

Article

Evaluating the Combined Effect of *Lactococcus lactis*, *Bacillus subtilis* and *Saccharomyces cerevisiae* in the Mixed Silage of Navel Orange Pomace and Rice Straw

Siyu Lu ¹, Vanajah Liyinthan ^{1,2}, Gang Liao ¹, Qinghua Qiu ¹ , Xianghui Zhao ¹, Yanjiao Li ¹ and Kehui Ouyang ^{1,*}

¹ College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang 330045, China; lsy13601655024@163.com (S.L.); vanajah@stu.jxau.edu.cn (V.L.); 18200131406@163.com (G.L.); rcuqqh@cau.edu.cn (Q.Q.); zhaoxh001@163.com (X.Z.); yanjiaoli221@163.com (Y.L.)

² Department of Animal Science, Faculty of Agriculture, Eastern University, Sri Lanka, Palacholai 30350, Sri Lanka

* Correspondence: ouyangkehui@sina.com

Abstract

A three-factor completely randomized design was used to evaluate the effects of *Lactococcus lactis*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* on the fermentation quality and nutritional composition of mixed silage prepared from navel orange pomace and rice straw. The addition of *L. lactis* increased lactic acid content and Flieg's score of the mixed silage. The addition of *B. subtilis* decreased the pH value and increased the lactic acid content of the mixed silage. The addition of *S. cerevisiae* decreased the lactic acid content and Flieg's score of the mixed silage. However, the interaction between the three strains significantly affected many parameters of the mixed silage. With respect to fermentation quality (Flieg's score), adding 0.3 g kg⁻¹ *L. lactis* and not adding *S. cerevisiae* achieved the best performance of the mixed silage. Under these conditions, increasing the level of *B. subtilis* resulted in a higher crude protein content of the mixed silage, whereas neutral detergent fiber and acid detergent fiber contents first decreased and then increased, and water-soluble carbohydrate content first increased and then decreased. Therefore, within the selected combination of adding 0.3 g kg⁻¹ *L. lactis* and not adding *S. cerevisiae*, the addition of 0.8 g kg⁻¹ *B. subtilis* gave the best nutritional quality.

Keywords: navel orange pomace; mixed silage; fermentation; *Lactococcus lactis*; *Bacillus subtilis*; *Saccharomyces cerevisiae*



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1. Introduction

Navel orange (*Citrus sinensis* Osb. var. *brassiliensis* Tanaka), a member of the Rutaceae family, is widely cultivated in China, Europe, and the Americas [1]. Gannan, particularly the Ganzhou region in Jiangxi Province of southern China, represents a major production area for this fruit. The region's planting area encompasses approximately 108,000 hectares, with an annual yield exceeding one million tons [2]. Fruit processing industries generate substantial quantities of by-products, including navel orange pomace, peel, and seeds [3]. Owing to their high moisture content and abundant carbohydrates, these by-products are difficult to manage and, if discarded directly, may pose environmental risks [4,5]. Navel orange pomace contains various nutrients, such as carbohydrates, vitamins, trace elements, and bioactive compounds (e.g., polyphenols and flavonoids) [6,7]. Consequently, research has increasingly focused on its potential application as animal feed. However, the presence

of bitter substances in citrus by-products reduces palatability, thereby limiting their use in animal production [8]. Studies have indicated that ensiling citrus pomace can alleviate bitterness, enhance nutrient content (e.g., crude protein), and improve digestibility [9,10]. Ensiling treatment thus represents an effective strategy for utilizing navel orange pomace. Nevertheless, direct ensiling often fails because of the excessively high moisture content of navel orange pomace. Rice straw, which is abundant in southern China and harvested around October, coincides with the navel orange harvest period. Given its high dry matter content and low water-soluble carbohydrate content, rice straw can be combined with navel orange pomace to produce high-quality silage [11].

Lactococcus lactis, *Bacillus subtilis*, and *Saccharomyces cerevisiae* are widely used as silage fermentation promoters. *Lactococcus lactis* rapidly produces lactic acid under anaerobic conditions, thereby lowering the pH of silage, inhibiting harmful microorganisms, and enhancing the antioxidant capacity of the silage feed [12]. *Bacillus subtilis* inhibits mold growth and improves aerobic stability [13]; additionally, it produces cellulases and hemicellulases, which degrade cellulose and hemicellulose to release fermentable sugars [14]. *Saccharomyces cerevisiae* rapidly consumes oxygen, establishing anaerobic conditions that favor lactic acid bacteria dominance and consequently enhance crude protein content [15–17]. Solid-state fermentation of citrus pomace with *Candida utilis* and *Bacillus subtilis* increases crude protein and soluble protein contents by 54.83% and 86.50%, respectively, while reducing pectin and crude fiber contents by 43.10% and 20.21%, respectively [17]. Hu et al. found that solid-state fermentation of citrus pomace with *Lactobacillus plantarum* and *Bacillus subtilis* increases dietary fiber and organic acid contents by 47.06% and 14.29%, respectively [18]. Despite these findings, most studies have used these microorganisms individually or in pairs, and few have investigated the combined use of all three for fermenting navel orange pomace. Wang et al. demonstrated that *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* constitute the most effective combination of fermentation substrates for apple pomace and millet bran [19]. In this experiment, rice straw was incorporated into navel orange pomace to counteract its excessive moisture. However, the inherently high fiber content and hollow structure [20] of rice straw pose considerable challenges to successful ensiling. Accordingly, we hypothesize that the combined inoculation with these three microbial strains in this mixed silage system can rapidly deplete oxygen, accelerate pH decline, enhance fiber digestibility, and increase crude protein content.

Therefore, to systematically evaluate the combined effects of the three microbial strains, this study employed a three-factor completely randomized design to determine the optimal combination and addition levels of microbial agents for the mixed silage of navel orange pomace and rice straw. The subsequent assessment of fermentation quality and nutritional composition provides a theoretical basis for the utilization of navel orange pomace in animal feed.

2. Materials and Methods

2.1. Preparation of Mixed Silage

Fresh navel orange pomace was sourced from a juice processing plant (Nongfu Spring Co., Ltd., Hangzhou, Zhejiang, China) located in Ganzhou, Jiangxi, China, in December 2023. It was a by-product produced by juicing fully matured navel oranges (mid-harvest fruit), consisting of pomace, peel, and seeds. Rice straw was manually harvested in Ganzhou, Jiangxi, China, in October and cut into 1–2 cm lengths prior to ensiling. Navel orange pomace and rice straw were homogeneously mixed at a weight ratio of 7:3, with the moisture content adjusted to approximately 65% and the water-soluble carbohydrate (WSC) content maintained at no less than 10% [21]. The nutritional composition of navel orange pomace and rice straw before ensiling is presented in Table 1. *Lactococcus lactis*,

Bacillus subtilis, and *Saccharomyces cerevisiae* were dissolved in distilled water at varying concentrations. The solutions were applied evenly to the prepared silage mixture with concurrent manual mixing, and the moisture level was finally adjusted to approximately 70% (*w/w*).

Table 1. Nutrient composition of navel orange pomace and rice straw before ensiling.

Item ¹	Navel Orange	Rice Straw
Dry matter (g kg ^{−1} FM)	125.13	796.76
Crude protein (g kg ^{−1} DM)	78.27	40.91
Ether extract (g kg ^{−1} DM)	32.89	26.06
Neutral detergent fiber (g kg ^{−1} DM)	140.34	605.53
Acid detergent fiber (g kg ^{−1} DM)	103.46	365.90
Water soluble carbohydrates (g kg ^{−1} DM)	235.21	55.27
Ash (g kg ^{−1} DM)	31.42	130.84
Ca (g kg ^{−1} DM)	3.66	8.68
P (g kg ^{−1} DM)	0.49	1.19

¹ FM: fresh matter. DM: dry matter. Same as below.

- (1) *Lactococcus lactis* (Jiangxi Qiangwei Microbial Technology Co., Ltd., Yichun, Jiangxi, China) was added at 0, 0.1, 0.2, and 0.3 g kg^{−1} fresh matter (FM), designated as A₁, A₂, A₃, and A₄, respectively. The viable count was at least 5 × 10⁸ colony-forming units (CFU) g^{−1}.
- (2) *Bacillus subtilis* (Guangzhou Duyi Biotechnology Co., Ltd., Guangzhou, Guangdong, China) was added at 0, 0.4, 0.8, and 1.2 g kg^{−1} FM, designated as B₁, B₂, B₃, and B₄, respectively. The viable count was at least 1 × 10⁹ CFU g^{−1}.
- (3) *Saccharomyces cerevisiae* (Angel Yeast Co., Ltd., Yichang, Hubei, China) was added at 0 and 0.03 g kg^{−1} FM, designated as C₁ and C₂, respectively. The viable count was at least 1 × 10⁹ CFU g^{−1}.

A three-factor completely randomized experimental design was used, comprising 32 treatments with five replicates per treatment. The prepared silage materials were packed into polyethylene bags (40 cm × 23 cm), each containing 350 g of sample, and sealed using a vacuum packaging machine (Mag, DZ-400/2S, Qingdao Mag Automation Equipment Co., Ltd., Qingdao, China). Each bag was considered an independent experimental unit. The silage density was approximately 420–450 kg m^{−3}. Samples were stored at room temperature (25 °C) and analyzed after 60 days.

2.2. Chemical Analyses

The quartering method was applied to each group. A 20 g subsample was uniformly collected and mixed with distilled water at a 1:9 (*w/v*) ratio [22]. The mixture was homogenized in a mixer tank, filtered through four layers of cheesecloth and filter paper, and allowed to stand for 30 min to obtain the leachate. The pH of the leachate was measured using a pH meter (Testo 206-pH 1, Testo SE & Co. KGaA, Titisee-Neustadt, Germany). The NH₃-N content was determined using the sodium hypochlorite and phenol method [23]. Organic acids, including lactic acid, acetic acid, propionic acid, and butyric acid, were quantified by high-performance liquid chromatography [24]. The Flieg's score was calculated according to the formula proposed by Kilic [25]. The quality of mixed silage feed was classified into five categories based on the score: excellent (80–100), good (60–80), fair (40–60), poor (20–40), and extremely poor (0–20).

The nutritional content of the raw materials (before ensiling) and the mixed silage feed (only the treatment groups with higher Flieg's scores) were measured. Samples of 300 g each of fresh navel orange pomace, fresh rice straw, and mixed silage were placed in

an oven at 120 °C for 15 min, then transferred to an oven at 65 °C and dried to constant weight to determine dry matter (DM) content [26]. The dried samples were then ground and sieved through a 40-mesh screen for chemical composition analysis. Crude protein (CP) and ether extract (EE) contents were determined using the Kjeldahl nitrogen method as recommended by AOAC [27]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were measured using Van Soest's fiber analysis method [28]. Ash content was determined by combustion at 550 °C [27]. Water-soluble carbohydrate (WSC) content was measured using the anthrone–sulfuric acid method [29].

2.3. Statistical Analysis

Statistical analyses were performed using SPSS version 25.0 (Chicago, IL, USA). After confirming residual normality via the Shapiro–Wilk test, fermentation quality data were subjected to a three-way fixed-effects ANOVA, whereas one-way ANOVA was applied to the nutritional composition data. When significant differences were found, post hoc pairwise comparisons were performed using Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Fermentation Quality of Mixed Silage

3.1.1. pH Value

The pH of the fermented orange pomace mixture ranged from 3.87 to 4.02 across all treatments (Table 2), indicating that an acidic environment was established regardless of the microbial inoculants used. Increasing levels of *Lactococcus lactis* (A₁–A₄) did not significantly change pH ($p = 0.27$). In contrast, a higher addition level of *Bacillus subtilis* resulted in a slight but consistent decrease in pH from B₁ to B₄ ($p = 0.02$). No significant interactions were observed between *L. lactis*, *B. subtilis*, and *S. cerevisiae* in any combination (A × B, A × C, B × C, and A × B × C; all $p > 0.05$). The low standard error of the mean (SEM = 0.01) indicates high consistency among replicates.

Table 2. Effect of three microbial agents on pH of mixed silage.

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	4.02	3.87	3.88	3.90	3.91	3.92	3.93	3.91	0.01
A ₂	3.99	3.91	3.89	3.91	3.93	3.92	3.90	3.90	
A ₃	3.93	3.90	3.92	3.92	3.91	3.89	3.90	3.87	
A ₄	3.92	3.90	3.92	3.92	3.88	3.87	3.87	3.91	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	0.27	0.02	0.06	0.53	0.53	0.08	0.41		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg^{−1} FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg^{−1} FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg^{−1} FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C.

3.1.2. NH₃-N

Table 3 indicates that the interaction among the three microbial agents (A, B, and C) did not have a significant effect on the NH₃-N content of the mixed silage ($p > 0.05$). Likewise, the application of each individual microbial agent did not significantly influence NH₃-N content ($p > 0.05$).

3.1.3. Lactic Acid

Table 4 shows that the interaction between microbial agents A and C significantly affected the lactic acid content of mixed silage ($p < 0.05$). At C₁ (0 g kg⁻¹ FM *S. cerevisiae*), A₃ and A₄ resulted in significantly higher lactic acid content compared to A₁ and A₂ ($p < 0.05$). In contrast, the addition of C₂ did not produce a significant effect at any level of agent A ($p > 0.05$). Similarly, the interaction between agents B and C significantly influenced lactic acid content ($p < 0.05$). At C₁ (0 g kg⁻¹ FM *S. cerevisiae*), B₂, B₃, and B₄ significantly increased lactic acid content compared to B₁ ($p < 0.05$), whereas the addition of C₂ resulted in no significant effect at any level of agent B ($p > 0.05$). Agents A and B significantly increased lactic acid content, whereas *S. cerevisiae* supplementation (C₂) significantly decreased it compared with its absence (C₁; $p < 0.05$). Specifically, A₃ and A₄, as well as B₂, B₃, and B₄, produced significantly higher lactic acid levels than their respective lower levels ($p < 0.05$). The absence of agent C resulted in higher lactic acid content than its presence ($p < 0.05$).

Table 3. Effect of three microbial agents on NH₃-N of mixed silage (g kg⁻¹ DM).

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	2.47	2.57	2.75	2.61	2.42	2.47	2.42	2.57	0.02
A ₂	2.48	2.60	2.58	2.68	2.52	2.49	2.56	2.55	
A ₃	2.53	2.64	2.61	2.66	2.45	2.48	2.62	2.60	
A ₄	2.63	2.47	2.54	2.51	2.54	2.61	2.63	2.57	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	0.86	0.20	0.12	0.96	0.29	0.99	0.71		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg⁻¹ FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg⁻¹ FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg⁻¹ FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C.

Table 4. Effect of three microbial agents on lactic acid of mixed silage (g kg⁻¹ DM).

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	7.09	16.47	15.48	16.19	10.10	12.52	13.38	11.31	0.26
A ₂	11.39	16.21	16.05	15.20	9.28	10.23	12.00	8.89	
A ₃	16.51	18.00	18.13	19.02	9.66	10.93	11.43	11.95	
A ₄	16.16	20.51	20.20	22.03	10.36	10.19	9.32	11.79	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	<0.01	<0.01	<0.01	0.32	<0.01	0.02	0.85		
Interaction ³	A × C	A ₁	A ₂	A ₃	A ₄	SEM	<i>p</i> -Value		
	C ₁	13.80 ^b	14.71 ^b	17.91 ^a	19.73 ^a	0.86	<0.001		
	C ₂	11.83	10.1	10.99	10.41	0.79	0.437		
	B × C	B ₁	B ₂	B ₃	B ₄				
	C ₁	12.79 ^b	17.80 ^a	17.46 ^a	18.11 ^a	0.90	<0.001		
	C ₂	9.85	10.97	11.53	10.99	0.79	0.502		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg⁻¹ FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg⁻¹ FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg⁻¹ FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C. ³ Values in the same row followed by different lowercase letters differ significantly ($p < 0.05$) by Duncan's multiple range test.

3.1.4. Acetic Acid

Table 5 indicates that neither the interaction among microbial agents nor the addition of individual agents significantly affected the acetic acid content of the mixed silage ($p > 0.05$).

Table 5. Effect of three microbial agents on acetic acid of mixed silage (g kg^{−1} DM).

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	4.02	4.58	4.90	4.39	4.23	4.22	4.32	4.27	0.06
A ₂	4.29	4.49	4.56	4.31	4.49	4.08	3.79	4.22	
A ₃	4.30	4.07	4.46	4.67	3.93	3.99	4.31	4.12	
A ₄	4.25	4.05	4.41	4.37	4.02	4.04	4.39	4.27	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	0.83	0.62	0.09	0.96	0.97	0.83	0.96		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg^{−1} FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg^{−1} FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg^{−1} FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C.

3.1.5. Propionic Acid

Table 6 shows that although the interaction between microbial agents A and C significantly affected propionic acid content ($p = 0.04$), no significant differences among A levels were detected within either C₁ or C₂ ($p > 0.05$). The application of individual microbial agents did not significantly affect propionic acid content ($p > 0.05$).

Table 6. Effect of three microbial agents on propionic acid of mixed silage (g kg^{−1} DM).

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	1.62	1.57	1.31	1.50	1.38	1.10	1.30	1.29	0.02
A ₂	1.41	1.42	1.28	1.43	1.30	1.18	1.49	1.33	
A ₃	1.35	1.48	1.71	1.34	1.22	1.54	1.35	1.58	
A ₄	1.35	1.28	1.16	1.45	1.42	1.34	1.29	1.54	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	0.30	0.55	0.11	0.11	0.04	0.43	0.11		
Interaction	A × C	A ₁	A ₂	A ₃	A ₄	SEM	<i>p</i> -Value		
	C ₁	1.50	1.38	1.47	1.31	0.06	0.106		
	C ₂	1.27	1.33	1.42	1.40	0.03	0.205		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg^{−1} FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg^{−1} FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg^{−1} FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C.

3.1.6. Butyric Acid

Table 7 shows that the interaction among the three microbial agents significantly affected the butyric acid content in the mixed silage ($p < 0.05$). The addition of agents A and B did not significantly influence butyric acid content ($p > 0.05$).

3.1.7. Flieg's Score

Table 8 demonstrates that the interaction between microbial agents A and C significantly affected the Flieg's score of the mixed silage ($p < 0.05$). In the absence of *S. cerevisiae* (C₁), A₄ resulted in a significantly higher Flieg's score compared to combinations with A₁ and A₂ ($p < 0.05$). Increasing the concentration of agent A alone also led to significant improvements in the Flieg's score, with higher levels producing greater effects ($p < 0.05$). The absence of microbial agent C resulted in higher Flieg's scores than its presence ($p < 0.05$). These findings suggest that the optimal combination for maximizing the Flieg's score is A₄ and C₁.

Table 7. Effect of three microbial agents on butyric acid of mixed silage (g kg⁻¹ DM).

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	0.19	0.35	0.23	0.37	0.26	0.00	0.26	0.16	0.02
A ₂	0.26	0.27	0.17	0.26	0.00	0.00	0.26	0.09	
A ₃	0.36	0.19	0.29	0.09	0.09	0.27	0.18	0.27	
A ₄	0.17	0.09	0.17	0.16	0.10	0.17	0.09	0.19	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	0.15	0.78	0.01	0.98	0.28	0.45	0.02		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg⁻¹ FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg⁻¹ FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg⁻¹ FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C.

Table 8. Effect of three microbial agents on Flieg's score of mixed silage.

Group	C ₁				C ₂				SEM ¹
	B ₁	B ₂	B ₃	B ₄	B ₁	B ₂	B ₃	B ₄	
A ₁	65.00	83.50	86.25	78.50	64.00	89.25	80.00	74.50	1.06
A ₂	74.25	86.50	89.00	82.75	77.25	84.25	74.25	72.75	
A ₃	84.75	93.00	91.25	97.50	78.50	70.50	76.00	70.75	
A ₄	94.00	98.75	98.75	99.25	80.25	74.00	75.00	75.75	
<i>p</i> -Value ²	A	B	C	A × B	A × C	B × C	A × B × C		
	0.02	0.07	<0.01	0.33	<0.01	0.24	0.94		
Interaction ³	A × C	A ₁	A ₂	A ₃	A ₄	SEM	<i>p</i> -Value		
	C ₁	78.31 ^b	83.13 ^b	91.63 ^a	97.69 ^a	1.48	<0.001		
	C ₂	76.94	77.13	73.94	76.25	1.63	0.894		

¹ SEM: standard error of the mean. ² A₁–A₄: Add 0, 0.1, 0.2, and 0.3 g kg⁻¹ FM *Lactococcus lactis*, respectively. B₁–B₄: Add 0, 0.4, 0.8, and 1.2 g kg⁻¹ FM *Bacillus subtilis*, respectively. C₁–C₂: Correspond to 0 and 0.03 g kg⁻¹ FM *Saccharomyces cerevisiae*, respectively. A × B: interaction effect between A and B; A × C: interaction effect between A and C; B × C: interaction effect between B and C; A × B × C: interaction effect among A, B, and C. ³ Values in the same row followed by different lowercase letters differ significantly ($p < 0.05$) by Duncan's multiple range test.

3.2. Nutritional Components of Mixed Silage

Four groups with higher Flieg's scores (A₄B₁C₁, A₄B₂C₁, A₄B₃C₁, and A₄B₄C₁) were selected for further nutritional analysis to evaluate fermentation effects (within the A₄C₁ subset). Table 9 presents the nutritional composition of the mixed silage. The crude protein (CP) content of mixed silage increased, with A₄B₄C₁ exhibiting significantly higher CP content than A₄B₁C₁ ($p < 0.05$). The neutral detergent fiber (NDF) content initially decreased and then increased, and the NDF content of A₄B₃C₁ was significantly lower than that of A₄B₁C₁ ($p < 0.05$). Compared to A₄B₁C₁, the NDF content of A₄B₂C₁, A₄B₃C₁, and A₄B₄C₁ decreased by 3.52%, 8.08%, and 4.85%, respectively. The acid detergent fiber (ADF) content showed a numerical tendency to first decrease and then increase; however, this effect was not statistically significant ($p = 0.064$). The water-soluble carbohydrate (WSC) content increased initially and then decreased, with A₄B₃C₁ showing a significantly higher WSC content than both A₄B₁C₁ and A₄B₄C₁ ($p < 0.05$). Compared to A₄B₁C₁, the WSC content of A₄B₂C₁, A₄B₃C₁, and A₄B₄C₁ increased by 6.90%, 16.09%, and 3.74%, respectively. The addition of microbial agent B did not significantly affect the dry matter (DM), ether extract (EE), or ash content of the mixed silage ($p > 0.05$). These results indicate that A₄B₃C₁ represents the optimal additive combination.

Table 9. Effect of different microbial agents on the nutritive composition of mixed silage.

Item	Group ¹				SEM ²	p-Value
	A ₄ B ₁ C ₁	A ₄ B ₂ C ₁	A ₄ B ₃ C ₁	A ₄ B ₄ C ₁		
Dry matter (g kg ⁻¹ FM)	281.96	290.62	290.02	291.11	0.35	0.935
Crude protein (g kg ⁻¹ DM)	61.10 ^b	62.52 ^{ab}	63.31 ^{ab}	65.49 ^a	0.06	<0.001
Ether extract (g kg ⁻¹ DM)	19.35	20.40	20.75	21.19	0.05	0.667
Neutral detergent fiber (g kg ⁻¹ DM)	476.71 ^b	459.99 ^{ab}	438.22 ^a	453.59 ^{ab}	3.15	0.047
Acid detergent fiber (g kg ⁻¹ DM)	303.60	295.55	285.67	296.03	0.24	0.064
Water soluble carbohydrate (g kg ⁻¹ DM)	34.79 ^b	37.28 ^{ab}	40.44 ^a	36.14 ^b	0.08	0.049
Ash (g kg ⁻¹ DM)	106.96	105.41	104.45	109.19	0.16	0.768

¹ SEM: standard error of the mean. ² Codes A₄B₁C₁, A₄B₂C₁, A₄B₃C₁, and A₄B₄C₁ indicate a fixed addition of 0.3 g kg⁻¹ FM *L. lactis* and no *S. cerevisiae*, with *B. subtilis* supplemented at 0, 0.4, 0.8, and 1.2 g kg⁻¹ FM, respectively. A₄: Add 0.3 g kg⁻¹ FM *L. lactis*. B₁-B₄: Add 0, 0.4, 0.8, and 1.2 g kg⁻¹ FM *B. subtilis*, respectively. C₁: Correspond to 0 g kg⁻¹ FM *S. cerevisiae*. Values in the same row followed by different lowercase letters differ significantly ($p < 0.05$) by Duncan's multiple range test.

4. Discussion

4.1. The Effect of Three Microbial Agents on the Fermentation Quality of Mixed Silage

The pH value, lactate content, and butyric acid content are crucial indicators for evaluating the quality of mixed silage. Generally, a lower pH value indicates better feed quality; however, excessively low pH has negative effects [30,31]. Low pH values can inhibit the activity of harmful microorganisms, thereby decreasing protein degradation and the production of harmful acids, which benefits the preservation of silage feed [32]. In this experiment, the pH of the mixed silage ranged from 3.8 to 4.2, indicating good silage quality [22]. The addition of *Bacillus subtilis* significantly decreased the pH of the mixed silage, consistent with the findings of Guo et al. [33]. *Bacillus subtilis* can create an anaerobic environment in silage by rapidly consuming oxygen during the early stages of ensiling, thereby inhibiting aerobic spoilage bacteria [13]. It also produces antimicrobial peptides to eliminate the main competitors of lactic acid bacteria [34]. Furthermore, it can degrade fibers and release sugars, promoting the rapid growth of anaerobic lactic acid bacteria, which subsequently produce large amounts of lactic acid and further lower the silage pH [35].

Lactic acid inhibits the growth and reproduction of harmful microorganisms, thereby ensuring the long-term preservation of silage feed [36]. In the present experiment, the addition of *Lactococcus lactis* and *Bacillus subtilis* significantly increased lactic acid content, consistent with previous studies [37–39]. Conversely, the addition of *Saccharomyces cerevisiae* significantly decreased lactic acid content, aligning with the findings of Zhou et al. [39]. Excessive supplementation with *Saccharomyces cerevisiae* may lead to competition with lactic acid bacteria for limited sugar resources, thereby increasing ethanol production [40,41]. Sofyan et al. reported that the simultaneous addition of *Lactococcus lactis* and *Saccharomyces cerevisiae* reduced both lactic acid content and Flieg's score in elephant grass silage [42]. Similarly, in this experiment, the combined addition of *Lactococcus lactis* and *Saccharomyces cerevisiae*, as well as *Bacillus subtilis* and *Saccharomyces cerevisiae*, reduced lactic acid content, corroborating previous research. Nutritional competition exists between *Saccharomyces cerevisiae* and *Lactococcus lactis*, as *Saccharomyces cerevisiae* preferentially utilizes sugars in silage raw materials for ethanol fermentation, thereby reducing the substrates available to *Lactococcus lactis* for lactic acid synthesis [42]. Additionally, metabolites produced by *Saccharomyces cerevisiae*, such as ethanol, can inhibit the growth and metabolic activity of certain lactic acid bacteria when accumulated to specific concentrations, further decreasing lactic acid production [43]. *Bacillus subtilis* can degrade structural carbohydrates into sugars,

which are subsequently utilized by yeast, thereby indirectly supporting *Saccharomyces cerevisiae* proliferation and metabolism and reducing lactic acid production [44].

Butyric acid is a harmful by-product generated through the fermentation activity of *Clostridium butyricum* [45]. Its pungent odor reduces silage palatability and, consequently, decreases animal feed intake [46]. In silage, *Clostridium* converts sugars and lactic acid into butyric acid [47]. The combined application of *Lactococcus lactis*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* decreases butyric acid content in silage, likely due to synergistic interactions among these microbial agents. *Bacillus subtilis* rapidly consumes oxygen to establish a strong anaerobic environment [13] and degrades fibers to release sugars [48], thereby supporting the growth of lactic acid bacteria and yeast, which collectively inhibit *Clostridium butyricum* proliferation.

In this study, supplementation with *Saccharomyces cerevisiae* significantly reduced lactic acid content and Flieg's score. The failure of yeast supplementation to improve fermentation quality under the present conditions may be attributed to the short straw length (2 cm), which allowed for the rapid dissipation of entrapped air from the hollow straw structure [20]. In practical production, straw may be crushed into longer fragments, leading to more pronounced oxygen entrapment. In such cases, *Saccharomyces cerevisiae* might still play a role in consuming residual oxygen, although this possibility remains speculative and warrants further investigation.

4.2. Effect of Microbial Agents on Nutritional Components of Mixed Silage

In this experiment, the crude protein (CP) content of the mixed silage increased with increasing amounts of *Bacillus subtilis* added. This can be explained by the fact that during ensiling, a decrease in pH can alleviate CP degradation by plant proteases and aerobic microorganisms, thereby reducing protein hydrolysis [49,50]. Furthermore, the addition of *Lactococcus lactis* and *Bacillus subtilis* can inhibit CP degradation by rapidly lowering pH, as rapid acidification reduces proteolysis and $\text{NH}_3\text{-N}$ formation, thereby helping preserve crude protein during ensiling [35]. The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were negatively correlated with animal feed intake and digestibility, respectively [51]. In this study, increasing *Bacillus subtilis* levels caused NDF content to first decrease and then increase; ADF content followed the same numerical pattern, but without statistical significance. This pattern was consistent with the findings of Li et al. [52]. Notably, the NDF and ADF contents were lowest when *Bacillus subtilis* was added at 0.8 g kg^{-1} .

During ensiling, *Bacillus subtilis* promotes the production of cellulolytic enzymes, which degrade plant cell walls and reduce the content of ADF and NDF in silage [53]. This reduction enhances the digestibility of silage for animals [53]. *Lactococcus lactis* lowers the pH, creating an acidic environment that further stimulates *Bacillus subtilis* to produce cellulose-related enzymes [54]. Consequently, a synergistic effect between *Lactococcus lactis* and *Bacillus subtilis* decreases NDF and ADF levels in mixed silage. However, excessive supplementation with *Bacillus subtilis* may result in insufficient fermentation substrates for lactic acid bacteria [55] and alter the microbial community structure, leading to suboptimal degradation of NDF and ADF.

Water-soluble carbohydrates (WSCs) serve as the primary energy source and substrate for lactic acid production by lactic acid bacteria during anaerobic fermentation [56]. Prolonged silage fermentation results in increased WSC consumption from the raw materials. In the present study, WSC content initially increased and then decreased as the addition level of *Bacillus subtilis* was raised, with 0.8 g kg^{-1} identified as the optimal level. Bai et al. reported that *Bacillus subtilis* supplementation significantly increased the WSC content of whole plant corn silage [57]. In contrast, another study demonstrated that a mixture

of *Lactobacillus plantarum* and *Bacillus subtilis* significantly decreased the WSC content of alfalfa silage [58]. *Bacillus subtilis* is capable of producing WSC by degrading plant cell walls [14]. However, excessive addition of *Bacillus subtilis* can result in substantial WSC consumption, as surplus bacteria utilize it for their metabolic activities. This consumption may surpass the rate of sugar production from fiber decomposition, leading to a reduction in WSC content. *Lactococcus lactis* can rapidly lower silage pH, thereby inhibiting WSC consumption by aerobic microorganisms [13]. An interaction between *Lactococcus lactis* and *Bacillus subtilis* was observed, which promoted WSC production in mixed silage. Nevertheless, when the proportion of these additives exceeds a certain threshold, WSC production in silage may decline.

5. Conclusions

The addition of *Lactococcus lactis* increased lactic acid content and Flieg's score. The addition of *Bacillus subtilis* decreased the pH value and increased the lactic acid content. The addition of *Saccharomyces cerevisiae* decreased the lactic acid content and Flieg's score. However, the interaction between the three strains significantly affected many parameters of the mixed silage. With respect to fermentation quality (Flieg's score), the A₄C₁ treatment achieved the best performance of the mixed silage. Under these conditions, increasing the level of *B. subtilis* resulted in a higher crude protein content of the mixed silage, whereas neutral detergent fiber and acid detergent fiber contents first decreased and then increased, and the water-soluble carbohydrate content first increased and then decreased. In brief, within the selected A₄C₁ combination, 0.8 g kg^{−1} *B. subtilis* improved the overall nutritional profile, particularly by reducing NDF and increasing WSCs.

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