillus plantarum* (LP) is a commonly used exogenous LAB for its rapid acidification during ensiling [9, 10], but it has been reported to be ineffective in improving the aerobic stability of silage [11]. Recently, *Bacillus spp* has gained attention and recognition for its use in food, medicine, and feed production. Research has shown that certain strains of *Bacillus spp* can quickly create an anaerobic environment and initiate lactic fermentation through a unique biological oxygen capture mechanism [12], thereby improving nutrient preservation during ensiling. In particular, *Bacillus coagulans* (BC) has been found to improve feed digestibility by secreting a-amylase, xylanase, and protease [13], and optimize the bacterial community by producing bacteriocin, which has activity against pathogens, such as yeasts, *Escherichia coli*, *Salmonella*, *Listeria*, and *Salmonella pullorum* [14]. These microorganisms are considered major contributors to aerobic deterioration. Additionally, BC possesses the characteristics of both *Bacillus* and LAB, metabolizing sugars to produce acid in large quantities and exhibiting greater tolerance to acid [14, 15]. It has primarily been used in probiotic dietary supplements and proven to be beneficial in animal husbandry [15, 16]. Therefore, it is recommended that BC be used as an alternative inoculant to optimize the microbial composition of silage and promote silage fermentation and aerobic stability.

It has been suggested that silage quality was affected by the alterations in microbial community structure, and the microbial community profile could provide insight into the fermentation process and guarantee effective silage conservation [7]. Nonetheless, to our knowledge, the effects of BC on bacterial communities during silage fermentation have not been extensively studied. Moreover, there is a lack of studies that assess the effect of BC and LP on silage.

We hypothesized that the use of triticale as raw material for silage could be improved by adding LP and BC, which would optimize the communities of microorganisms and potentially lead to synergistic effects when used together.

Methods

Forage and ensiling

Triticale was harvested from three randomly selected experimental plots (13 m × 10 m) located in a semiarid irrigated area of Ningxia (107°N, 37°N, Yanchi, China) on May 19, 2021. Fresh triticale had been cut into 1–2 cm pieces and wilted to a dry matter (DM) content of around 350 g/kg.

Chopped triticale was randomly split into five subsamples, and treated with (1) control (Control; sterile water only, 10 mL); (2) commercial *Lactobacillus plantarum* (LP; Tianjin Yunli Star Co., LTD, Tian-jin, China); (3) *Bacillus coagulans* 21,735 (BC; China center of industrial

culture collection, accession number: CICC21735, Beijing, China); or (4) a combination of commercial *Lactobacillus plantarum* with *Bacillus coagulans* 21735 (LP + BC). The rest subsample was stored at −80 °C as raw material. Strain BC and LP were dissolved in 10 mL of sterile distilled water and sprayed evenly onto the forage for each treatment at an application rate of 1×10^6 cfu/g fresh weight of chopped triticale. For the control, the same amount of distilled water was added. Here, the BC strain could produce more organic acids in the silage environment. Before the study began, strains LP and BC were cultivated for 24 h each in MRS agar (HB0384, Hope Bio-Technology Co., Ltd, Qingdao, China) and nutrient agar (NA, HB0109, Hope Bio-Technology Co., Ltd, Qingdao, China), respectively. Then the number of live bacteria was determined using the plate-count method.

Around 500 g of mixed forage was packed into a polyethylene bag (300 × 270 mm; Embossed Food Saver Bag Co., Ltd., Chengdu, China) for each group, which was then vacuum sealed (DZ-400, Shandong Zhucheng Yizhong Machinery Co., Ltd., Zhucheng, China) and kept at 24–26 °C for 60 days. Each group was ensiled in triplicates.

Fermentation characteristic analysis

Twenty grams of fresh or silage material and 180 mL of distilled water (1:9 ratio) were homogenized for 2 min and filtered through 4 layers of cheesecloth. The pH was then measured using a pH meter (S-3G, Shanghai INESA Scientific Instrument Co., Ltd., China). The supernatant was divided into smaller portions and centrifuged at 2500 rpm for 10 min before being filtered through a 0.22 m microporous filter. Organic acids were then determined by high-performance liquid chromatography (HPLC, KC-811 column, Shodex; Shimadzu: Japan) at the oven temperature of 50 °C, the flow rate of 1 mL/min, and SPD of 210 nm [17].

Chemical composition analysis

The collected materials and silage samples were dried at 105 °C for 15 min before being reduced to 65 °C for 48 h in a forced-air oven. The samples were then ground and sieved through a 1-mm screen, followed by reheating at 105 °C until the DM was stable [18]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed using an ANKOM A2000i fiber analyzer (A2000i, ANKOM Technology, New York, USA) according to the methods of Van Soest and Goering [19, 20]. Crude protein (CP) was calculated by multiplying 6.25 with the content of total nitrogen (TN), which was determined using the Kjeldahl apparatus (K-360, BUCHI laboratory equipment trade Co., Ltd., Shanghai, China) [18]. Ammonia nitrogen (NH_3-N) and water-soluble carbohydrates (WSC) were measured via Cai's method [21]. Ether extract (EE) and crude ash were analyzed according to the methods of the Association of Official Analytical Chemists (AOAC) [22]. The data were expressed in g/kg DM.

Aerobic stability analysis

After 60 d of ensiling, each polyethylene bag was replaced with a new, perforated polyethylene bag (270 mm × 300 mm; Embossed Food saving bag; Changyang, Chengdu, China) that held around 300 g of silage. At the geometric center of each bag, a HOBO Pendant Temperature Data Logger (manufactured by Onset Ltd, Massachusetts, USA) was installed to record the temperature every 30 min. The silages were kept at a temperature of 24–26 °C, and aerobic stability was expressed as the time cost for the temperature of the silage exceeded the room temperature by 2 °C.

Bacterial community analysis

The total DNA extraction was performed according to the method of Zheng et al. [23]. Twenty grams of sample was collected, mixed with 80 mL of sterile water, and stirred at 120 rpm and 4 °C for 2 h. The samples were filtered through two layers of sterile gauze and then centrifuged at 10,000 × g for 15 min at 4℃. The DNA of fresh and silage materials was extracted (MP Biomedicals, Solon, USA), using the Fast DNA SPIN for soil Kit. The NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA) was used to measure the final DNA concentration and purity. Primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were employed to amplify the V3-V4 hypervariable portions of the bacteria's 16S rRNA gene using the Gene Amp 9700 (ABI, USA).

A PCR protocol was employed to analyze the DNA of fresh and silages. This protocol consisted of a 3-min denaturation at 95 °C, followed by 27 cycles of 30 s at 95 °C for denaturation, 30 s at 55 °C for annealing, and 45 s at 72 °C for elongation, and a final extension at 72 °C for 10 min. A 20 μL mixture was prepared for the PCR reactions, which included 4 μL of 5 FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 M), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Using the Illumina MiSeq platform (Majorbio, Shanghai). Prior to sequencing, the PCR samples underwent purification using Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, USA). The less than 50 bp reads were discarded in order to obtain clear reads. In order to examine the species diversity of all the samples, the clean reads were then grouped into operational taxonomic units

(OTUs) using the Uparse software at a similarity of 97%. The data were analyzed using the online platform of the Majorbio Cloud Platform (www.majorbio.com).

Statistical analysis

The general linear model of SPSS 18.0 (SPSS Inc., Chicago, USA) was used to analyze the data according to the model for a 2×2 factorial treatment design: $Y_{ij} = \mu + V_i + G_j + (V \times G)_{ij} + e_{ij}$, where μ = overall mean; V_i = effect of inoculation of commercial *Lactobacillus plantarum* ($i = 1, 2$); G_j = effect of inoculation of *Bacillus coagulans* 21,735 ($j = 1, 2$); $(V \times G)_{ij}$ = effect of interaction between inoculation of commercial *Lactobacillus plantarum* j and inoculation of *Bacillus coagulans* 21735 k, and e_{ij} was the residual error. The main effects were LP, BC, and their interaction. Tukey's HSD was employed to separate the differences between treatment means when at least one of the contrasts of LP × BC was significant. The difference was considered significant at $P < 0.05$.

Table 1 Characteristics of freshly chopped whole-plant triticale before ensiling (means ± standard deviation)

Item[1]	Content
DM, g/kg	261 ± 0.31
pH	6.61 ± 0.20
CP, g/kg DM	132 ± 0.34
NH_3-N, g/kg TN	48.1 ± 0.01
NDF, g/kg DM	584 ± 1.80
ADF, g/kg DM	323 ± 1.79
WSC, g/kg DM	139 ± 1.20
Crude ash, g/kg DM	36.4 ± 0.17
EE, g/kg DM	25.5 ± 0.30

[1] *DM* dry matter, *CP* crude protein, *NH_3-N* ammonia nitrogen, *TN* total nitrogen, *NDF* neutral detergent fiber, *WSC* water-soluble carbohydrate, *ADF* acid detergent fiber, *EE* ether extraction

Results

Chemical composition before ensiling

The chemical analysis of the fresh triticale is presented in Table 1. The fresh triticale was wilted to a DM content of 261 g/kg, with a relatively high pH (6.61). The CP, WSC, NDF, and ADF concentrations were 132, 139, 584, and 323 g/kg DM, respectively.

Fermentation characteristics analysis

The characteristics of triticale silage's fermentation are presented in Table 2. Inoculation with LP resulted in decreased pH values and acetic acid (AA) concentrations in treated silages ($P < 0.01$). There was an LP × BC interaction ($P < 0.05$) for lactic acid (LA) concentrations in treated silages. Silage treated with BC alone had greater LA concentration when compared to the control silage, while further application of BC did not have any effects on silage treated with LP + BC relative to silage treated with LP alone. Propionic acid (PA) ($P < 0.01$) was higher in silage with BC in comparison to those not treated with this organism, and the addition of LP significantly increased the concentration of PA in silage with BC ($P < 0.05$).

Chemical composition of triticale silage

The chemical composition of the silage was not significantly altered by the inoculation with LP or BC (Table 3). However, WSC concentrations were lower in silages inoculated with LP when compared to those not treated with inoculant. The WSC concentrations were lower in silages treated with BC, but more when no LP was applied. Inoculation with BC or LP resulted in significantly lower NDF and NH_3-N, especially when the two were combined. The aerobic stability of triticale silage was improved in all inoculated groups compared to those not treated with

Table 2 Fermentation characteristics of triticale silages ensiled for 60 d

Item[1]	No LP		LP[3]		SEM[5]	Effect (*p*-value)		
	No BC	BC[2]	No BC	BC		LP	BC	LP × BC[4]
pH	4.68[a]	4.39[ab]	4.19[b]	4.08[b]	0.05	< 0.01	0.10	0.42
LA, g/kg DM	26.56[c]	37.36[b]	41.53[ab]	44.13[a]	0.91	< 0.01	< 0.01	< 0.05
AA, g/kg DM	13.70[a]	13.17[a]	10.93[b]	10.46[b]	0.22	< 0.01	0.16	0.92
PA, g/kg DM	1.26[b]	1.30[b]	1.33[b]	1.56[a]	0.49	< 0.05	< 0.01	0.35
LA/AA	1.94[c]	2.84[b]	3.80[a]	4.21[a]	0.08	< 0.01	< 0.01	0.15

[1] *LA* lactic acid, *AA* acetic acid, *PA* propionic acid

[2] BC, *Bacillus coagulans* 21735 (China center of industrial culture collection, CICC 21735)

[3] LP, commercial *Lactobacillus plantarum*

[4] LP × BC, an interaction effect of inoculated LP and BC

[5] SEM, standard error of the means

[a–d] Means of inoculation treatments within a row with different superscripts differ ($P < 0.05$)

those inoculants ($P < 0.05$), with LP + BC-treated silage showing the greatest aerobic stability ($P < 0.05$).

Bacterial community analysis

Principal coordinate analysis (PCoA) based on Bray–Curtis distances was used to identify variations in the bacterial communities of triticale silage, as presented in Fig. 1a. The results demonstrate that the treated silages were clearly distinct from one another, with the LP + BC and control silages being the least divided. Additionally, the fresh materials were grouped together and separated from samples from other categories. PC1 and PC2 took up 22.52% and 17.6% of the overall variation, respectively. The shared and unique OTUs of the bacterial communities in triticale silages were visualized in a Venn diagram (Fig. 1b). There were 360, 130, 184, 498, and 115 OTUs in the FM, control, LP, BC, and LP + BC silages, respectively, with 198, 6, 6, 286, and 5 being unique. Additionally, all silages shared 34 OTUs.

Table 4 reveals a decrease in the Shannon index ($P < 0.01$) and an increase in the Ace and Chao1 indices ($P < 0.01$) for triticale silages treated with LP compared to

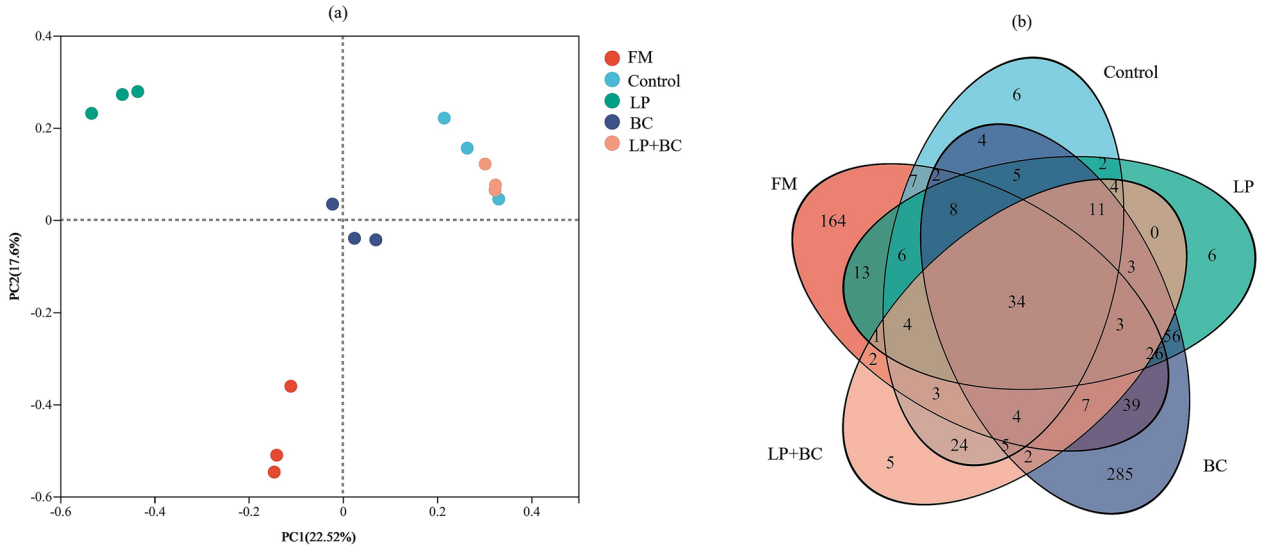


Fig. 1 Analysis of the bacterial community of triticale silage at the operational taxonomic unit (OTU) level by principal coordinate analysis (PCoA) (**a**) and Venn diagram (**b**). *FM* fresh materials, *LP* commercial *Lactobacillus plantarum*, *BC Bacillus coagulans* 21,735; LP + BC, silage treated with the mixture of *Bacillus coagulans* 21735 and commercial *Lactobacillus plantarum*

Table 3 Chemical composition of triticale silages ensiled for 60 d

Item[1]	No LP		LP[3]		SEM[4]	Effect (*p*-value)		
	No BC	BC[2]	No BC	BC		LP	BC	LP × BC[5]
DM, g/kg	289	292	297	298	2.01	0.08	0.49	0.69
CP, g/kg DM	110	110	111	111	0.52	0.91	0.75	0.93
NH_3-N, g/kg TN	46.00[a]	41.13[b]	39.20[b]	36.67[c]	0.42	< 0.01	< 0.01	0.17
WSC, g/kg DM	71.06[a]	64.90[b]	51.73[c]	47.90[d]	0.21	< 0.01	< 0.01	< 0.05
ADF, g/kg DM	356	347	337	330	2.72	0.12	0.46	0.93
NDF, g/kg DM	539[a]	519[b]	500[c]	494[d]	1.92	< 0.01	< 0.01	0.09
Crude ash, g/kg DM	30.80	31.33	29.47	29.33	0.26	< 0.05	0.76	0.62
Aerobic stability, h	44.50[d]	70.83[c]	81.67[b]	99.76[a]	1.46	< 0.01	< 0.01	0.19

[1] *DM* dry matter, *CP* crude protein, *NH_3-N* ammonia nitrogen, *TN* total nitrogen, *WSC* water-soluble carbohydrate, *ADF* acid detergent fiber, *NDF* neutral detergent fiber

[2] *BC Bacillus coagulans* 21,735 (China center of industrial culture collection, CICC 21735)

[3] *LP* commercial *Lactobacillus plantarum*

[4] *SEM* standard error of the means

[5] LP × BC, an interaction effect of inoculated LP and BC

[a–d] Means of inoculation treatments within a row with different superscripts differ ($P < 0.05$)

Table 4 Analysis of triticale silage's alpha diversity after ensiling

Items	No LP[2]		LP		SEM[4]	Effect (*p*-value)		
	No BC[1]	BC	No BC	BC		LP	BC	LP × BC[3]
Shannon	1.90[a]	1.35[c]	0.89[d]	1.79[b]	0.01	< 0.01	< 0.01	< 0.01
Ace	127[c]	400[a]	306[b]	105[c]	12.97	0.20	0.06	< 0.01
Chao 1	113[c]	315[a]	205[b]	93.6[c]	3.38	< 0.01	< 0.01	< 0.01
Coverage	0.99	0.99	0.99	0.99	0.00	0.24	0.12	< 0.01

[1] *BC Bacillus coagulans* 21,735 (China center of industrial culture collection, CICC 21735)

[2] *LP* commercial *Lactobacillus plantarum*

[3] LP × BC, an interaction effect of inoculated LP and BC

[4] *SEM* standard error of the means

[a] [−d]Means of inoculation treatments within a row with different superscripts differ ($P < 0.05$)

those without. Moreover, there were LP × BC interactions ($P < 0.01$) for all indices, with LP resulting in lower Ace and Chao1 and higher Shannon indices in silages treated with BC compared to those not treated with BC. The coverage was relatively high for all samples (> 0.99).

The major communities of the bacteria in the triticale silages comprised 4 phyla and 17 genera (Fig. 2). Fresh triticale was dominated by the *Cyanobacteria* (85.36%), while triticale silages were mainly composed of *Firmicutes* (75.51 ~ 96.45%). The control silage had a greater relative abundance of *Firmicutes* and a lower abundance of *Proteobacteria* when compared to the treated silages. Additionally, the control silage had a lower relative abundance of *Actinobacteriota* than the LP and BC-treated silages, but higher than the LP + BC-treated silage. The BC-treated silage had a lower relative abundance of *Firmicutes* and a higher abundance of *Actinobacteriota* than the LP and LP + BC-treated silages.

In terms of the genus (Fig. 2b), the control silage was mainly composed of *Pediococcus* (28.81%), *Enterococcus* (27.59%), *Weissella* (13.71%), and *Lactobacillus* (13.53%). The highest proportions of *Lactobacillus* (71.02%), and *Bacillus* (45.23%) were observed in the LP and BC-treated silages, respectively. Application of LP or BC alone resulted in lower *Weissella* and *Enterococcus* in treated silages. Compared with silages treated with LP alone, LP + BC-treated silage had a lower relative abundance of *Lactobacillus*, and greater relative abundances of *Enterococcus* (38.36%), *Bacillus* (12.34%), and *Serratia* (7.12%).

Correlation analysis of fermentation characteristics and bacterial community

As shown in Fig. 3, Spearman's rank correlation analysis indicated a negative correlation between the relative abundance of *Bacillus* and NDF/AA concentrations ($P < 0.05$). Furthermore, there was a negative relationship between the PA concentration with the abundance of *Weissella* ($P < 0.05$) and *Pediococcus* ($P < 0.01$). Additionally, *Lactobacillus* was observed negatively correlated with pH and NH_3-N/TN ($P < 0.05$) and positively associated with LA/AA and LA concentration ($P < 0.05$).

Discussion

Characteristics of triticale before and after ensiling

The quality of silage depends upon the characteristics of the raw materials used. Triticale is a highly nutritious option for ensiling, however, it is prone to aerobic deterioration due to its hollow stem. To protect silage from the growth of harmful microorganisms, the use of suitable additives is necessary. Bacterial inoculants have been developed to reduce the duration of the primary fermentation process and achieve good fermentation quality. *Bacillus spp*, a facultative anaerobic bacterium, can consume oxygen sources to create an anaerobic environment, which stimulates the growth of LAB [12] and the acidification of silages, thus suppressing the growth of spoilage microbes and minimizing nutrient loss by inhibiting. The degree of protein degradation in silage is frequently determined by the accumulation of NH_3-N (typically less than 10–15% of total nitrogen) during the ensiling process [24, 25]. In this study, the inoculants used decreased the concentration of NH_3-N. Proteolysis of ensiled triticale was inhibited most by the combination of LP and BC. This rapid decrease in pH may have inhibited the activity of plant proteases and decomposable protein strains [26], as well as the bacteriostatic effect of BC in suppressing mold activity and reducing the conversion efficiency of protein substances to NH_3-N. The decrease of NDF and ADF contents suggested that plant structural carbohydrates had been degraded, likely due to the *Bacillus*' production of fibrinolytic enzymes during the silage fermentation process [27]. The WSC concentration of the LP-treated straws was lower than that of the control silage [28], which was in accordance with our study where silages treated with LP or BC had lower

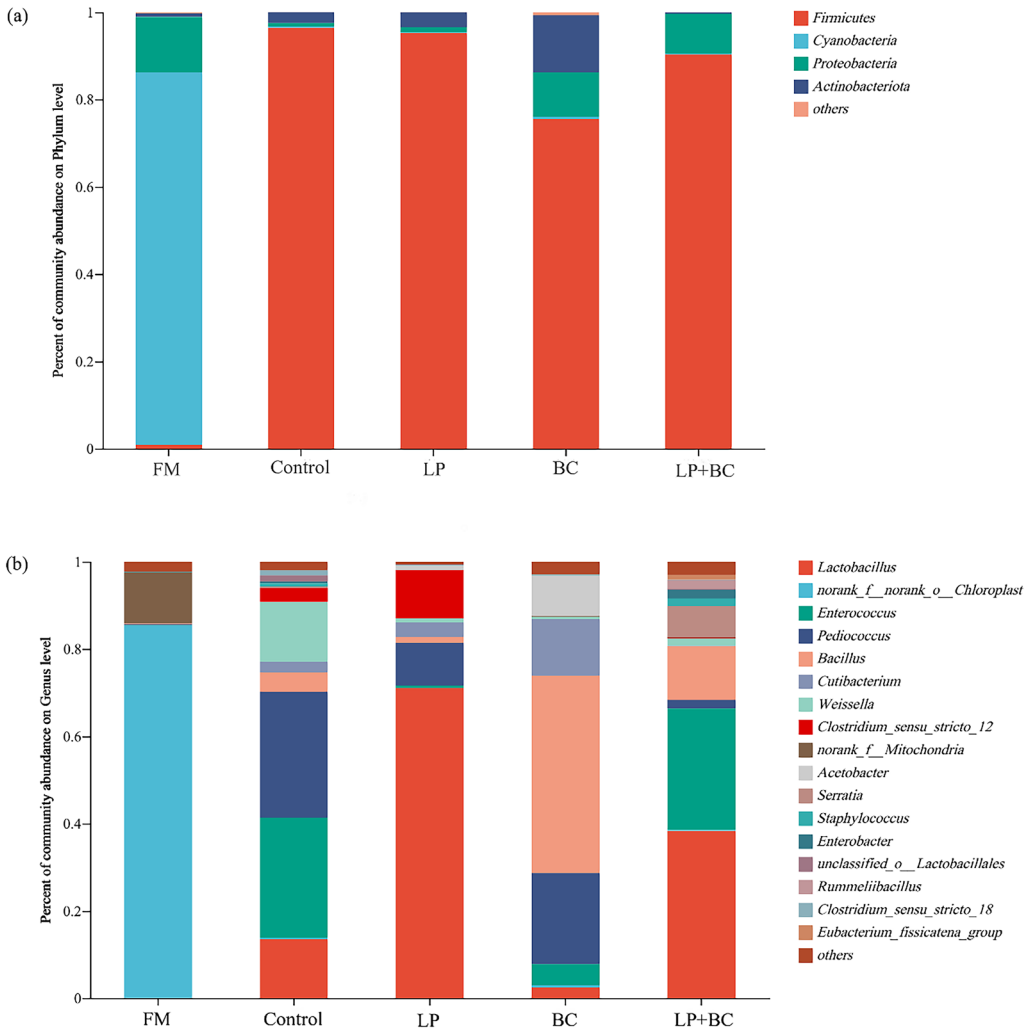


Fig. 2 Bacterial communities and relative abundance of triticale materials and their silages at the phylum level (**a**) and genus level (**b**)

WSC residues. Furthermore, anaerobic bacteria decompose the soluble sugar into lactic acid during the second stage of fermentation, resulting in the synergistic effect of the inoculants being observed to reduce the concentrations of WSC [29].

A rapid pH drop of ensiling plays a crucial role in inhibiting undesirable microorganisms and decreasing nutrient losses during ensiling [30]. The pH of the LP-treated groups was all below 4.2, which is the criteria for high-quality silage. *Lactobacillus plantarum*, a homofermentative bacterium, can encourage the formation of lactic acid, which is compatible with the results of this study [31]. The highest concentration of LA was observed in the LP + BC-treated silage, likely due to the accelerating growth of homofermentative LAB during fermentation by BC. However, the decrease in AA observed in ensiled triticale after LP inoculation is likely attributable to the suppressive effect of homofermentative LAB on heterofermentative LAB. The growth and reproduction of molds and fungi can be effectively inhibited by PA, which also has the capability to curtail the occurrence of secondary fermentation. This acid can promote the growth of LAB to a certain extent, thereby regulating the silage process and minimizing the decomposition of proteins [32, 33]. The combination of LP and BC was better for enhancing the silage quality of triticale silage in terms of nutritional content and fermentation characteristics.

Inoculation with either LP or BC alone, or a combination of both, has been proven to prolong the aerobic stabilization time of triticale silage. This is likely due to LP's ability to establish a stable acidic environment, which impedes the growth of spoilage bacteria and prevents yeast from metabolizing lactic acid and WSC [34]. Additionally, BC has been observed to produce antibiotics, which inhibit the proliferation of harmful microorganisms such as *Escherichia coli*, *Staphylococcus aureus*,

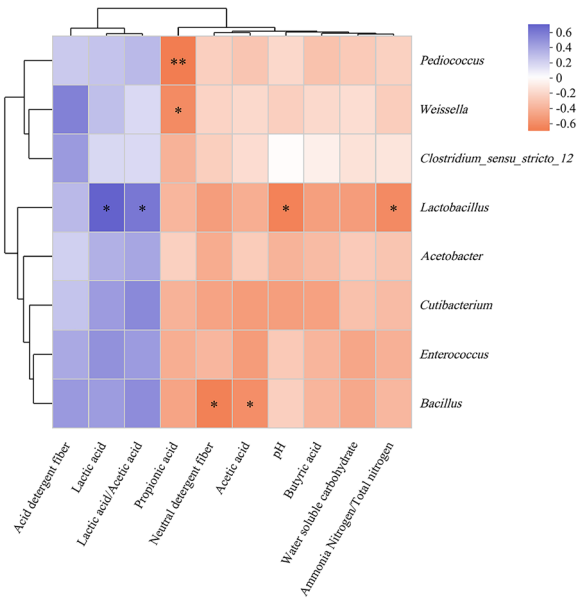


Fig. 3 The correlation between microorganisms and fermentation parameters at the genus level using Spearman's correlation analysis. * represents $P < 0.05$ and ** represent $P < 0.01$. Different color ranges represent different correlation coefficients in the right legend

Salmonella, and *Candida albicans* [35]. The LP + BC-treated silage exhibited the greatest aerobic stability, indicating a synergistic effect between the two.

Microbial communities of triticale silage

The microbial community and its metabolites play an essential role in the fermentation quality of silage. Based on our findings, it can be inferred that the sequencing data were found to be sufficient for a detailed analysis of the bacterial population, with a coverage value of 0.99 for each treatment. The Shannon and Chao1 indices serve as indicators of bacterial community diversity and richness, respectively [36]. Results showed that the bacterial diversity was lower and the richness was higher in the silages treated with LP or BC alone, which is in line with the findings of Liu et al. [6]. This may be due to the antibacterial activity of BC and lower pH [35].

The β-diversity analysis was utilized to measure the difference of bacterial communities across samples, and it was found that the microbial composition of triticale silage was significantly altered by ensiling in this study. The PCoA results indicated that a single additive could significantly modify the bacterial composition of triticale silage. Moreover, the inoculation with LP was found to be the primary factor of alterations during anaerobic fermentation. The Venn diagram revealed that BC augmented the total and unique OTU counts in comparison to the control silage. This could be attributed to the inhibited activity of BC and reduced production of bacteriocin [14]. The interaction between LP and BC prevented the growth of certain microorganisms in triticale silage by enhancing the production of lactic acid, thus decreasing the pH of the silage and creating an acidic environment, further suppressing the proliferation of *Clostridium* and other undesirable bacteria.

The ensiling process is primarily driven by bacterial fermentation, whereby the microflora present on the plant material plays a crucial role in determining the quality of silage produced. In this study, it was observed that *Cyanobacteria* dominated the microbial community on triticale prior to ensiling, with a subsequent shift towards *Firmicutes* during the ensiling process. This finding is consistent with the findings of a previous study conducted by Yuan et al. [37], which reported a marked increase in the abundance of *Firmicutes* in Napier grass silage with prolonged storage time. *Firmicutes* are Gram-positive bacteria that could break down a variety of macromolecules, including cellulose, protein, and starch. After silage fermentation, there is an increase in *Firmicutes* species such as *Lactobacillus* and *Enterococcus*, which can lead to a decrease in NDF, ADF, and NH_3-N in silage.

We also assessed bacterial communities at the genus level in order to future reveal the compositions of the bacterial communities in triticale silage inoculated with LP and BC. It has been previously observed that *Enterococcus*, *Pediococcus*, and *Weissella* are found in untreated barley silage [38], oat silage [39], and rice straw silage [40]. To date, it has been identified that *Pediococcus* and *Weissella* are regarded as unwanted in silage and encourage the development of microbes that cause spoilage. The addition of LP or BC resulted in a decrease in the relative abundance of *Pediococcus* and *Weissella* compared to a control group. In addition, the most abundant genus was *Lactobacillus*, which is essential for the decrease in pH during the later stages of ensiling and provides a stable environment [41]. These results indicated that exogenous microorganisms could modify the composition of the bacterial community in triticale silage. In this study, the dominant bacteria composition in the treatment group was similar, but there was an observable difference in the degree of relative abundance. The LP + BC-treated silage had a lower relative abundance of *Lactobacillus* than LP-treated silage, yet the fermentation quality was higher due to the addition of BC, which rapidly consumes oxygen and initiates fermentation, and acid-resistant LP, which helps to further reduce the pH. Additionally, a higher abundance of *Enterococcus* and *Serratia* was found in the LP + BC-treated silage, which might suggest that the synergy of the two additions is good for the growth of *Enterococcus* and *Serratia*. *Enterococcus* is a kind of cumulative anaerobic bacterium that mostly produces L (+)-LA, which contributes to

enhancing the fermentation quality of silage. Prodigiosin, a secondary metabolite produced by *Serratia*, may inhibit the growth of the fungi [42]. The greater aerobic steadiness in the LP + BC-treated silage could be attributable to this.

Ensiling promoted *Lactobacillus*, *Enterococcus*, and *Bacillus* proportion, which may have contributed to the fermentation process by creating an acidic environment that inhibited most microorganisms, as suggested by Cai et al. [43]. *Acetobacter aceti* is suitable for growing in an environment with a pH of about 7.0, inhibiting the growth of LAB and leading to silage fermentation [44], which may be one of the reasons for the high pH and AA of the BC-treated silage.

The correlation between fermentation characteristics and microflora

A strong association between the top 8 known bacterial genera and 10 environmental parameters was found using Spearman analysis. *Lactobacillus* was found to have a positive effect on LA and LA/AA, yet a negative effect on NH_3-N, as reported by Yang et al. [45]. Additionally, propionic acid was found to be highly soluble and permeable, thus altering the microbial composition of silage during the early stages of fermentation, resulting in an increase in lactic acid bacteria content and promoting silage fermentation [46, 47]. This study revealed that propionic acid could effectively inhibit the spoilage and growth of undesirable microorganisms during fermentation. *Bacillus* had negative effects on neutral detergent fiber and acetic acid. This could be due to the fact that *Bacillus* bacteria can synthesize a range of digestive enzymes, such as galase, glucanase and cellulase, which help in the degradation of complex carbohydrates in feed [48]. Additionally, *Bacillus* could also produce a variety of metabolites, including LA, antibiotics, and coagulin, which inhibit the growth of pathogenic and spoilage bacteria.

Conclusions

The application of BC or LP resulted in higher LA and lower WSC, AA, BA, NH_3-N, and NDF concentrations. This, in turn, leads to improved aerobic stability, increased relative abundances of *Lactobacillus*, *Enterococcus*, and *Bacillus*, and decreased relative abundance of *Weissella*. Furthermore, this combination inhibited protein degradation, making it a viable option for the reasonable utilization of triticale resources. Compared with LP alone, the combination of LP and BC has been shown to have a synergistic effect on optimizing bacterial communities in triticale silage, thereby improving fermentation quality and aerobic stability, which is vital for the reasonable utilization of triticale resources.

Abbreviations

LP	Commercial *Lactobacillus plantarum*
BC	*Bacillus coagulans* 21735
DM	Dry matter
CP	Crude protein
LA	Lactic acid
AA	Acetic acid
PA	Propionic acid
NH_3-N	Ammonia nitrogen
TN	Total nitrogen
WSC	Water-soluble carbohydrate
ADF	Acid detergent fiber
NDF	Neutral detergent fiber
EE	Ether extraction
LAB	Lactic acid bacteria

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Author contributions

Conceptualization, GZ; methodology, SL and WK; software, SL; formal analysis, WK; investigation, WK; resources, GZ; data curation, SL; writing—original draft preparation, SL and WK; writing—review and editing, QZ, WK, Dan Undersander and GZ; visualization, WK; supervision, GZ; project administration, GZ; funding acquisition, GZ. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The total sequencing data are accessible at NCBI (accession number PRJNA944439). The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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