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Impact of inoculating various lactic acid bacteria on vitamin A levels in total mixed ration silage

Yumeng Cheng, Zidie Wu, Kaijian Bi, Xiqing Wang, Haizhong Yu & Pengjiao Tian✉

This study investigated the impact of different strains of lactic acid bacteria (LAB) on the vitamin A content in total mixed ration (TMR) silage. The TMRs were inoculated with *Lactiplantibacillus plantarum* (LP), *Lacticaseibacillus casei* (LC), *Enterococcus faecium* (EF) or left without LAB as control (CK) and subsequently ensiled under dark, anaerobic conditions for 56 days. Throughout the ensiling process, the fermentation quality, chemical composition, microbial community, and vitamin A content of the TMR silage were monitored. Additionally, the free radical scavenging ability, total antioxidant capacity (T-AOC), and activities of antioxidant enzymes (SOD, GSH-Px, CAT) in the three LAB strains were assessed. After 56 days of ensiling, the antioxidant enzyme activities of T-AOC, SOD, GSH-Px and CAT of TMR silage were also analyzed. The results indicated that LAB inoculation had a significant effect on both the fermentation quality and vitamin A content of TMR silage ($p < 0.05$). Among the LAB strains, LP and LC enhanced the fermentation quality of the TMR silage. Vitamin A loss rates in decreasing order were LC, CK, EF and LP groups. Notably, LAB strains with higher antioxidant properties were found to improve the antioxidant capacity of the TMR silage, thereby mitigating the degradation of vitamin A.

Keywords LAB, Vitamin A, TMR silage, Antioxidant activity

In recent decades, the livestock industry has undergone significant transformations in response to evolving consumer demands, environmental concerns, and scientific advancements. Among the various aspects of livestock management, nutrition stands out as a critical determinant of animal health, productivity, and the quality of animal-derived products. Within this field, the potential of silage, particularly total mixed ration (TMR) silage, to optimize feed efficiency, enhance nutrient utilization and mitigate feed spoilage has garnered considerable attention[1,2]. TMR silage is a fermented feed commonly used in animal husbandry, particularly for the feeding of ruminants. It is scientifically formulated with multiple ingredients based on the animal's nutritional needs, including roughage, concentrates, minerals, vitamins and other additives[3]. Therefore, TMR silage ensures that every bite of feed consumed by the animal is a complete diet, maintaining a stable ratio of concentrate to roughage and a consistent nutrient concentration. Furthermore, TMR silage undergoes fermentation by lactic acid bacteria (LAB) under anaerobic conditions, playing a pivotal role in converting sugars into organic acids, primarily lactic acid. This fermentation process lowers the pH of the TMR silage, inhibiting the growth of undesirable microorganisms and preserving nutrient integrity[4]. In addition to its role in fermentation, recent research has begun to highlight the impact of LAB on the nutritional composition of TMR silage, with particular emphasis on essential micronutrients such as vitamin A. Vitamin A, a fat-soluble micronutrient, is critical for various physiological functions in animals, including vision, immune response, and reproduction[5]. Since ruminant animals cannot synthesize vitamin A, they must obtain it from their feed, making it essential to minimize vitamin A loss during TMR silage fermentation.

In a previous study, Tian et al. found that LAB are associated with the loss of vitamin A during TMR fermentation[6]. The acidic environment resulting from LAB's metabolic activities has been shown to degrade vitamin A in TMR silage[7]. However, given the complexity of the TMR fermentation process, it remains unclear whether other factors contribute to the loss of vitamin A caused by LAB. Thus, the objective of this paper is to investigated the influence of various LAB on vitamin A in TMR silage.

The effectiveness of LAB as additives in TMR silage applications is widely recognized[8]. *Lactiplantibacillus plantarum* (LP) is commonly incorporated into TMR silage to enhance fermentation quality, due to its rapid growth and ability to quickly reduce pH levels[9,10]. *Lacticaseibacillus casei* (LC) exhibits excellent acid resistance and can significantly reduce the ammonia nitrogen (NH_3-N) content in silage[11]. *Enterococcus fae*cium (EF) has a

College of Food Science and Chemical Engineering, Hubei University of Arts and Science, Xiangyang 441053, PR China. ✉email: 13120018505@163.com

good ability to degrade carbohydrates, improving the carbohydrates utilization efficiency in ruminants[12]. These LAB strains exhibit distinct functionalities, which may lead to varying effects on the vitamin A content in TMR silage. This study hypothesizes that different LAB species exert variable influences on vitamin A preservation in TMR silage, with the aim of elucidating the underlying mechanisms through which LAB affect vitamin A stability.

Materials and methods

LAB strains and growth conditions

The strains LP (GenBank accession number: MN658809), LC (GenBank accession number: MN658813), and EF (GenBank accession number: MN658816), derived from TMR silage, were cryopreserved at -80 °C in a cryoprotectant solution containing 10% glycerol. To prepare for inoculation, each strain was individually transferred into 5 milliliters of de Man, Rogosa, and Sharpe (MRS) broth (Nissui-Seiyaku Ltd., Japan), and incubated at 37 °C for 24 h in incubator shaker (Yiheng THZ-100, Shanghai, China). After the initial incubation, each culture underwent two subsequent passages. They were then transferred into 50 milliliters of fresh MRS broth and incubated under the same conditions for another 24-hour period. The population of LAB was quantified using the serial dilution technique, enabling the calculation of the appropriate inoculum dosage for subsequent applications.

TMR and TMR silage Preparation

TMR was prepared by combining alfalfa hay, soy milk residue, soybean meal, maize flour, vitamin-mineral supplements, and salt in the ratio of 36:21:5:32.5:5:0.5 based on dry matter (DM) content. After the mixture was prepared, the strains LP, LC, and EF were introduced into the TMR at a concentration of 10^6 colony-forming units per gram of fresh weight (cfu/g FW). A control group (CK) received an equivalent volume of sterile water. Each treatment was replicated three times, with 300 g of the mixture placed into vacuum-sealable bags (initial packaging density is 510 kg/m^3). The fermentation process for all treatments was conducted at ambient temperature, ranging from 24 to 28 °C, in a dark environment.

Chemical analysis

The DM content was determined by drying the sample at 65 °C for 48 h, following the protocol outlined in the 930.15 method[13]. Post-drying, the samples were mechanically pulverized to a particle size that could pass through a 1-mm mesh, utilizing a laboratory-grade knife mill. The resulting fine powder was subsequently used to quantify water-soluble carbohydrates (WSC), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF). The WSC was evaluated using the anthrone assay as described by John et al.[14]. The CP was quantified according to the analytical procedures set forth by the Association of Official Analytical Communities (AOAC), as detailed in Yang et al.[15]. NDF and ADF were determined using the method described by Van Soest et al.[16]. Vitamin A was analyzed in fresh samples after freeze-drying with a vacuum freeze-dryer, in accordance with the method of Tian et al.[7].

Fermentation quality analysis

Fermentation quality indicators were determined as follows: 90 mL of sterile water was added to 10 g of fresh silage sample and the mixture was homogenized for 30 s using a vortex shaker (QL-866, Kylin-Bell, Haimen, China). The filtered extract was used to determine the pH, organic acids and NH_3-N. The pH of TMR silage was measured using a glass electrode pH meter (S20K, Mettler Toledo, Greifensee, Switzerland). The concentrations of lactic acid, acetic acid, propionic acid and butyric acid were analyzed by high-performance liquid chromatography (HPLC, Showa Denko K.K., Kawasaki, Japan, with Shodex RS Pak KC-811 column, detector: DAD, 210 nm, eluent: 3 mmol/L $HCLO_4$, 1.0 mL/min; column temperature: 50 °C). NH_3-N content was determined using the method described by Broderick & Kang[17].

Microbial analysis

To enumerate LAB, aerobic bacteria, and yeasts, serial dilutions (ranging from 10^{-1} to 10^{-7}) were prepared from the water extract. The enumeration of LAB was executed on MRS agar medium (Nissui-Seiyaku Ltd., Japan) and incubated anaerobically at 37 °C for 48 h. Aerobic bacteria were enumerated using nutrient agar medium (Nissui, Tokyo, Japan) and incubated at 30 °C for 24 h. Yeast counts were performed on potato dextrose agar (Nissui, Tokyo, Japan), with incubation at 30 °C for 48 h.

LAB antioxidant activity determination

Preparation of cells and intracellular cell-free extracts

LAB were diluted to a 10-fold concentration with phosphate-buffered saline (PBS) solution, homogenized for 1 min using a turbine mixer, and subsequently inoculated into MRS broth at a 2% volume-to-volume ratio under conditions devoid of oxygen and maintained at 37 °C for 12 h. After incubation, the bacterial cells were harvested by centrifugation at 6000 g for 10 min at 4 °C. The pellets were washed twice with deionized water and resuspended in the same. The bacterial suspension was adjusted to a final concentration of 10^9 cfu/mL.

To prepare intracellular cell-free extracts, a procedure was adapted with minor alterations from Lin & Yen[18]. The cells were treated with lysozyme (1 mg/mL) and incubated at 37 °C for 30 min. Subsequently, the cells were disrupted by ultrasonic treatment using a sonicator (Fisher Scientific 120, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sonication consisted of five 1-minute cycles with ice bath intervals to prevent overheating. The resulting cell debris was removed by centrifugation at 8000 g for 10 min at 4 °C, and the supernatant was collected as the intracellular cell-free extract.

Scavenging of DPPH free radical

The ability of LAB to neutralize DPPH radicals was evaluated using a modified method based on Kao & Chen[19]. Briefly, 1.0 mL of LAB suspension at a concentration of 10^9 cfu/mL was mixed with 2.0 mL of an ethanolic solution of DPPH radicals (0.05 mmol/L). The resulting mixture was thoroughly mixed and incubated at ambient temperature in the dark for 30 min. Control samples consisted of deionized water and the DPPH solution, while blank samples contained ethanol and cells without DPPH. Post incubation, the mixture was centrifuged at 8000 g for 10 min to separate the components. The absorbance of the supernatant was measured in triplicate at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). The calculation of the scavenging capacity was executed utilizing the formula:

Scavenging activity (%)=[1- (A_s-A_b)/A_c] × 100.

where A_s, A_b, and A_c represent the absorbance values of the sample, blank, and control, respectively.

Scavenging of hydroxyl radical (·OH)

The ·OH scavenging capacity was evaluated using the Fenton reaction approach. Briefly, the reaction solution consisted of 1.0 mL of a brilliant green solution (0.435 mmol/L), 2.0 mL of 0.5 mmol/L ferrous sulfate ($FeSO^4$), 1.5 mL of 3.0% (w/v) hydrogen peroxide (H_2O_2), and 1.0 mL of the intracellular cell-free extract. This mixture was incubated at ambient temperature for 20 min. After incubation, the absorbance of the reaction mixture was measured at 624 nm. The change in absorbance was used as an indicator of the LAB strains' capacity to neutralize ·OH. The efficacy of ·OH scavenging was determined by applying the following formula:

Scavenging activity (%) = [(As-A_C)/(A -A_C)] ×100.

Where A_S is the absorbance in the presence of the sample; A_C is the absorbance of the control group (without the sample); and A represents the absorbance in the absence of both the sample and the Fenton reaction system.

Superoxide anion (O_2^-) scavenging ability

A mixture was prepared by combining Tris-HCl buffer (150 mmol/L, pH 8.2), EDTA (3 mmol/L), 1,2,3-benzenetriol (1.2 mmol/L), and cell-free extract (0.5 mL) to achieve a cumulative reaction volume of 3.5 mL, as described by Oyaizu[20]. The mixture was incubated at 25 °C for 10 min. After incubation, the absorbance of the mixture was measured at 325 nm using a spectrophotometer. The capability of the cell-free extract to counteract O_2^- was quantified by calculating the percentage of inhibition as follows:

Scavenging effect (%) =[1- (A_D -A_B)/(A_A- A_C)] ×100.

Where A_A represents the absorbance of a sample devoid of both cell-free extract and 1,2,3-benzenetriol; A_C signifies the absorbance of a sample without cell-free extract but in the presence of 1,2,3-benzenetriol; A_B represents the absorbance of a sample containing cell-free extract yet absent of 1,2,3-benzenetriol; and A_D is indicative of the absorbance of a sample containing both cell-free extract and 1,2,3-benzenetriol.

Tolerance to hydrogen peroxide

LAB were inoculated in the medium containing H_2O_2 (2 mmol/L) and incubated at 37 °C for 24 h. The cell concentration (expressed as OD_{600} value) was determined according to Van Niel et al.[21].

Determination of antioxidant activity of LAB

The total antioxidant capacity (T-AOC) and the activity of superoxide dismutase (SOD) activity, catalase (CAT), and glutathione peroxidase (GSH-Px) of LAB were analyzed using a reagent kit (Nanjing Jiancheng Bioengineering Institute, China) according to the instructions[22]. The cells were washed three times with cold sterile PBS, collected, and then centrifuged at 2000 g for 10 min at 4 °C. The supernatant was discarded, and the cells were washed with 1 mL of cold PBS. The cells were then subjected to centrifugation at 2000 g for 10 min at 4 °C. Subsequently, 1 mL of 1% Triton X-100 was added, and the sample was thoroughly mixed. The cells were then centrifuged at 4000 g for 15 min at 4 °C, and the supernatant was collected. The T-AOC, SOD, CAT and GSH-Px activities of the supernatants from the various treatment groups were measured separately.

Determination of antioxidant activity of TMR fermentation

Following the procedure described by Zhang et al.[23], the TMR silage samples were centrifuged at 4 °C for 10 min at 12,000 g. The supernatant obtained from this process was subsequently utilized for the assessment of T-AOC, SOD activity, GSH-Px activity, and CAT activity.

Statistical analysis

Each test was conducted in triplicate to ensure accuracy and reliability of the results. Statistical comparisons were performed using SPSS20.0 software.

To ascertain the statistical significance of the differences among the various treatments, an analysis of variance (ANOVA) was carried out. Subsequently, Tukey's multiple comparison test was utilized to identify the specific differences between treatments. A significance level of $p < 0.05$ was applied throughout. The correlation between vitamin A and the array of measured variables was evaluated using the Pearson correlation coefficient. The criterion for statistical significance was set at $p < 0.05$.

Results

Fermentation quality and microbial composition of TMR

The variations in fermentation quality and the microbial profile throughout the ensiling process of TMR supplemented with different LAB strains were summarized in Tables 1 and 2, respectively. The type of LAB, ensiling time, and their interaction had significant effects on pH, acetic acid, NH_3-N, and LAB count ($p < 0.05$). Lactic acid levels were influenced by both the LAB type and ensiling time ($p < 0.05$). Throughout fermentation,

Item	Treatment	Ensiling time(d)					SEM	*p*-value		
		0	7	14	28	56		S	T	SXT
pH	CK	6.72^{Aa}	5.00^{Ba}	4.90^{Ca}	4.63^{Db}	4.55^{Ea}	0.105	< 0.001	< 0.001	< 0.001
	LP	6.75^{Aa}	4.93^{Bb}	4.79^{Cb}	4.58^{Dc}	4.52^{Eb}				
	LC	6.68^{Aa}	4.91^{Bc}	4.78^{Cb}	4.54^{Dd}	4.50^{Dc}				
	EF	6.69^{Aa}	4.99^{Ba}	4.90^{Ca}	4.65^{Da}	4.54^{Ea}				
Lactic acid (g/kg DM)	CK	10.8^{Da}	51.8^{Cb}	58.1^{Bab}	69.2^{Aa}	73.9^{Aa}	3.011	< 0.001	< 0.001	0.055
	LP	9.8^{Da}	55.1^{Cab}	63.8^{Bab}	71.2^{Aa}	74.5^{Aa}				
	LC	9.4^{Da}	57.1^{Ca}	64.3^{Ba}	72.2^{Aa}	75.2^{Aa}				
	EF	10.4^{Ca}	52.5^{Bb}	57.1^{Bb}	68.4^{Aa}	72.6^{Aa}				
Acetic acid (g/kg DM)	CK	0^{Bb}	6.90^{Aa}	6.41^{Aa}	6.19^{Aa}	7.06^{Aa}	0.241	0.016	< 0.001	< 0.001
	LP	2.32^{Ca}	5.76^{Ba}	5.97^{ABa}	6.31^{Aa}	6.36^{Aab}				
	LC	2.53^{Ca}	5.89^{ABa}	5.95^{ABa}	6.11^{Aa}	5.64^{Bb}				
	EF	2.98^{Ba}	6.44^{Aa}	6.27^{Aa}	6.30^{Aa}	6.97^{Aa}				
Propionic acid (g/kg DM)	CK	ND	ND	ND	ND	1.90	-	-	-	-
	LP	ND	ND	ND	1.88	1.88				
	LC	ND	ND	ND	1.85	1.87				
	EF	ND	ND	0.61	1.85	1.92				
Butyric acid (g/kg DM)	CK	ND	ND	ND	ND	ND	-	-	-	-
	LP	ND	ND	ND	ND	ND				
	LC	ND	ND	ND	ND	ND				
	EF	ND	ND	ND	ND	ND				
NH_3-N (% TN)	CK	0.74^{Da}	1.82^{Ca}	2.84^{Ba}	3.45^{Aa}	3.62^{Aa}	0.133	< 0.001	< 0.001	0.002
	LP	0.65^{Da}	1.41^{Cc}	2.19^{Bb}	2.92^{Ab}	3.15^{Ab}				
	LC	0.64^{Da}	1.57^{Cb}	2.22^{Bb}	2.91^{Ab}	3.14^{Ab}				
	EF	0.73^{Da}	1.84^{Ca}	2.77^{Ba}	3.41^{Aa}	3.59^{Aa}				

Table 1. Fermentation quality of TMR during ensiling. DM, dry matter; TN, total nitrogen; S, LAB strains; T, ensiling time; SXT, Interaction between LAB and ensiling time; ND, not detected; SEM, standard error of the means; LP, *L. plantarum*; LC, *L. casei*; EF, *E. faecium*; CK, control; Means with different letters in the same column (a–c) differ ($p < 0.05$); Means with different letters in the same row (A–E) differ ($p < 0.05$).

Items	Treatment	Ensiling time(d)					SEM	*p*-value		
		0	7	14	28	56		S	T	SXT
LAB (log_{10} cfu/g FW)	CK	6.51^{Db}	8.85^{Aa}	7.98^{Ca}	8.23^{BCa}	8.52^{ABab}	0.080	< 0.001	< 0.001	< 0.001
	LP	7.48^{Ca}	8.94^{Aa}	8.16^{Ba}	8.18^{Ba}	8.34^{Bb}				
	LC	7.44^{Ca}	8.96^{Aa}	8.28^{Ba}	8.28^{Ba}	8.68^{ABa}				
	EF	7.31^{Ca}	8.86^{Aa}	7.99^{Ba}	8.14^{Ba}	8.39^{ABb}				
Yeast (log_{10} cfu/g FW)	CK	3.92	2.5	ND	ND	ND	-	-	-	-
	LP	3.75	ND	ND	ND	ND				
	LC	3.89	ND	ND	ND	ND				
	EF	3.87	ND	ND	ND	ND				
Aerobic bacteria (log_{10} cfu/g FW)	CK	7.33^{Aa}	3.28^{Ba}	3.27^{Ba}	3.78^{Ba}	3.24^{Ba}	0.218	0.173	< 0.001	0.538
	LP	7.06^{Aa}	3.16^{Ba}	3.06^{Ba}	3.56^{Ba}	2.76^{Ba}				
	LC	7.53^{Aa}	2.80^{Ca}	2.96^{Ca}	3.44^{Ba}	3.25^{BCa}				
	EF	7.29^{Aa}	2.94^{Ca}	3.15^{Ca}	3.79^{Ba}	3.03^{Ca}				

Table 2. Microbial composition during ensiling of TMR. FW, fresh weight; S, LAB strains; T, ensiling time; SXT, Interaction between LAB and ensiling time; ND, not detected; SEM, standard error of the means; LP, *L. plantarum*; LC, *L. casei*; EF, *E. faecium*; CK, control; Means with different letters in the same column (a–c) differ ($p < 0.05$); Means with different letters in the same row (A–E) differ ($p < 0.05$).

the pH of all TMRs decreased significantly, particularly in the LP and LC groups. Concurrently, the content of lactic acid and acetic acid increased, while butyric acid was undetectable. The NH_3-N content of all TMRs gradually increased during the fermentation, but remained below 5% TN by days 56. Notably, the NH_3-N content in the LP and LC groups was consistently lower than that in the CK and EF groups during ensiling. During the first 7 days, LAB numbers increased significantly and then stabilized. Except for the CK group, yeast counts in

all other groups were suppressed by day 7 and remained below the detection limit thereafter. Aerobic bacteria were only significantly affected by ensiling time ($p < 0.05$) and were inhibited 7 days before ensiling, while their numbers remain around 10^3 cfu/g.

Chemical composition of TMR silage

The influence of different LAB strains on the chemical composition of TMR silage throughout the ensiling process was presented in Table 3. The type of LAB, ensiling time, and their interaction significantly affected on the content of WSC, CP, NDF, and ADF ($p < 0.05$), while the content of DM was only affected by ensiling time ($p < 0.001$). With the extension of ensiling time, the DM content significantly decreased in all groups except for the LC and EF groups, while the LP group showing the most substantial reduction in DM content. The WSC content of all TMR silages decreased rapidly within the first 7 days, followed by a slower decline. After 56 days of ensiling, the CP and ADF contents of all TMR silages increased slightly, and the NDF and ADF contents of the CK, LP and LC groups also increased slightly, but the NDF content of the EF group decreased slightly.

Vitamin A changes in TMR silage with different LAB inoculations

As shown in Fig. 1, there was a significant difference ($p < 0.05$) in the effect of different LABs on vitamin A loss during TMR ensiling. After 56 days of ensiling, the LC group exhibited the highest loss rate of vitamin A, reaching 58.9%. In contrast, the LP group had the lowest loss rate of vitamin A at 44.3%. The CK and EF groups showed intermediate loss rates of 51.3% and 47.5%, respectively. These results suggest that inoculation with LP and EF reduced the loss of vitamin A in TMR silage, while inoculation with LC did not effectively reduce vitamin A loss.

The tolerance of LAB to H_2O_2

The tolerance of different LAB to H_2O_2 is shown in Fig. 2a. Under the condition of adding 2 mM H_2O_2, the growth of LC, EF, and LP was inhibited. After 24 h of cultivation, the OD_{600} values of LP, LC and EF were 1.1, 0.86, and 0.75, respectively. Further analysis revealed significant differences in H_2O_2 tolerance among LC, EF and LP ($p < 0.05$). Among them, LP exhibited the strongest tolerance to H_2O_2, followed by LC, while EF displayed the weakest tolerance.

DPPH radical scavenging activity

As shown in Fig. 2b, this study measured the scavenging activity of the fermentation broth, cells, and cell-free extracts of LC, EF, and LP against DPPH radicals. The results demonstrated that all components (fermentation broth, cells, and cell-free extracts) from LC, EF, and LP exhibited scavenging activity against DPPH radicals. However, significant differences in scavenging capacity were observed ($p < 0.05$). The highest DPPH radical

Items	Treatment	Ensiling time(d)					SEM	*p*-value		
		0	7	14	28	56		S	T	SXT
DM (g/kg FW)	CK	581^{Aa}	568^{ABa}	563^{Ba}	565^{ABa}	566^{ABa}	1.357	0.072	< 0.001	0.955
	LP	576^{Aa}	571^{ABa}	557^{ABa}	556^{ABa}	553^{Bb}				
	LC	580^{Aa}	574^{Aa}	568^{Aa}	564^{Aa}	565^{Aa}				
	EF	574^{Aa}	565^{Aa}	560^{Aa}	560^{Aa}	563^{Aa}				
WSC (g/kg DM)	CK	99.7^{Aa}	69.3^{Ba}	65.9^{Bb}	62.0^{BCb}	54.5^{Cb}	2.570	< 0.001	< 0.001	< 0.001
	LP	100.4^{Aa}	62.2^{Ba}	55.6^{BCc}	48.3^{BCc}	43.4^{Cc}				
	LC	109.6^{Aa}	60.7^{Ba}	55.9^{Bc}	47.1^{Cc}	43.5^{Cc}				
	EF	99.2^{Aa}	65.0^{Ba}	77.3^{Ba}	72.5^{Ba}	74.0^{Ba}				
CP (g/kg DM)	CK	164^{Cb}	164^{Cb}	181^{Aab}	174^{Bb}	181^{Ab}	0.090	< 0.001	< 0.001	< 0.001
	LP	166^{Db}	175^{Ca}	185^{ABa}	178^{BCab}	188^{Aa}				
	LC	178^{ABa}	172^{Bab}	183^{Aab}	181^{Aa}	182^{Ab}				
	EF	179^{ABa}	179^{Ba}	178^{Bb}	177^{Bab}	185^{Aab}				
NDF (g/kg DM)	CK	286^{Aa}	270^{Aa}	276^{Ab}	245^{Ba}	286^{Aa}	1.858	0.001	0.003	< 0.001
	LP	263^{CDb}	278^{ABa}	254^{Dc}	268^{BCa}	284^{Aa}				
	LC	263^{Ab}	278^{Aa}	286^{Aa}	283^{Aa}	271^{Aab}				
	EF	271^{ABb}	260^{BCb}	282^{Aab}	248^{Da}	256^{CDb}				
ADF (g/kg DM)	CK	202^{Aa}	188^{Bb}	192^{Ba}	173^{Ca}	202^{Aa}	1.612	< 0.001	< 0.001	< 0.001
	LP	176^{Cc}	191^{ABb}	172^{Cb}	188^{Ba}	198^{Aa}				
	LC	183^{Ab}	208^{Aa}	191^{Aa}	200^{Aa}	202^{Aa}				
	EF	184^{Bb}	177^{Cb}	195^{Aa}	170^{Da}	183^{Bb}				

Table 3. Chemical composition of TMR during ensiling. DM, dry matter; FW, fresh weight; WSC, water soluble carbohydrates; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; S, LAB strains; T, ensiling time; SXT, Interaction between LAB and ensiling time; ND, not detected; SEM, standard error of the means; LP, *L. plantarum*; LC, *L. casei*; EF, *E. faecium*; CK, control; Means with different letters in the same column (a–c) differ ($p < 0.05$); Means with different letters in the same row (A–D) differ ($p < 0.05$).

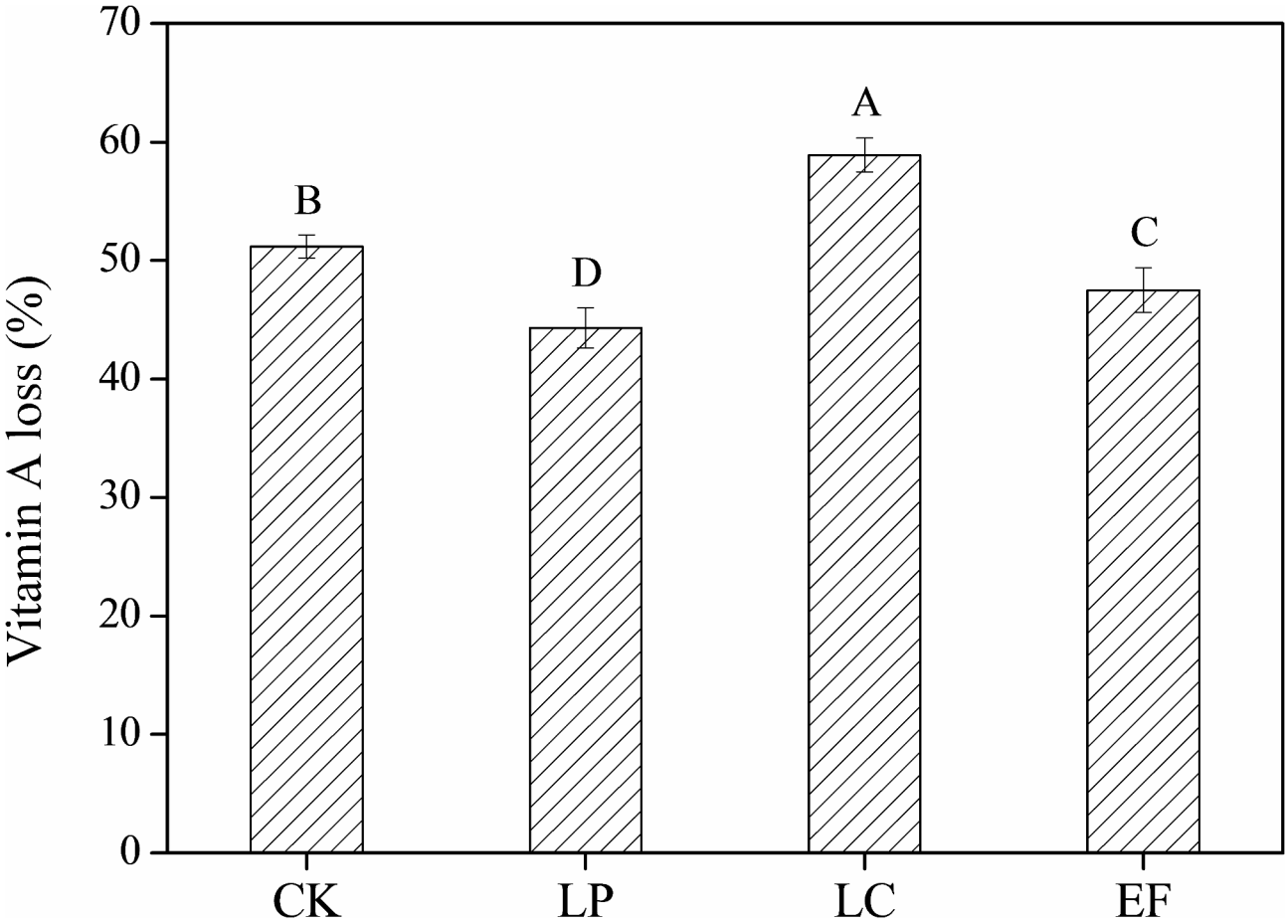

Fig. 1. Vitamin A loss rate of TMR inoculated with different LAB after 56 days of ensiling. Capital letters (A-D) represent the level of significant difference between samples ($p < 0.05$). The values are the mean ± standard deviation of three replicates. LP, *L. plantarum*; LC, *L. casei*; EF, *E. faecium*; CK, control.

scavenging activity was observed in the fermentation broth, with LP, LC, and EF showing scavenging capacities of 85%, 79%, and 62%, respectively. This was followed by the cells, with scavenging capacities of 40%, 38%, and 26%, respectively. The weakest scavenging activity was observed in the cell-free extracts, with scavenging capacities of 16%, 12%, and 10%, respectively.

Furthermore, a comparison of the scavenging rates of the same components of LP, LC, and EF against DPPH radicals revealed significant differences ($p < 0.05$) among the three strains. Among them, LP exhibited the strongest scavenging activity against DPPH radicals in all components, followed by LC intermediate scavenging activity and EF with the weakest scavenging activity against DPPH radicals.

Hydroxyl radical (·OH) scavenging ability

Figure 2c illustrates that all three LAB strains exhibit scavenging abilities against ·OH radicals, with notable differences between them. Significant differences were observed both among the components of the same LAB strain and among the same components across different LAB strains ($p < 0.05$). Compared to the fermentation broth and cells, the cell-free extracts of LP, LC, and EF exhibited stronger ·OH scavenging abilities. Among them, the cell-free extract of EF demonstrated the highest scavenging capability against ·OH radicals. Furthermore, significant differences were observed in the ·OH scavenging abilities between the fermentation broth and cell components of each strain. The order of scavenging abilities in the fermentation broth was EF > LC > LP, while the same order was observed in the cells.

Superoxide radical (O_2^-) scavenging ability determination

As depicted in Fig. 2d, all components of the three LAB strains exhibited O_2^- scavenging abilities. The fermentation broth of LP showed the strongest capability in scavenging O_2^- radicals at 70.3%. Significant differences were observed in the O_2^- scavenging abilities among the various constituent parts of the same LAB strain, with the cells and cell-free extracts demonstrating reduced capabilities compared to the fermentation broth. Significant discrepancies were also identified in the O_2^- scavenging capabilities among the same components of disparate LAB ($p < 0.05$). Among the cells, LC exhibited the strongest O_2^- scavenging ability, while among the cell-free extracts, LP showed the strongest O_2^- scavenging ability.

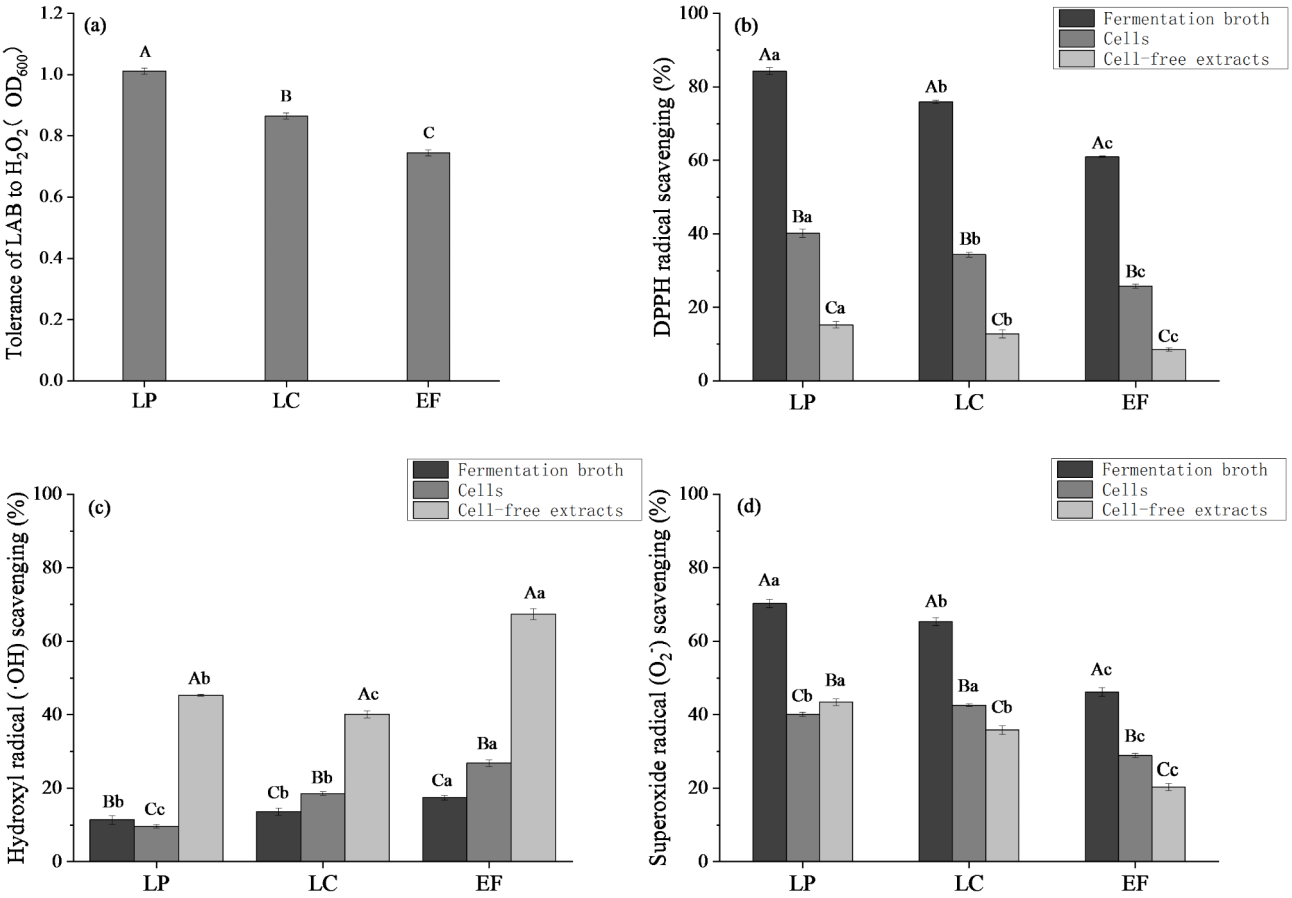

Fig. 2. Antioxidant activity of LAB. Capital letters (A-C) represent the level of significant difference between different components of the same LAB ($p < 0.05$). Lowercase letters (a-c) represent the level of significant difference between different LAB of the same component ($p < 0.05$). The values are the mean ± standard deviation of three replicates. LP, *L. plantarum*. LC, *L. casei*. EF, *E. faecium*. CK, control.

	Fermentation broth(U/mL)				Cells(U/mL)				Cell-free extracts(U/mL)			
Strain	T-AOC	SOD	GSH-Px	CAT	T-AOC	SOD	GSH-Px	CAT	T-AOC	SOD	GSH-Px	CAT
LP	38.87	57.64	19.35	0.41	1.54	16.52	11.84	2.54	0.78	9.89	12.75	2.38
LC	30.69	42.88	14.56	0.21	0.94	10.37	5.63	0.89	0.84	7.25	9.47	0.86
EF	21.32	36.36	10.35	0.34	0.38	7.59	2.43	0.96	0.34	2.54	5.58	0.36

Table 4. T-AOC, SOD, GSH Px and CAT activities of LAB. LP, *L. plantarum*; LC, *L. casei*; EF, *E. faecium*; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase.

Antioxidant activity of LAB

A comparative analysis of the T-AOC and the enzymatic activities of SOD, GSH-Px, and CAT was conducted for the LP, LC, and EF groups. These results were displayed in Table 4. The T-AOC levels in the fermentation broth of all three LAB strains were higher than in their cells and cell-free extracts. The LP fermentation broth exhibited the highest T-AOC level. All three LAB strains exhibited antioxidant enzyme activities. The fermentation broth of LP exhibited the highest SOD activity, the cell exhibited the highest GSH-Px activity and the cell-free extract exhibited the highest CAT activity. However, the cell-free extract exhibited the lowest SOD activity. In summary, the antioxidant activity of LAB was ranked from highest to lowest as follows: LP > LC > EF.

Antioxidant activity of fermented TMR

As shown in Table 5, after 56-day of ensiling TMR inoculated with various LAB strains, significant differences in antioxidant activity were observed among the treatment groups ($p < 0.05$). The T-AOC values of TMR fermented with LP and LC were significantly higher than those of the CK and EF groups, with the highest T-AOC value observed in the LP group. However, the SOD activity in TMR fermented with LP and LC was lower than that

Items	CK	LP	LC	EF	SEM	*p*-value
T-AOC, U/g FW	265^{C}	307^{A}	289^{B}	274^{C}	4.842	< 0.001
SOD, U/g FW	489^{A}	456^{B}	454^{B}	481^{A}	4.902	0.001
GSH-Px, U/g FW	1432^{D}	1992^{A}	1725^{B}	1689^{C}	5.930	< 0.001
CAT, U/g FW	18.4^{D}	42.2^{A}	34.6^{B}	23.9^{C}	0.746	< 0.001

Table 5. Antioxidant activity of TMR after 56 days of ensiling. LP, *L. plantarum*; LC, *L. casei*; EF, *E. faecium*; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; FW, Fresh matter; SEM, standard error of the means; Means with different letters in the same row (A–D) differ ($p < 0.05$).

in the CK and EF groups. The GSH-Px and CAT activities in the TMR fermented with LAB strains were higher than those in the CK, with the highest GSH-Px and CAT activities found in the TMR fermented with LP. Consequently, it can be concluded that the antioxidant activity of the TMR fermented with LP is greater than that of the TMR fermented with the other two LAB.

Discussion

The impact of LAB on the fermentation quality and chemical composition of TMR silage

This study explored the effect of different strains of LAB on the fermentation characteristics of TMR as detailed in Tables 1 and 2. After 56-day of ensiling, the pH values across all treatment groups remained within the narrow range of 4.50 to 4.55. These findings are consistent with those reported by Chen et al.[24], which indicated that at elevated DM concentrations exceeding 40%, the pH typically fluctuated between 4.0 and 5.0. Furthermore, Nishino & Hattori reported that the pH decreased to 4.09 after 60 days of ensiling of TMR silage supplemented with LC[25]. However, in this study, the pH of the TMR in the LC group only decreased to 4.50 after 56 days of ensiling, which may be due to differences in the composition of the TMR ingredients. LP, a homofermentative LAB, is known to promote acid production during ensiling, resulting in significantly lower pH values throughout the fermentation process compared to the CK and EF groups. The pH values of CK and EF groups showed no significant differences during ensiling, indicating that EF did not significant affect pH variation of TMR silage. Consistent with previous studies, LP and LC strains were found to increase lactic acid content while reducing acetic acid content[26], which was also observed in this study. Moreover, LP and LC were also observed to reduce the propionic acid content of TMR.

The NH_3-N content of TMR silage in all groups remained relatively low, with values below 5% TN. This may be attributed to the higher DM content (> 40%) inhibiting the growth of clostridium, as NH_3-N is mainly generated by the metabolism of amino acids by clostridium[27]. Additionally, no butyric acid content was detected in any group, further supporting the inhibition of clostridia fermentation. The NH_3-N content in the CK group was higher than that in the LP and LC groups, which may be attributed to the slower pH decline in the CK group, which has been shown to favour the hydrolytic action of microbial proteases. In this experiment, the addition of LP, LC, and EF did not significantly affect the quantity of LAB and aerobic bacteria. However, they were found to rapidly inhibit yeast growth on the 7th day. This finding is consistent with the findings of Jiang et al., which reported that yeast was below the detection limit after 14 days of fermentation with LP-inoculated TMR[10]. This indicates that LAB inoculation in TMR inhibits yeast growth.

The impact of LAB inoculation on DM content was not statistically significant in this experiment, with a loss of less than 2.3%. The LP and LC groups exhibited a significant reduction in WSC, while the WSC content in EF showed an upward trend by day 14. This may be attributed to EF's ability to degrade starch into soluble sugars during the later stages of fermentation, as previously described in reference[12].

After 56 days of ensiling, the loss of DM led to an increase in CP, NDF and ADF. However, the reduction in NDF content in the EF group may be due to the ability of EF to degrade certain components of NDF.

Vitamin A content influenced by LAB

Based on the previous studies, LAB may be correlated with the loss of vitamin A[6]. This study aimed to investigated the effect of different LAB strains on vitamin A content in TMR silage. The results showed that the rate of vitamin A loss followed this descending order: LC > CK > EF > LP (Fig. 1). Previous studies have indicated that a reduction in pH can accelerate the degradation of vitamin A[7]. In this study, the pH levels of both the LP and LC groups experienced a parallel decline. Consequently, one might expect no significant difference in vitamin A degradation between these two groups. However, the vitamin A loss rate in the LC group was significantly higher than that in the LP group, as shown in Fig. 1. Similarly, no significant difference in pH levels was observed between the EF group and CK group throughout the ensiling process. Despite this, the rate of vitamin A degradation in the CK group was considerably higher than that in the EF group. These findings suggest that, in addition to pH, other factors related to LAB may influence the changes in vitamin A content. Studies by Siedler et al.[28] and Kurzer et al.[29] have shown that the addition of antioxidants to skim milk or poultry feed can improve its antioxidant properties, thereby reducing vitamin A loss. Likewise, Feng & Wang have shown that LAB can produce antioxidant enzymes and therefore have certain antioxidant properties[30]. Therefore, we speculate that different LAB strains may exert varying effects on the antioxidant capacity of TMR silage, which could contribute to the observed differences in vitamin A loss between the groups.

Antioxidant activity of LAB

During metabolic reactions, the body produces many reactive oxygen species, such as·OH, O_2^- and H_2O_2, etc. When the content of free radicals in the body exceeds a certain level, it can disrupt normal metabolic processes, leading to oxidative stress and causing a series of damages to the body. Many research has demonstrated a direct correlation between the antioxidant activity of LAB and its capacity to scavenge free radicals[31,32]. The effect of the antioxidant activity of LAB on the TMR silage was investigated. The results demonstrated that LP exhibited a higher scavenging capacity for DPPH radicals and O_2^-radicals, as well as a higher tolerance for H_2O_2 (Fig. 2). These findings indicate that LP has the strongest ability to scavenge free radicals. Furthermore, a multitude of experiments have demonstrated that LAB possesses antioxidant activity. The mechanism by which it exerts antioxidant effects may be related to its ability to produce a variety of antioxidant enzymes, including SOD, GSH-Px and CAT[33]. In this experiment, the SOD, GSH-Px and CAT activities of all fractions of LP were found to be higher than those of LC and EF (Table 4). This may be responsible for the higher T-AOC of LP. While LC exhibited higher T-AOC and SOD activities in all fractions than EF, the GSH-Px activities of fermentation broth and cells were slightly lower than those of EF. Therefore, in a comprehensive view, the antioxidant activity of LC was higher than that of EF. Consequently, the antioxidant activities of LAB in descending order were LP > LC > EF.

Antioxidant activity of TMR silage influenced by LAB

Supplementing with additional antioxidants can increase the antioxidant capacity of a substance, thereby preventing oxidative losses from occurring. Previous research has indicated that adding antioxidants to poultry feed or skim milk can improve the stability of vitamin A[28,29]. It was inferred that an increase in antioxidant capacity promotes the preservation of vitamin A, protecting it from oxidative losses. Therefore, the antioxidant capacity of TMR silage inoculated with LAB was also determined (Table 5). The results showed that different LAB improved the antioxidant capacity of TMR silage differently. The most elevated levels of T-AOC and activities of GSH-Px, as well as CAT, were detected in the LP group, succeeded by the LC group, and ultimately the EF group. In contrast, the SOD activity in the TMR silage of the LP and LC groups was notably reduced in comparison to the CK and EF groups. Nonetheless, the T-AOC of the TMR silage in the LP and LC groups was significantly superior to that of the CK and EF groups. This suggests that the enhancement of the antioxidant capacity of TMR silage was mainly due to the role of GSH-Px and CAT[23].

The impact of antioxidant activity on vitamin A in relation to LAB

Based on the results of 4.2, the loss of vitamin A caused by inoculation with different LAB in TMR was variable. Inoculation with LP and EF reduced the loss of vitamin A in TMR silage, while inoculation with LC did not reduce vitamin A loss. This discrepancy may be attributed to the differing fermentation characteristics of TMR influenced by LAB, and the varying impacts of these LAB on the antioxidant activity of TMR silage. TMR silage from the LP group exhibited the highest T-AOC, GSH-Px and CAT activities. This suggests that the inoculation of LP may have enhanced the antioxidant capacity of TMR silage, thereby reducing vitamin A loss. The SOD activity of the TMR silage in the LP and LC groups was lower than that of the CK and EF groups. This can be attributed to the fact that the main role of SOD is to catalyse the conversion of O_2^- to H_2O_2, and then GSH-Px and CAT decompose H_2O_2 to H_2O by donating electrons to H_2O_2 and ·OH[29,34]. However, it is important to note that O_2^- is produced in electron transfer reactions during the transport of oxygen in alkaline and aerobic environments[35,36]. This suggests that the lower pH of TMR silage in the LP and LC groups may have inhibited SOD activity. Additionally, although the T-AOC of TMR silage in the LC group was higher than that of EF and CK, the loss of vitamin A was greater. Previous studies have indicated that vitamin A is sensitive to acidic conditions[7]. Therefore, the greater vitamin A loss in the LC group could be attributed to the lower pH of the TMR silage, where the antioxidant capacities of GSH-Px and CAT were insufficient to counteract the degradation of vitamin A caused by the acidic environment. Consequently, inoculation with LC did not result in a reduction in the loss of vitamin A from TMR silage. The T-AOC, GSH-Px and CAT activities of fermented TMR in the EF group were higher than those in the CK group, while the pH reduction was similar to that of the CK group. This may explain why the EF group exhibited a lower vitamin A loss compared to the CK group.

Conclusion

The fermentation quality of TMR inoculated with LP and LC was significantly superior to that of the CK group, while the fermentation quality of TMR inoculated with EF showed no significant difference from the CK group. The loss of vitamin A in TMR silage varied among the treatment groups, with LC exhibiting the greatest loss, followed by CK, EF, and LP. In addition, an analysis of the antioxidant capacity of the different LAB strains and their impact on vitamin A loss revealed a correlation between the strength of the LAB's antioxidant capacity and the extent of vitamin A loss. Inoculating TMR silage with LAB strains that have higher antioxidant capacity significantly enhanced the antioxidant potential of the silage, thereby reducing the loss of vitamin A. However, when the pH drop was severe, even the enhanced antioxidant capacity was insufficient to prevent vitamin A degradation.

Data availability

The datasets generated and/or analysed during the current study are available in the GenBank, and accession numbers are MN658816, MN658813,MN658809.

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Author contributions

Yumeng Cheng: Experiment investigation, writing-Original draft preparation, sampling and analysis. Zidie Wu: Data curation, formal analysis. Kaijian Bi: Investigation, validation. Haizhong Yu: Resources, Software. Xiqing Wang and Pengjiao Tian: Writing- Reviewing and Editing, Project administration, Funding acquisition.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to P.T.

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