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RESEARCH ARTICLE

Synergistic Effects of Wheat Bran and Inoculants on Fermentation and Nutritional Quality of Soybean Straw Silage

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Abstract: The efficient valorisation of lignocellulosic biomass from soybean straw into high-quality silage remains a pressing challenge for sustainable livestock production. This study employed a 3×3 factorial design to systematically investigate the interactive effects between wheat bran additions (0%, 10%, 20% of fresh weight) and microbial inoculant regimes (control, Effective Microorganisms, EM+*Trichoderma longibrachiatum*) on soybean straw silage quality. Two-way ANOVA revealed significant main effects and interactions between these factors across multiple quality parameters, providing novel insights into their mechanistic relationships. The strategic combination of these additives significantly enhanced silage quality, with crude protein content increasing by up to 12% (reaching 18.52% in optimal treatments), fiber fractions decreasing significantly (NDF reduced by 8.7%), and pH declining from 5.44 to 4.43, thereby elevating relative feed value by 16 points ($P<0.05$). Most notably, all 10% wheat bran treatments completely suppressed butyric acid production. The 10% wheat bran plus EM inoculant (W2T2) treatment emerged as particularly effective, achieving optimal lactic:acetic acid ratio (4.5:1) while maintaining negligible butyric acid production. Unexpectedly, 20% wheat bran without inoculant (W3T1) yielded the highest relative feed value (142.73), suggesting that higher wheat bran levels provide both sufficient fermentable carbohydrates and an indigenous microbial community capable of effective fermentation. This research advances our understanding of enzymatic bioconversion of recalcitrant lignocellulosic structures through specific substrate-inoculant threshold combinations that optimize silage quality via synergistic interactions rather than additive effects alone. These findings offer a scientifically grounded approach to transform abundant low-quality crop residues into high-value animal feed, simultaneously addressing feed security challenges, mitigating agricultural waste, and promoting circular agricultural systems.

Keywords: Soybean Straw, Silage, EM Bacteria, *Trichoderma longibrachiatum,* Synergistic Effects

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Introduction

At the intersection of waste management, food security, and sustainable agriculture lies an overlooked treasure: soybean (*Glycine max*) straw, produced at aw1w staggering rate of 1.5 kilograms per kilogram of harvested beans, with China's annual production alone surpassing 25 million tons [1]. However, the utilization of soybean straw as direct feed is limited by its

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structural characteristics: high cellulose and lignin content forms a complex biochemical barrier that impedes efficient nutrient utilization [2].

From a biochemical perspective, the primary structural obstacle in soybean straw is the tight network formed by cellulose, hemicellulose, and lignin. Lignin forms covalent cross-links with polysaccharides, creating complexes highly resistant to microbial enzymatic systems [3]. This structural complexity significantly limits the bioavailability and digestibility of nutrients in soybean straw, restricting its direct feeding value. Therefore, developing effective biotransformation strategies to decipher this lignocellulosic structural barrier has become the core challenge to improving soybean straw resource utilization efficiency.

Conventional agricultural practices often treat straw as a mere by-product, resulting in suboptimal utilization [4]. The high crude fiber content, lignification, low digestibility, and poor palatability of soybean straw limit its direct use as animal feed [2]. However, its composition including cellulose, hemicellulose, insoluble lignin [3], ash, protein, and pectin [5, 6] represents a significant renewable biomass resource with potential for ruminant feed production. Developing enhancement technologies for soybean straw is therefore critically important for effective resource utilization.

Silage fermentation offers a promising biotechnological solution with numerous advantages, including minimal nutrient loss, climate resilience, improved palatability, and extended storage capability [7, 8], which has stimulated increased research interest. However, the relatively low soluble carbohydrate content in soybean straw limits the growth of lactic acid bacteria and the production of organic acids, thereby affecting the rate of pH decline and ultimate fermentation quality [5, 6]. This challenge necessitates combined intervention strategies. Previous research indicates that adding materials rich in fermentable carbohydrates (such as bran) can provide energy substrates for fermentation microorganisms. [9] demonstrated that bran flakes, lactobacilli, and cellulolytic bacteria improve both fermentation quality and nutrient composition of soybean straw silage. [10] established that lactic acid bacteria and cellulase enhance soybean straw preservation, with their combination yielding optimal results. Further research by [11] showed that adding 2 kg/t urea and 10% bran significantly improves the nutritional and fermentation quality of soybean straw silage. Moreover, *Trichoderma longibrachiatum*, a fungus isolated from the rumen, has been found to produce lignocellulolytic enzymes that aid in fiber degradation [12]. These enzymes include endoglucanases, exoglucanases, xylanases, and various auxiliary enzymes that work synergistically to disassemble the complex structure of plant cell walls.

Despite these advances, a key research gap remains in systematically exploring the synergistic effects of combining multiple enhancement strategies. Specifically, the interaction between complex microbial inoculants (e.g., effective microorganisms, EM), specialized fiber-degrading fungi (e.g., *T. longibrachiatum*), and nutritional additives like wheat bran is not well-understood. This study proposes the following biotechnological hypothesis: The addition of wheat bran as a substrate, combined with specific microbial inoculants (EM bacteria and *T. longibrachiatum*), will synergistically enhance key biochemical pathways (such as enzymatic hydrolysis and acidogenesis) to effectively improve the fermentation efficiency and nutritional value of soybean straw silage. This synergistic action is expected to be achieved through the following mechanisms:

1) Wheat bran provides readily fermentable carbohydrates, promoting lactic acid bacteria growth
2) The EM microbial complex offers diverse metabolic pathways
3) T. longibrachiatum secretes cellulases and xylanases that disrupt plant cell wall structures, releasing more fermentable substrates

This study is designed to address this gap by investigating the individual and interactive effects of these additives on the fermentation kinetics and nutritional profile of soybean straw silage. The objective is to identify an optimal combination that leverages synergistic interactions to maximize the feed value of this underutilized resource.

Materials and Methods

Materials

The study utilized soybean variety Nong 60, with straw collected immediately after harvest on October 11, 2023, from Xiatang Town, Changfeng County, Anhui Province, China (31°44'N, 117°11'E). Straw was harvested at physiological maturity (R8 stage) when beans were fully dried in pods and moisture content of straw was approximately 35%. Plants were cut at ground level, beans were removed, and straw (including stems, leaves, and pod husks) was transported to the laboratory within 6 hours of harvest to minimize nutrient losses and undesired fermentation. The nutritional composition of raw soybean straw is outlined in Table 1.

Table 1: Nutritional Components of Raw Soybean Straw

Items	Value
Dry matter (%DM)	90.857
Crude protein (%DM)	15.421
Ether extract (%DM)	1.116
Acid detergent fiber (%DM)	32.883
Neutral detergent fiber (%DM)	51.016
Relative Feed Value	115

Experimental Design

Harvested soybean straw was air-dried and cut into uniform 1-2 cm sections. The moisture content was subsequently adjusted to 65±2% by adding sterile water to ensure optimal conditions for silage fermentation. The treatments comprised three levels of wheat bran (0%, W1; 10%, W2; and 20%, W3) in combination with three inoculant treatments (none, T1; Effective Microorganisms (EM), T2; and EM + *Trichoderma longibrachiatum*, T3). This resulted in a total of nine treatment groups, with three replicates each (see Table 2 for details).

Table 2: Experimental design of adding bacteria treatment and bran

Group	Wheatbran ($g\text{-}kg^{-1}$)	Inoculanttreatments	
		EM($g\text{-}kg^{-1}$)	*T.longibrachiatum* ($g\text{-}kg^{-1}$)
W1T1(CK)	0	0	0
W1T2	0	2	0
W1T3	0	2	2
W2T1	200	0	0
W2T2	200	2	0
W2T3	200	2	2
W3T1	400	0	0
W3T2	400	2	0
W3T3	400	2	2

The EM bacterial consortium (sourced from Henan Nanhua Microbiology Co., Ltd., Zhengzhou, China) contained Lactobacillus plantarum, L. casei, L. fermentum, Saccharomyces cerevisiae, and Rhodopseudomonas palustris at a minimum concentration of 1×10^9 $cfu\cdot g^{-1}$. The inoculant was activated by incubation in molasses solution (1:20 w/v) for 24 hours at 30°C before application. The final applied concentration was 1×10^7 $cfu\cdot g^{-1}$ of fresh straw. The dose of 2 $g\cdot kg^{-1}$ was selected based on preliminary experiments that demonstrated optimal fermentation outcomes at this concentration.

Trichoderma longibrachiatum (strain CICC 40340, sourced from China Center of Industrial Culture Collection) was cultured on potato dextrose agar at 28°C for 7 days. Spores were harvested by flooding the plates with sterile 0.85% NaCl solution containing 0.01% Tween 80, filtered through sterile cheesecloth, and adjusted to 1×10^7 $spores\cdot g^{-1}$ using a hemocytometer. The dose of 2 $g\cdot kg^{-1}$ was chosen based on literature recommendations for optimal cellulolytic activity in plant biomass.

Silage materials were vacuum-sealed in polyethylene bags, stored for 45 days, and then analyzed for nutritional and fermentation parameters.

Measurement Indicators and Methods

Sensory Evaluation

After opening the silage bags, sensory evaluation followed German Agricultural Society (DLG) Feed Industry Standards [13]. Evaluation was performed by five trained panelists. Panelists were trained through standardized reference samples and calibration sessions prior to evaluation. Samples were presented in randomized, coded containers without identification of treatment to ensure blinding. Inter-rater reliability was assessed using Cronbach's alpha coefficient ($\alpha = 0.87$), indicating strong consistency among evaluators.

Evaluation included color (0-2 points), odor (0-14 points), and texture (0-4 points). Mean scores from three replicates were calculated for each treatment. Overall silage quality categories based on total score were: 20-16, excellent; 15-10, good; 9-5, moderate; and 4-0, poor.

Nutritional Analysis

After 30 days of ensiling, 30 g samples were collected from each silage bag, dried at 100°C for 16 h, and cooled to a constant weight to determine Dry Matter (DM) content. Dried samples were ground, passed through a 40-mesh sieve, and stored in sealed glass bottles for analysis.

Crude Protein (CP) was determined according to GB/T 6432-2018 [14], Ether Extract (EE) according to GB/T 6433-2006 [15], Acid Detergent Fiber (ADF) according to NY/T 1459-2022 [16], and Neutral Detergent Fiber (NDF) according to GB/T 20806-2022 [17]. Relative Feed Value (RFV) was calculated as [18]:

$$\mathrm{RFV} = (88.9 - 0.779 \times \mathrm{ADF}) \times (120/\mathrm{NDF})/1.29 \quad (1)$$

Fermentation Quality Analysis

For fermentation quality analysis, 3.000 g of silage sample was weighed into a 100 mL volumetric flask with 60 mL of ultrapure water. Samples underwent ultrasonic extraction in a 50°C water bath for 20 min, cooled to room temperature, diluted to volume, and filtered to obtain the extract. pH and organic acids (lactic acid, acetic acid, propionic acid, and butyric acid) were determined within 24 h of extraction. pH was measured using a PHS-25 digital pH meter [19]. Organic acids were quantified using a DUG-20A high-performance liquid chromatography system [20] equipped with a ShimNex UPC18 column.

Data Analysis

Data were initially processed in Excel 2019. Data were analyzed by two-way ANOVA to evaluate the main effects of wheat bran level and inoculant type as well as their interactions using SPSS 27.0, followed by Duncan's multiple range test to detect significant differences among treatment means. Statistical significance was set at $\alpha=0.05$. Exact P-values are reported in tables alongside mean comparisons, with significance levels denoted as: not significant (^{ns}), $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). Figures were generated using Origin 2024.

Results and Analysis

Effects on Sensory Evaluation of Soybean Straw Silage

As demonstrated in Table 3, sensory scores for all additive treatments were found to be significantly higher than for the control (W1T1) (P<0.05). Groups W1T1, W1T2, and W1T3 were evaluated as good quality, while all other treatment groups were categorized as excellent quality. In W1T1, W1T2, and W1T3, silages exhibited a slight butyric acid odor, strong acidic notes with mild fruity aroma, and color similar to raw straw (light brown after drying with intact stem structure). The remaining treatment groups exhibited no butyric acid odor, a pleasant fruity aroma, light brown coloration after drying, and well-preserved stem and leaf structures. The W3T2 group achieved the highest mean sensory score (17.5).

Table 3: Effect of adding different combinations of bacteria treatments and different contents of wheat bran treatments on the sensory evaluation of soybean straw silage

Group	Index score			Total score	Grade
	Odor	Texture	Color		
W1T1	7.0	4.0	2.0	12.0±0.58d	Well
W1T2	8.7	4.0	2.0	14.7±0.3c	Well
W1T3	8.3	4.0	2.0	14.3±0.88c	Well
W2T1	9.3	4.0	2.0	15.3±0.88bc	Excellent
W2T2	9.7	4.0	2.0	15.7±0.33bc	Excellent
W2T3	9.7	4.0	2.0	15.7±0.33bc	Excellent
W3T1	10.3	4.0	2.0	16.3±0.33ab	Excellent
W3T2	11.5	4.0	2.0	17.5±0.50a	Excellent
W3T3	11.0	4.0	2.0	17.0±0.33a	Excellent

Note: Different lowercase letters in the same column indicate significant differences (P<0.05); same letters or absence of markers indicate non-significant differences (P>0.05)

Two-way ANOVA (Table 4) revealed that both wheat bran level (P<0.001) and inoculant type (P<0.05) significantly influenced sensory scores, with no significant interaction effect (P = 0.391). This suggests that the factors independently contributed to improving sensory attributes, with wheat bran having a particularly strong effect, as evidenced by the 3× higher F-value for wheat bran (33.125) compared to inoculant type (4.271). The highest sensory scores were observed in treatments W3T2 and W3T3 (17.5 and 17.0, respectively), indicating that 20% wheat bran combined with microbial inoculation yields optimal sensory outcomes.

This lack of significant interaction effect (P = 0.391) suggests that wheat bran and microbial inoculants enhance sensory quality through different biochemical pathways. Wheat bran primarily improves sensory attributes by providing readily fermentable substrates that promote lactic acid production, generating pleasant fruity-acidic aromas. Simultaneously, its physical properties may alter the texture and moisture retention capacity of the silage material, further enhancing the sensory experience. Meanwhile, microbial inoculants contribute to sensory quality through directed fermentation that produces specific metabolites, such as ethanol, ethyl acetate, and other volatile compounds that impart distinct flavor characteristics.

Table 4: Two-way ANOVA results for sensory evaluation scores

Effect	F-value	P-value	Significance
Wheat bran level	33.125	<0.001	***
Inoculant type	4.271	0.028	*
Interaction	1.083	0.391	ns

Note: ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001

Effects on Nutritional Quality of Soybean Straw Silage

Our two-way ANOVA showed that wheat bran level significantly affected CP content (P=0.014), with 10% and 20% additions yielding significantly higher CP values than 0% wheat bran. However, neither inoculant type (P=0.775) nor the interaction between factors (P = 0.157) significantly influenced CP content.

The mechanism behind increased crude protein content in wheat bran-supplemented silages likely involves multiple processes:

(1) Direct protein contribution from wheat bran itself, which contains approximately 15-17% protein

(2) Enhanced microbial protein synthesis due to improved availability of fermentable carbohydrates and particularly through fixation of non-protein nitrogen

(3) Reduced proteolysis during fermentation due to more rapid pH decline, which inhibits protease activity [21]

The nonsignificant effect of inoculants on protein content contradicts our initial hypothesis that *T. longibrachiatum* would enhance protein availability through fiber degradation, suggesting that its cellulolytic enzyme activity may have been insufficient to significantly impact total protein content within the 45-day fermentation period, or was counterbalanced by other factors.

Two-way ANOVA showed that wheat bran level had a significant effect on ADF content ($P<0.05$), with a significant interaction between wheat bran and inoculant type ($P<0.05$). For NDF content, while the main effects of wheat bran level and inoculant type were not significant, their interaction was significant ($P<0.05$), indicating that specific combinations effectively reduced fiber content.

From an enzymological perspective, the reduction in NDF can be attributed to the synergistic action of cellulase and hemicellulase complexes produced by T. longibrachiatum. These enzyme systems include endoglucanases (random internal hydrolysis of β-1,4-glycosidic bonds), cellobiohydrolases (releasing glucose from cellobiose), and β-glucosidases (converting oligosaccharides to glucose). Simultaneously, the xylanase complex (including endoxylanases and β-xylosidases) degrades hemicellulose backbones, releasing xylose, arabinose, and acetyl groups. Additionally, non-enzymatic hydrolysis in acidic conditions may have contributed to partial degradation of fiber components. The treatment group with the greatest NDF reduction (W3T1) likely benefited from the synergistic action of endogenous enzymatic activity provided by high levels of wheat bran and the indigenous fermentation microbial community it promoted.

Two-way ANOVA revealed that wheat bran level significantly affected RFV ($P = 0.019$), with both 10% and 20% additions yielding higher values than the control. Interestingly, there was also a significant interaction effect between wheat bran and inoculant type ($P = 0.032$), indicating that specific combinations performed better than would be expected from their individual effects.

This synergistic effect was particularly evident in the W3T1 treatment, which achieved the highest RFV value (142.73) despite having no bacterial inoculant. The higher RFV values in treated groups compared to the control indicate that combined application of EM bacteria, *T. longibrachiatum*, and wheat bran promoted effective fermentation and nutrient transformation, improving overall forage quality.

The unexpected superior performance of W3T1 (20% wheat bran without inoculants) in terms of RFV likely occurred because the 20% wheat bran level provided both sufficient fermentable carbohydrates and an indigenous microbial community robust enough to initiate rapid fermentation, thus explaining its high RFV even without additional inoculants.

A nutritional analysis was conducted, which revealed significant differences among the treatments, as illustrated in Table 5. Compared with the control (W1T1), Dry Matter (DM) contents of W2T2, W2T3, W3T1, and W3T2 were significantly reduced ($P<0.05$), reaching 18.52% compared to 17.13% in the control–an increase of 8.1%. Crude Protein (CP) content in W2T2 was significantly higher than W1T1 ($P<0.05$), Our two-way ANOVA showed that wheat bran level significantly affected CP content ($P=0.014$), with 10% and 20% additions yielding significantly higher CP values than 0% wheat bran (Table 6 and Table 7). However, neither inoculant type ($P=0.775$) nor the interaction between factors ($P=0.157$) significantly influenced CP content. Ether extract (EE) contents of W2T2 and W3T1 were numerically higher, while those of other treatments were lower than W1T1, though differences were not significant ($P>0.05$). Acid detergent fiber (ADF) contents in W2T2, W2T3, W3T1, W3T2, and W3T3 were lower than W1T1 ($P>0.05$), with W3T1 showing the greatest reduction (from 32.19% to 29.70%, a decrease of 7.7%). Neutral detergent fiber (NDF) contents in W1T3, W2T3, and W3T1 were significantly lower than W1T1 ($P<0.05$), with the largest reduction observed in W3T1 (from 46.97% to 42.87%, a decrease of 8.7%).

Two-way ANOVA revealed that wheat bran level significantly affected RFV ($P = 0.019$), with both 10% and 20% additions yielding higher values than the control. Interestingly, there was also a significant interaction effect between wheat bran and inoculant type ($P=0.032$), indicating that specific combinations performed better than would be expected from their individual effects. This synergistic effect was particularly evident in the W3T1 treatment, which achieved the highest RFV value (142.73) despite having no bacterial inoculant. The higher RFV values in treated groups compared to the control indicate that combined application of EM bacteria, T. longibrachiatum, and wheat bran promoted effective fermentation and nutrient transformation, improving overall forage quality.

Table 5: Effects of adding different combinations of bacterial treatments and different contents of bran treatments on the nutritional quality of soybean straw silage

Treatments	DM (%FM)	CP (%DM)	EE (%DM)	ADF(%DM)	NDF (%DM)	RFV
W1T1	38.36±0.32a	17.13±0.10bc	2.50±0.27ab	32.19±0.31abcd	46.97±0.90a	126.71±3.16c
W1T2	37.14±0.53abc	17.56±0.30abc	2.11±0.09b	33.17±1.52abc	44.31±0.87ab	132.47±3.05bc
W1T3	38.36±0.94a	16.51±0.45c	2.18±0.09ab	34.59±1.42a	43.84±0.69b	131.50±2.50bc
W2T1	37.41±0.63ab	17.51±0.27abc	2.25±0.15ab	33.77±0.48ab	44.08±0.69ab	132.19±2.82bc
W2T2	35.76±0.35bcd	18.52±0.87a	2.69±0.25a	30.55±0.85cd	44.46±0.76ab	136.34±3.67abc
W2T3	35.65±0.28bcd	17.84±0.33ab	2.38±0.21ab	31.00±0.79bcd	43.63±0.81b	138.19±3.71ab
W3T1	35.42±0.17cd	18.08±0.67ab	2.62±0.01ab	29.70±0.53d	42.87±0.18b	142.73±1.49a
W3T2	35.34±0.81d	17.60±0.22abc	2.25±0.01ab	31.67±0.42abcd	44.33±1.10ab	134.93±2.87abc
W3T3	37.30±0.29ab	18.33±0.34ab	2.39±0.11ab	31.48±1.12bcd	45.47±1.48ab	131.89±3.29bc

Note: FM denotes fresh matter, DM denotes dry matter

Table 6: Two-way ANOVA results for nutritional quality parameters

Index	Effect	F-value	Significance	Index	Effect	F-value	Significance
CP	Wheat bran	5.317	*	NDF	Wheat bran	1.678	ns
	Inoculant	0.258	ns		Inoculant	0.6410	ns
	Interaction	1.849	ns		Interaction	3.544	*
RFV	Wheat bran	4.873	*	ADF	Wheat bran	5.06	*
	Inoculant	0.426	ns		Inoculant	0.320	ns
	Interaction	3.257	*		Interaction	3.156	*

Note: ns: not significant; *: P<0.05; **: P<0.01; ***: P<0.001

Table 7: Main effects of wheat bran levels and inoculant treatments on key silage parameters

	Bran treatments		Inoculation treatments		Interaction effects			
					Inoculation treatments	Bran treatments		
						1	2	3
CP	1	17.09a	1	17.59a	1	17.19bc	17.51abc	18.08ab
	2	17.96b	2	17.90a	2	17.56abc	18.52a	17.60ab
	3	18.00b	3	17.56a	3	16.51c	17.84abc	18.33ab
RFV	1	127.27a	1	130.92a	1	117.83b	132.19ab	142.73a
	2	135.57b	2	134.58a	2	132.47ab	136.34ab	134.93ab
	3	136.52b	3	133.86a	3	131.50ab	138.19ab	131.89ab
pH	1	4.99b	1	4.92b	1	5.44a	4.76b	4.58bc
	2	4.78a	2	4.66a	2	4.72b	4.83b	4.43c
	3	4.53c	3	4.72a	3	4.82b	4.75b	4.60bc
LA	1	3.78a	1	3.63a	1	3.33c	3.77bc	3.80abc
	2	3.90a	2	3.86b	2	3.70c	4.27ab	3.60c
	3	3.73a	3	3.92b	3	4.29a	3.65c	3.79abc
AA	1	0.22534b	1	0.42291a	1	0.27bc	0.82a	0.186c
	2	0.72899a	2	0.40101a	2	0.18c	0.95a	0.07c
	3	0.12743b	3	0.25784b	3	0.23bc	0.419b	0.13c

	1	0.65192a	1	0.40042b	1	0.08ab	ND	0.66ab
BA	2	ND c	2	0.60421a	2	1.13a	ND	0.14ab
	3	0.48616b	3	0.46992b	3	0.22ab	ND	0.67ab

Effects on Fermentation Quality of Soybean Straw Silage

Two-way ANOVA revealed that both wheat bran level and inoculant type exerted extremely significant effects on pH ($P<0.001$), with a significant interaction between these factors ($P<0.001$). As shown in Table 7, pH progressively decreased with increasing wheat bran levels, with the lowest pH (4.53) observed in the 20% wheat bran treatment. Similarly, both inoculant treatments significantly reduced pH ($P<0.001$).

This pronounced interaction effect ($F = 9.975$, $P<0.001$) reveals complex dynamics between substrate availability and microbial activity. On one hand, microbial inoculants require sufficient fermentable carbohydrates to grow efficiently and produce organic acids; on the other hand, high levels of substrate require appropriate microbial communities for effective utilization. In the W3T2 treatment (20% wheat bran + EM), the EM complex contains multiple lactic acid bacterial species with complementary metabolic capabilities that can efficiently utilize the diverse carbon sources provided by wheat bran, resulting in a stronger acidification effect than predicted by single factors alone.

In terms of F-values (Table 8), the effect of wheat bran level on pH ($F = 19.753$) was more pronounced than that of inoculant type ($F = 15.694$), indicating substrate supply is the primary determinant of silage pH. However, the high interaction F-value ($F = 9.975$) indicates that specific bran-inoculant combinations produced synergistic effects, resulting in greater pH reduction than predicted by the individual main effects alone. The W3T2 treatment (20% wheat bran + EM) achieved the lowest pH value (4.43), significantly below levels predicted by main effects alone. This synergistic effect likely stems from an optimal match between substrate availability and microbial fermentation capacity−the EM microbial consortium contains multiple lactic acid bacteria strains capable of rapidly utilising fermentable carbohydrates from wheat bran, thereby lowering pH more substantially than either factor alone.

Our factorial analysis revealed significant effects of inoculant type ($P = 0.004$) on lactic acid production, with both EM and EM+T. longibrachiatum treatments producing significantly higher lactic acid levels than treatments without inoculants. Furthermore, a strong interaction effect ($P<0.001$) was observed between wheat bran level and inoculant type, indicating that specific combinations performed particularly well for lactic acid production. The W1T3 and W2T2 treatments yielded the highest lactic acid concentrations (4.29 and 4.27 g·kg^{-1}, respectively), demonstrating how proper combinations of substrate and microorganisms can optimize fermentation outcomes.

From a molecular perspective, lactic acid fermentation involves multi-step reactions catalyzed by phosphoenolpyruvate carboxykinase (PEPCK) and Lactate Dehydrogenase (LDH). The lactic acid bacteria in the EM microbial complex, through these key enzymes, convert glucose to lactic acid under limited oxygen conditions, while regenerating NAD+ to maintain continued glycolysis.

Notably, all treatments with 10% wheat bran (W2T1, W2T2, W2T3) completely suppressed butyric acid production, suggesting an optimal substrate level for inhibiting clostridial fermentation.

Two-way ANOVA demonstrated significant effects of inoculant type ($P=0.004$) on lactic acid production, with both EM and EM+*T. longibrachiatum* treatments producing significantly higher lactic acid levels than treatments without inoculants. Furthermore, a strong interaction effect ($P<0.001$) was observed between wheat bran level and inoculant type, indicating that specific combinations performed particularly well for lactic acid production. The W1T3 and W2T2 treatments yielded the highest lactic acid concentrations (4.29 and 4.27 g·kg^{-1}, respectively), demonstrating how proper combinations of substrate and microorganisms can optimize fermentation outcomes.

Notably, wheat bran level did not have a significant main effect on lactic acid production ($P=0.165$), suggesting that merely adding wheat bran without appropriate microbial inoculants may be insufficient to significantly increase lactic acid yield. However, the strong interaction effect ($F = 16.872$) indicates that the key lies in the matching between substrate and microorganisms, rather than simple substrate addition.

Two-way ANOVA of organic acid data revealed that wheat bran level and inoculant type significantly affected acetic acid content, with particularly high acetic acid levels observed in the 10% wheat bran treatments (W2T1, W2T2). Most notably, all treatments with 10% wheat bran (W2T1, W2T2, W2T3) completely suppressed butyric acid production, suggesting an optimal substrate level for inhibiting clostridial fermentation. The 10% wheat bran level likely provides an optimal balance: sufficient fermentable carbohydrates to support rapid lactic acid production and pH decline below the growth threshold for Clostridium species (~4.8), without excessive substrate that could lead to secondary fermentation pathways. This finding has significant implications for optimizing additive formulations for soybean straw silage.

From a microbial ecology perspective, this inhibition might be achieved through multiple mechanisms:

(1) Rapid acidification creating a pH environment unfavorable for clostridial growth

(2) Antimicrobial substances such as bacteriocins produced by lactic acid bacteria directly inhibiting clostridial growth

(3) Competitive exclusion through rapid consumption of available nutrients

(4) Establishment of a dominant lactic acid bacterial community that restricts the ecological niche for clostridia

The 10% wheat bran level likely provides sufficient fermentable carbohydrates to stimulate lactic acid bacterial growth without excessive substrate that could lead to heterolactic or secondary fermentation pathways.

As demonstrated in Table 9, The pH values of all additive treatments were significantly lower than those of the control ($P<0.05$). Organic acid analysis showed that Lactic Acid (LA) contents were higher in all treated groups compared to W1T1, with significantly higher levels observed in W1T2, W1T3, W2T1, W2T2, W3T1, and W3T3 ($P<0.05$). Acetic Acid (AA) contents in W2T1 and W2T2 were significantly higher than W1T1 ($P<0.05$). Propionic Acid (PA) contents in W1T3 and W2T1 were lower than W1T1, though differences were not significant. Butyric acid (BA) levels were reduced in all treatments compared with W1T1.

Table 8: Two-way ANOVA results for key fermentation quality parameters

Index	Effect	F-value	Significance	Index	Effect	F-value	Significance
pH	Wheat bran	19.753	***	AA	Wheat bran	19.753	***
	Inoculant	15.694	***		Inoculant	15.694	*
	Interaction	9.975	***		Interaction	9.975	*
LA	Wheat bran	1.956	ns	BA	Wheat bran	1.956	***
	Inoculant	7.349	**		Inoculant	7.349	*
	Interaction	16.872	***		Interaction	16.872	***

Table 9: Effects of adding different combinations of bacterial treatments and different contents of bran treatments on fermentation quality of soybean straw silage fermentation

Treatment	pH	Lacticacid (g-kg-1FM)	Aceticacid (g-kg-1FM)	Propionicacid (g-kg-1FM)	Butyricacid (g-kg-1FM)
W1T1	5.44±0.01a	3.33± 0.08c	0.27±0.02bc	1.60±0.21abc	0.08±0.02ab
W1T2	4.72±0.03bc	3.70±0.14b	0.18±0.01c	1.64±0.13abc	1.13±0.04a
W1T3	4.82±0.06b	4.29±0.10a	0.23±0.02bc	1.25±0.16c	0.22±0.61ab
W2T1	4.76±0.12bc	3.77±0.14b	0.82±0.12a	1.39±0.12bc	ND
W2T2	4.83±0.06b	4.27±0.11a	0.95±0.12a	1.70±0.11abc	ND
W2T3	4.75±0.05bc	3.65±0.12bc	0.41±0.09b	1.86±0.08ab	ND
W3T1	4.58±0.02cd	3.80±0.07b	0.18±0.06c	1.94±0.20a	0.66±0.07ab
W3T2	4.43±0.06cd	3.60±0.07bc	0.07±0.02c	1.66±0.17abc	0.14±0.04ab
W3T3	4.60±0.02cd	3.79±0.04b	0.13±0.01c	2.05±0.22a	0.67±0.11ab

Note: ND indicates not detected

Discussion

In this study, we investigated the complex interplay between substrate availability and microbial inoculation in transforming low-quality soybean straw into nutritionally enhanced silage. The unique contribution of our work lies not merely in confirming individual additive effects but in establishing a comprehensive optimization framework that identifies specific thresholds (e.g., 10% wheat bran for complete butyric acid suppression) critical for developing practical, cost-effective silage production protocols.

Effects on Sensory Evaluation of Soybean Straw Silage

Sensory evaluation provides a rapid, intuitive assessment of silage quality, relying on human senses to evaluate color, texture, and odor [22]. This preliminary evaluation offers valuable insights into feed acceptability by livestock. In our study, all additive treatments achieved higher total sensory scores than the control, indicating that wheat bran and bacterial inoculants effectively enhanced the sensory quality of soybean straw silage. Two-way ANOVA revealed that both wheat bran level ($P<0.001$) and inoculant type ($P<0.05$) significantly influenced sensory scores, with no significant interaction effect ($P = 0.391$). The lack of a significant interaction effect ($p = 0.391$) suggests that these factors improve sensory quality through independent mechanisms. This suggests that the factors independently contributed to improving sensory attributes, with wheat bran having a particularly strong effect, as evidenced by the 3× higher F-value for wheat bran (33.125) compared to inoculant type (4.271).

From a biochemical perspective, wheat bran promotes lactic acid fermentation, lowering ph values, inhibiting protein degradation and ammonia generation, thus reducing putrid odors. Simultaneously, soluble sugars from bran are converted to lactic acid and other organic acids, along with small quantities of esters, producing pleasant fruity aromas. Inoculant treatments further enhance these sensory characteristics by accelerating fermentation initiation and suppressing spoilage microorganism growth.

These findings align with previous research where probiotics and enzyme complexes improved corn silage sensory traits by enhancing acidic aroma and increasing total sensory scores [23]. However, treatments with and without *T. longibrachiatum* showed no significant differences in sensory properties, suggesting that this fungal inoculant provided limited additional benefits to the sensory characteristics of soybean straw silage.

Effects on Nutritional Quality of Soybean Straw Silage

During silage fermentation, biochemical reactions catalyzed by microorganisms and enzymes cause partial Dry Matter (DM) losses [24]. In our experiment, DM levels declined in all treated groups compared to the control, though losses were less pronounced in treatments containing wheat bran. This suggests that wheat bran supplementation supports more efficient microbial fermentation. Crude Protein (CP), critical for animal growth, reproduction, and productivity [25], increased in all treatment groups compared to the control. This improvement likely resulted from lactic acid bacteria-mediated rapid acidification during early fermentation, which inhibited undesirable microbes and reduced nutrient degradation [26].

The mechanism behind increased crude protein content in wheat bran-supplemented silages likely involves multiple processes:

(1) Direct protein contribution from wheat bran itself, which contains approximately 15-17% protein
(2) Enhanced microbial protein synthesis due to improved availability of fermentable carbohydrates
(3) Reduced proteolysis during fermentation due to more rapid pH decline, which inhibits protease activity [21]

The nonsignificant effect of inoculants on protein content contradicts our initial hypothesis that *T. longibrachiatum* would enhance protein availability through fiber degradation, suggesting that its cellulolytic action was either insufficient or counterbalanced by other factors.

Forage digestibility is inversely related to Acid Detergent Fiber (ADF) content [27] while Neutral Detergent Fiber (NDF) strongly influences feed utilization efficiency—lower NDF indicates greater proportions of digestible nutrients [28]. In this study, although ADF reductions were not statistically significant, NDF levels decreased in treatments containing EM inoculant, *T. longibrachiatum*, and wheat bran. These improvements likely resulted from hemicellulose degradation [29] and partial breakdown of cell wall components by fermentation-derived organic acids [30].

Relative Feed Value (RFV), developed by the Hay Market Task Force of the U.S. Forage and Grassland Council, integrates forage quality parameters relative to animal feeding standards. All silage treatments in our study achieved RFV values exceeding 100, confirming soybean straw silage as a suitable forage resource with considerable utilization potential. Fig. 1 illustrates the interaction effects of wheat bran level and inoculant type on RFV. Bran significantly enhanced the RFV of soybean straw silage ($P<0.05$), with both 10% and 20% additions yielding higher values than the control. However, the effects of inoculant treatments were found to be less significant ($P>0.05$). Interestingly, there was also a significant interaction effect between wheat bran and inoculant type ($P<0.05$), indicating that specific combinations performed better than would be expected from their individual effects. This synergistic effect was particularly evident in the W3T1 treatment, which achieved the highest RFV value (142.73) despite having no bacterial inoculant. This suggests that high wheat bran levels provide both sufficient fermentable carbohydrates and indigenous microbial populations robust enough to initiate rapid fermentation, thus explaining its high RFV even without additional inoculants.

Silage additives are known to improve fermentation characteristics [31], typically assessed through pH and organic acid profiles [32]. Lower pH suppresses undesirable microbial growth, minimizes nutrient loss, and preserves silage quality [33]. In our study, as illustrated in Fig. 2, all treated groups exhibited significantly lower pH values than the control, consistent with findings by Guo et al. who reported similar effects of lactic acid bacteria in pear silage [34]. Two-way ANOVA demonstrated that both wheat bran level and inoculant type had highly significant effects on pH ($P<0.001$), with a strong interaction between these factors ($P<0.001$). As shown in Table 7 and Fig. 2, increasing wheat bran levels progressively reduced pH values, with 20% wheat bran yielding the lowest pH (4.53). Both inoculant treatments significantly reduced pH compared to no inoculant ($P<0.001$). Demonstrated as Fig. 2, the interaction plot reveals highly significant bran × inoculant effects ($P<0.001$), with 20% bran combined with EM treatment (W3T2) achieving the lowest pH (4.43). This synergistic effect indicates that when high levels of fermentable substrate are combined with efficient microbial inoculants, optimal pH reduction can be achieved, providing better preservation conditions.

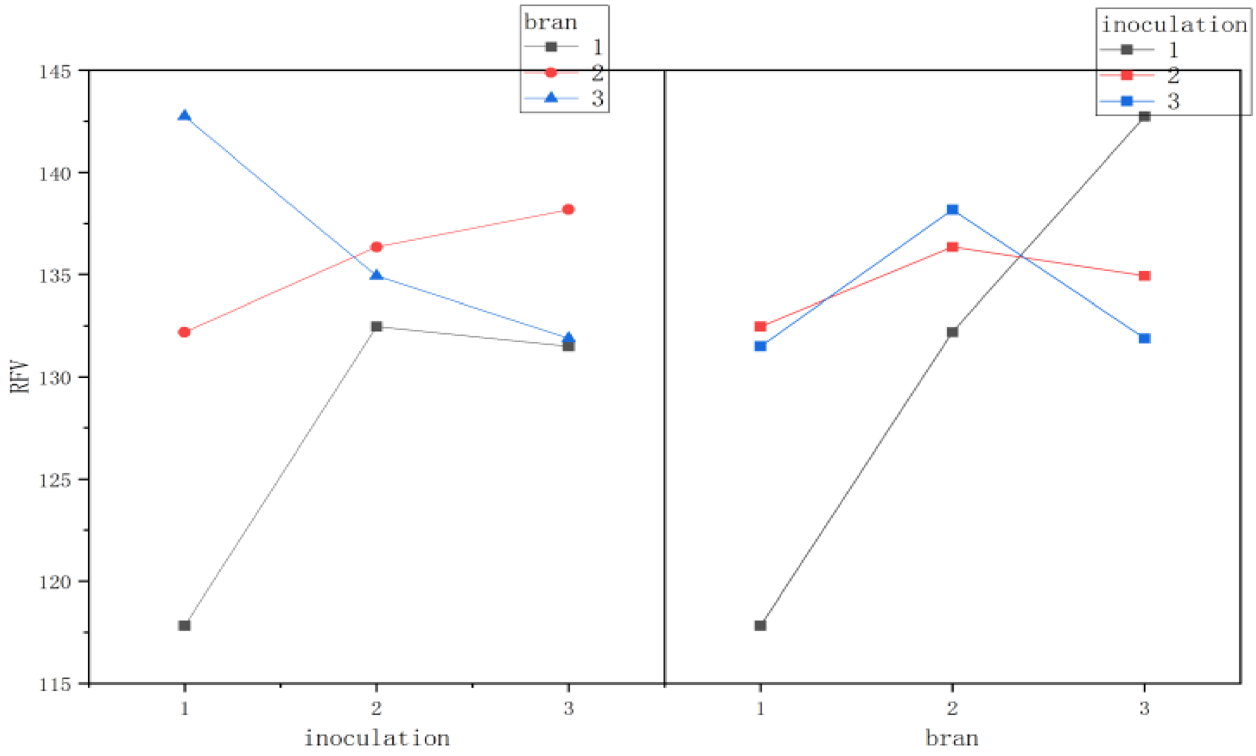

Fig. 1: Wheat bran and inoculant interaction effects on RFV

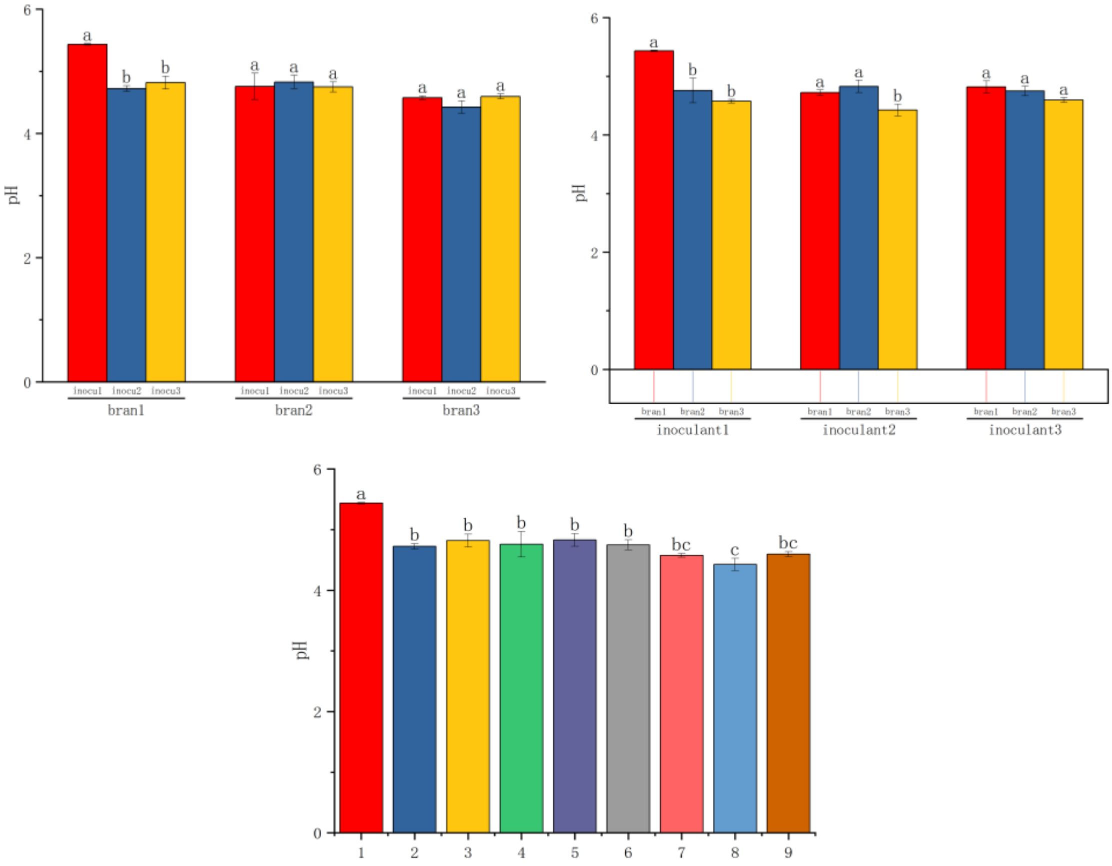

Fig. 2: Effects of bran and inoculants on silage pH

The pronounced interaction effect on pH (F = 9.975, P<0.001) reveals complex dynamics between substrate availability and microbial activity. For instance, while wheat bran generally lowered pH, the magnitude of this effect varied substantially depending on the inoculant treatment. The W3T2 treatment (20% wheat bran + EM) achieved the lowest pH (4.43), significantly lower than would be predicted from the main effects alone. This synergistic effect likely stems from optimal matching of substrate availability with microbial fermentation capacity—the EM consortium contains multiple lactic acid bacteria species that can rapidly utilize the readily fermentable carbohydrates provided by wheat bran, driving pH lower than either factor alone.

This pH reduction can be attributed primarily to increased production of lactic acid and acetic acid during fermentation [35]. Lactic acid, the primary fermentation product of lactic acid bacteria, ensures rapid pH decline, while acetic acid contributes to improved aerobic stability by inhibiting fungal proliferation [36]. Our factorial analysis revealed significant effects of inoculant type (P<0.01) on lactic acid production, with both EM and EM+*T. longibrachiatum* treatments producing significantly higher lactic acid levels than treatments without inoculants. Furthermore, a strong interaction effect (P<0.001) was observed between wheat bran level and inoculant type, indicating that specific combinations performed particularly well for lactic acid production. The W1T3 and W2T2 treatments yielded the highest lactic acid concentrations (4.29 and 4.27 $g\cdot kg^{-1}$, respectively), demonstrating how proper combinations of substrate and microorganisms can optimize fermentation outcomes.

From a metabolic pathway perspective, this pH reduction can be primarily attributed to increased production of lactic and acetic acids during fermentation [37]. Lactic acid, the primary product of lactic acid bacterial fermentation, is produced via the homolactic pathway, generating 2 moles of lactic acid per mole of glucose, while regenerating 2 moles of NAD+. This ensures rapid ph decline, while acetic acid contributes to improved aerobic stability by inhibiting fungal growth [36]. This metabolic regulation mechanism forms a synergistic network in the microbial community: lactic acid bacteria lower environmental ph, inhibiting competing microorganisms; meanwhile, cellulolytic enzymes from T. longibrachiatum and other organisms in the EM increase fermentable substrate availability, further promoting lactic acid production.

[17] also demonstrated that probiotic addition in mixed silages enhanced lactic and acetic acid production while reducing pH, thereby markedly improving silage stability. In our study, lactic acid levels were consistently higher in treated groups than in the control. Acetic acid was particularly elevated in treatments with 10% wheat bran addition, while propionic acid showed no significant changes across groups. Butyric acid content remained very low and was undetectable in some treatments. Notably, all treatments with 10% wheat bran (W2T1, W2T2, W2T3) completely suppressed butyric acid production, suggesting an optimal substrate level for inhibiting clostridial fermentation. These results suggest that EM bacteria and optimal wheat bran supplementation supplied sufficient fermentable substrates to stimulate lactic acid bacteria growth, reduce butyric fermentation, and improve overall fermentation quality.

Our factorial analysis revealed significant effects of inoculant type (P = 0.004) on lactic acid production, with both EM and EM+T. longibrachiatum treatments producing significantly higher lactic acid levels than treatments without inoculants. Furthermore, a strong interaction effect (P<0.001) was observed between wheat bran level and inoculant type, indicating that specific combinations performed particularly well for lactic acid production. The W1T3 and W2T2 treatments yielded the highest lactic acid concentrations (4.29 and 4.27 $g\cdot kg^{-1}$, respectively), demonstrating how proper combinations of substrate and microorganisms can optimize fermentation outcomes.

In this complex fermentation ecosystem, interactions between different microbial strains significantly influenced the final organic acid profile. Under limited oxygen conditions, homofermentative lactic acid bacteria (such as L. plantarum) convert glucose to lactic acid via the Embden-Meyerhof-Parnas pathway, while heterofermentative lactic acid bacteria produce a mixture of lactic acid, acetic acid, ethanol, and CO2. Simultaneously, yeasts may participate in partial conversion of lactic acid, affecting the final acid ratio. The optimal balance between lactic and acetic acids is crucial for both ensuring rapid pH decline and maintaining aerobic stability.

Surprisingly, the addition of T. longibrachiatum to the EM consortium (comparing T2 vs. T3 treatments) produced inconsistent effects on fermentation parameters, showing benefits at 0% wheat bran (W1T3 > W1T2 for lactic acid) but no advantages or even slight decreases in fermentation quality at higher wheat bran levels. This suggests that the fungal inoculant's cellulolytic activity may be most beneficial in substrate-limited conditions, where liberation of additional fermentable sugars from fiber is crucial. When readily available carbohydrates are abundant (as in the wheat bran treatments), the fungal contribution appears redundant or potentially competitive with the lactic acid bacteria for nutrients.

From an enzymological perspective, T. longibrachiatum secretes multiple hydrolytic enzymes that play key roles in lignocellulosic biomass degradation. These enzymes include endo-β-1,4-glucanase, exo-β-1,4-glucosidase, β-glucosidase, as well as hemicellulases such as xylanase and β-xylosidase. However, in environments rich with readily fermentable carbon sources, the production of these enzymes may be suppressed by Carbon Catabolite Repression (CCR), which may explain why the contribution of T. longibrachiatum was limited in high wheat bran treatments.

The complete suppression of butyric acid at the 10% wheat bran level represents a key finding with significant practical implications. Butyric acid is associated with clostridial fermentation and indicates protein degradation, nutrient loss, and reduced palatability. The 10% wheat bran level likely provides an optimal balance: sufficient fermentable carbohydrates to support rapid lactic acid production and pH decline below the growth threshold for Clostridium species (~4.8), without excessive substrate that could lead to secondary fermentation pathways. This represents a critical threshold for silage additive formulations targeting soybean straw.

The 10% wheat bran level likely provides sufficient fermentable carbohydrates to stimulate lactic acid bacteria growth without excessive substrate that could lead to heterolactic or secondary fermentation pathways. This optimal balance maintains the lactic-to-acetic acid ratio in a range that supports both rapid pH decline and aerobic stability.

Future Research Directions

Building upon the demonstrated efficacy of optimized wheat bran and microbial inoculant combinations in transforming soybean straw into nutritionally enhanced silage, several promising research avenues warrant further investigation. First, farm-scale validation trials across multiple geographical locations and seasons would establish the robustness of these results under variable environmental conditions. Longitudinal sampling at multiple timepoints (7, 14, 30, 60, and 90 days) would provide valuable insights into fermentation kinetics and help establish optimal storage duration guidelines.

The lack of direct microbiological data represents another limitation of our current work. Future research should incorporate advanced molecular techniques such as metagenomic sequencing and qPCR to track the survival and dominance of inoculated species versus indigenous microflora throughout the fermentation process. This would help resolve the unexpected findings regarding T. longibrachiatum's contribution and explain the efficacy of the indigenous microflora in the W3T1 treatment.

Comprehensive animal feeding trials are critically needed to validate whether the observed nutritional improvements translate to enhanced dry matter intake, digestibility, and production performance in ruminants [38]. Additionally, economic analyses incorporating regional substrate availability, application costs, and resulting feed value would help establish practical implementation thresholds for producers [39]. Future studies should also explore the molecular mechanisms underlying the synergistic effects between substrate availability and microbial activity using metagenomic and metabolomic approaches to identify key microbial species and metabolic pathways responsible for enhanced fermentation [40].

In-depth biochemical studies of the fermentation process could elucidate why 10% wheat bran was optimal for butyric acid suppression while 20% was more effective for other parameters. Understanding the threshold effects of substrate concentration on microbial competition and metabolic pathways would enable more precise optimization of silage formulations. Enzyme kinetics and inhibition studies might reveal rate-limiting steps in key biochemical conversions, while proteomic analysis could uncover differential enzyme systems expressed by fermentation microbial communities under different treatments.

Finally, comparative evaluation of alternative agricultural byproducts with varying carbohydrate profiles (e.g., rice bran, corn bran, molasses) could identify even more cost-effective substrate options for regions where wheat bran availability is limited [8]. This systematic optimization approach would further advance the valorization of lignocellulosic biomass, providing sustainable solutions for livestock production in resource-limited regions.

Conclusion

This study provides compelling evidence that strategic combinations of wheat bran and microbial inoculants significantly enhance soybean straw silage quality through synergistic interactions. Our factorial analysis of nine treatment combinations yielded several key findings with important practical implications.

First, two-way ANOVA revealed distinct mechanistic pathways by which wheat bran and inoculants improve silage quality. Wheat bran primarily enhances nutritional value by contributing fermentable carbohydrates and protein, while inoculants accelerate fermentation kinetics and organic acid production. The significant interactions between these factors across multiple parameters demonstrate that their combined effects exceed simple additive benefits, confirming true synergistic action. From a molecular perspective, the diverse oligosaccharides and monosaccharides provided by wheat bran serve as substrates for differential metabolism by the inoculated strains, while the diversity of enzyme systems in the inoculants accelerates the conversion of these substrates, creating a mutually enhancing biochemical network.

Second, we identified critical concentration thresholds for optimal silage production. Notably, 10% wheat bran completely suppressed butyric acid production across all inoculant treatments, representing an essential breakthrough for preventing clostridial fermentation. This threshold provides a precisely defined target for cost-effective commercial formulations, balancing minimal input with maximum quality improvement. From a microbial ecology perspective, this concentration achieves the optimal balance point for competitive exclusion in the fermentation ecosystem: providing sufficient nutrition to support rapid lactic acid bacterial growth and dominance, while not generating excess carbon sources that could shift fermentation direction.

Third, our unexpected finding that 20% wheat bran without inoculants (W3T1) yielded the highest relative feed value (142.73, $P<0.05$) reveals the previously unrecognized potential of indigenous microflora when provided with adequate substrate. This challenges conventional wisdom that microbial inoculation is always necessary for quality silage production. This finding suggests that in some scenarios, optimizing the substrate environment may be more critical than introducing exogenous microorganisms, offering a simplified implementation strategy for resource-limited regions.

Fourth, the W2T2 treatment (10% wheat bran + EM) emerged as the most balanced approach, achieving significant protein enrichment (18.52% CP), optimal lactic:acetic acid ratio (4.5:1), complete butyric acid suppression, and superior sensory properties. This specific combination represents a practical, economically viable solution for transforming abundant soybean straw into valuable livestock feed. The success of this combination lies in its synergistic mechanism: the lactic acid bacterial community in EM efficiently utilizes the diverse carbon sources provided by wheat bran, producing an ideal spectrum of fermentation metabolites while suppressing undesirable fermentation microorganisms.

Finally, this research advances circular agriculture by providing a scientifically validated methodology for converting agricultural waste into high-value animal feed, simultaneously addressing feed security challenges and reducing environmental impact. The optimization framework established herein can be adapted to other crop residues, contributing to more sustainable agricultural systems. From a broader biorefinery perspective, this research provides new insights into efficient valorization of agricultural residues, transforming lignocellulosic biomass from an environmental burden into a valuable bioresource, achieving a "waste-to-wealth" transformation.

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Author’s Contributions

Lie Yang and Rundong Qin: Designed the experiment, collated the data, and prepared the paper.

Jiahuan Liu and Dongmei Ma: Performed the experiment, analyzed the data, and wrote the paper.

Lingyu Li and Bin Yang: Participated in the experiment, collated the paper and revised the manuscript.

Ethics

The authors declare that this manuscript entails no ethical issues.

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