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Effects of fermented and unfermented aging corn on ruminal fermentation, bacterial communities, lactation performance and plasma metabolites in Holstein cows

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ABSTRACT

Aging reduces the nutritional value of corn grain, which may be improved by fermentation prior to use. This study investigated the effects of replacing conventional corn (**CC**) with aging corn (**AC**) and fermented aging corn (**FAC**) in the diets of lactating Holstein cows. Six healthy third-parity Holstein cows were used in a replicated 3 × 3 Latin square experiment with 21-day periods. Cows were fed twice and milked twice daily. The cows were randomly divided into three treatment groups: (1) CC diet: a diet with 23.6% starch in diet DM containing 24.0% conventional corn; (2) AC diet: a diet with 23.5% starch in diet DM containing 24.0% aging corn replacing conventional corn; and (3) FAC diet: a diet with 23.2% starch in diet DM containing 24.6% fermented aging corn replacing conventional corn. The diets were formulated to be isonitrogenous and contained identical roughage. The FAC treatment increased the starch digestibility compared with AC. Feeding FAC increased the concentrations of total volatile fatty acid compared with CC and AC and decreased the molar proportion of acetate compared with AC. At the family level, the relative abundance of *Prevotellaceae* was higher on FAC than CC. The relative abundance of *Succinivibrionaceae* was lower on FAC than on CC and AC. Besides, at the genera level, the relative abundance of *Succinivibrionaceae_UCG_002* in the rumen was higher on AC than CC and FAC. The relative abundance of *Prevotella* and *Ruminococcus* was higher on FAC than CC and AC. The relative abundance of *Succinivibrionaceae_UCG_001* was lower on FAC than CC. The Simpson index was lower on FAC than CC and AC. The FAC treatment increased the milk yield (34.0, 33.7, and 35.2 kg/d for CC, AC, and FAC group, respectively) and protein yield, and thus, energy-corrected milk production was increased, and at the same time, decreased the somatic cell score compared with CC and AC. The AC treatment increased the malondialdehyde concentration in plasma compared with CC and FAC. The concentrations in plasma of triglyceride and malondialdehyde were lower on FAC than AC. The immunoglobulin G concentration in plasma was higher on FAC than CC and AC. Overall, feeding AC resulted in decreased plasma antioxidant capacity compared with CC, whereas feeding FAC altered the relative abundance of bacteria in the rumen and improved starch digestibility, ruminal bacterial diversity, lactation performance, plasma antioxidant capacity and immune competence compared with AC in dairy cows.

Implications

Aging corn is the product of long-term storage of corn and can not be widely used in animal feeding. The beneficial role of solid-state fermentation in improving the nutritional quality of animal feed has been reported across studies. In this study, although feeding aging corn did not affect the performance of dairy cows, it decreased the antioxidant capacity, whereas, feeding fermented aging corn had positive effects on milk performance and the overall health of dairy cows. Our results provide new insight for improving the utilisation of non-conventional feed resources and the health of dairy cows.

Introduction

Corn (maize, *Zea mays*), one of the most important crops in the world, is primarily used for food, animal feed, and biofuel. In China, the production of corn reached 272.6 million tonnes in 2021, which accounted for 43% of Chinese cereal crop production (NBS, 2022). Corn, both the grain and whole crop silage, is the predominant energy feedstuff for ruminants in China. It contains large

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amounts of starch and contributes to meeting the energy needs of ruminants. As one of the important reserve grains in China, corn can be stored for as long as 2 or more years (Yin et al., 2017). With longer storage, free amino groups in protein bind with the reducing sugars in the Maillard reaction, and this combined product, is not easily utilised by the proteolytic enzyme and starch hydrolase in the corn itself, which leads to nutritional loss (Rehman, 2006). The relative content of amylose increases while amylopectin decreases during storage, which may adversely affect the digestion and utilisation of starch, and further limits the wide application of aging corn (**AC**) in animal feed (Huang and Lai, 2014; Wang et al., 2022). Furthermore, during prolonged storage and aging, high levels of polyunsaturated fatty acids in corn can easily be oxidised with the generation of hydrogen peroxide, which leads to decreased activities of catalase and peroxidase as well as causes a negative impact on the oxidative status of the animal (Zhou et al., 2007; Rehman, 2006). Therefore, finding a processing technology for aging corn for wide use in animal production is a prime concern that needs to be addressed.

In recent years, several studies have demonstrated that solid-state fermentation of feedstuff, a traditional fermentation method done under low-moisture conditions to optimise the growth and metabolism of the several organisms used, can enhance its nutritional value, amino acid profile, and palatability, as well as facilitate nutrient digestion and absorption in the organism (Lambo et al., 2024; Kim et al., 2012; Saylor et al., 2020). Fermentation can also reduce the presence of mycotoxins in cereals and enhance their safety (Wacoo et al., 2019). As the key microorganism used in various fermented food and animal feed products, lactic acid bacteria (**LAB**) have proven efficient in enhancing the nutritive quality, digestibility, and other biological functions like antioxidant and antibacterial activities (Da Silva et al., 2018; Zhang et al., 2020b; Gaisawat et al., 2019). A previous study by our research team also showed that LAB improved corn gluten-wheat bran mixture antioxidant capacity and bioavailability, which largely improved the feed quality (Jiang et al., 2021b). Furthermore, adding proteases facilitates the prolamine degradation process, which exposes the starch within the hydrophobic starch-protein matrix and increases the contact area of starch in the rumen, thereby promoting the digestion and utilisation of starch (Kung et al., 2014).

Several studies have reported the effects of AC on growth and production performance, antioxidant capacity, and intestinal health in commercial laying hens, broilers, and weaned piglets (Yin et al., 2017; Luo et al., 2019; Zhou et al., 2019). However, to our knowledge, there are no studies published on the effects of feeding AC on production performance, rumen fermentation, and antioxidant capacity in dairy cows. The ameliorative influence of fermentation on the negative effects of AC is also unclear. Our hypothesis for this study is that feeding AC may diminish the production performance and antioxidant capacity of dairy cows, while fermented aging corn (**FAC**) can be used in the diets of dairy cows to replace conventional corn (**CC**) and improve these indicators. Therefore, this study aims to determine the effects of replacing CC with AC and FAC on nutrient digestibility, plasma metabolites, milk yield and composition, ruminal fermentation, and microflora of dairy cows.

Material and methods

Preparation of conventional corn, aging corn, and fermented aging corn

The CC was obtained from a grain depot in Harbin, Heilongjiang province. This grain depot is also the source of AC. The AC samples were kept under a low temperature, humidity, and good ventilation to avoid mycotoxin contamination. The age of the corn used for CC was 6 months, while the AC and FAC were 4 years. The particle size of the corn sample (CC and AC) in the grain depot is less than 2 cm. The AC samples were subjected to solid-state fermentation by adding lactic acid bacteria and acid protease. The LAB (*Lactobacillus rhamnosus* CICC 23119) was provided by the China Centre of Industrial Culture Collection. Acid protease (60 000 U/g) was purchased from Xiasheng Industrial Group Co., Ltd. (Ningxia, China), which can be used effectively at a pH of 2.0–6.0 and has good stability at high temperatures (85 °C). Fermentation to produce FAC was carried out by Sanhua Biotechnology Co., Ltd. (Harbin, China). The AC samples were ground in a hammer mill before the fermentation was started to obtain corn samples with an average particle size of about 1 500 μm (The CC samples were similarly handled). A total of 7.5 kg of acid protease and 20 L of LAB (5×10^9 CFU/mL) were added to 60 L of water and then subjected to constant agitation until they completely dissolved, resulting in a homogeneous solution in full contact with the AC through the form of a spray. Following this, 925 L of water was transferred to the tank gradually, and the mixture containing AC and water was stirred constantly at the same time until it was mixed evenly. The final moisture content of the AC was adjusted to approximately 40%. Finally, the treated corn was loaded into polyethylene bags and vacuumed to achieve anaerobic fermentative conditions. Polyethylene bags were placed in the thermostatic chamber at a constant temperature (36 °C) and stored for 20 days to form FAC. Both FAC and AC came from the same batch, and five samples were extracted from each treatment (CC, AC and FAC) for subsequent determination of chemical composition and antioxidant enzymatic activity, and each sample had three parallel samples. All feed was stored in a cool warehouse for subsequent feeding trials. When the room temperature was high, the warehouse was cooled by air conditioning to reduce the influence of temperature on the stability of fermented feed.

Animals, diets, and experimental design

The experiment was conducted from May to August 2022 at the Songhuajiang Dairy Farm (Harbin, China). All experimental animal procedures conformed to the guidelines for animal experiments of Northeast Agricultural University, and all procedures involving animal care were done under the approval of the Animal Care Advisory Committee of Northeast Agricultural University. Six healthy third-parity Holstein cows with similar BW (614 ± 39.4 kg), days in milk (146 ± 21.5), and milk yield (33.7 ± 2.26 kg/d; all mean ± SD) were used in a replicated 3 × 3 Latin square design with three periods of 21 d. For each period, the initial 14 d were used for diet adaptation, and the final 7 d were used for sampling and data collection. Cows were fed three different diets: (1) basal diet with CC; (2) replacement of CC with AC; and (3) replacement of CC with FAC. Three isonitrogenous experimental diets for dairy cows (Table 1) were prepared using the Cornell-Penn-Miner dairy nutrition model, version 3.0.10 (Boston et al., 2000) to meet the nutritional needs of dairy cows producing 39.0 kg/d milk with 3.90% fat and 3.25% protein (Tedeschi et al., 2008). Cows were kept in individual pens throughout the experimental period, fed twice daily (0600 and 1800 h) at between 105 and 110% *ad libitum* intake, and milked twice daily at 0530 and 1730 h. The feeds were pushed at least 10 times daily, and freshwater was allowed access freely by cows at all times throughout the study period.

Data collection and sampling

On days 15–21 of each period, the total mixed ration (**TMR**) offered and refused was weighed for seven consecutive days to calculate DM intake (**DMI**), and they were also collected and stored at

Table 1
Ingredients and chemical composition of diets offered to lactating Holstein cows.

Item	CC	AC	FAC
Ingredient composition, % of DM			
Corn silage	27.1	27.1	26.9
Alfalfa hay	16.8	16.8	16.7
Oat hay	3.80	3.80	3.75
Wet brewers grains	5.40	5.40	5.35
CC	24.0	0	0
AC	0	24.0	0
FAC	0	0	24.6
Sodium bicarbonate	0.80	0.80	0.80
Concentrate[1]	22.1	22.1	21.9
Chemical composition, % of DM			
CP	16.8	16.9	17.1
NDF	35.7	35.6	34.9
ADF	19.3	19.3	19.0
Ether extract	3.85	3.91	3.96
NFC[2]	38.4	38.6	39.5
Starch	23.6	23.5	23.2

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn; NFC = non-fibrous carbohydrate.

[1] The ingredient composition of concentrate was corn husk (27%), corn germ meal (16%), DDGS (10%), soybean meal (32%), beet molasses (3.5%), limestone (4%), calcium bicarbonate (0.07%), sodium bicarbonate (1.6%), extruded urea (4%), sodium chloride (2%); contained per kilogram of concentrate: Ca 142.5 g, P 54.0 g, S 3.7 g, Mg 49.3 g, K 500 mg, Na 106.4 g, Co 12 mg, Cu 500 mg, Cl 29.5 g, Zn 1.8 g, Fe 4.858 g, Mn 800 mg, I 25 mg, Se 10 mg, vitamin A 180 000 IU, vitamin D 55 000 IU, and vitamin E 1 500 IU.

[2] NFC = 100 − % NDF − % CP − % ether extract − % ash.

−20 °C during the final 3 d of each period (d 19, 20, and 21), then were pooled by periods and cows. Feed samples containing feed ingredients and TMR were dried at 55 °C for 48 h, then pulverised, sieved using a 1 mm mesh, and stored in closed plastic bags at 4 °C for nutritional composition determinations. The milk yield was recorded daily on d 15–21, milk samples were harvested on d 19, 20, and 21 of each period, and 24-h milk samples (50 mL) to be tested were prepared according to the proportions of cows' real milk yield at each milking. The milk samples to be tested were mixed with potassium dichromate, stored at 4 °C, and used for subsequent determinations of milk composition. On d 19, 20, and 21, approximately, 500 g of faeces were spot sampled from the rectum at 0600 and 1800 h and mixed according to periods and cows. One part of the sample was dried at 55 °C for 48 h, then pulverised and sieved using a 1 mm mesh and stored in closed plastic bags at 4 °C, which were used for nutrient digestibility determinations.

Blood samples were collected into sodium heparin tubes from the coccygeal vessels of cows 3 h after morning feeding on d 15, 16, and 17 of each period. Plasma was obtained by centrifuging blood samples at 3 000 × g for 15 min at 4 °C and stored at −20 °C. Ruminal fluid samples were collected via oral tubing from cows approximately 3 h after the morning feeding on d 15, 16, and 17 of each period. To decrease the risk of contamination of ruminal fluid samples with saliva, the first 100–200 mL of collected ruminal fluid was discarded. The samples were filtered through four layers of cheesecloth, and 5 mL of filtrate was added to 1 mL of metaphosphoric acid (25%, wt/vol) and mixed uniformly. The mixed solution was centrifuged at 3 000 × g for 15 min at 4 °C, and the supernatant was separated and stored at −20 °C, which was used for volatile fatty acid (**VFA**) determinations. Furthermore, 3 mL of the filtrate was loaded into cryopreservation tubes and then stored in liquid nitrogen, which was used to determine bacterial communities.

Laboratory analysis

Chemical composition

The DM, CP, ash, and ether extract contents of feed samples were determined according to the method described by AOAC International (2000). Heat-stable amylase was used to determine the NDF and ADF content of the feed samples following a previously reported method (Van Soest et al., 1991). Content of non-fibrous carbohydrates was calculated as 100 − % NDF − % CP − % ether extract − % ash. The starch content of the feed samples was measured using the Megazyme Total Starch Assay Kit (product no: K-TSTA; Megazyme International Ireland Ltd., Wicklow, Ireland). The DM, CP, NDF, ADF, and starch of CC, AC, and FAC were also measured according to the above method.

Antioxidant enzymatic activity

Catalase, superoxide dismutase, glutathione peroxidase activity, total antioxidant capacity, and malondialdehyde concentration of CC, AC, and FAC were measured using an antioxidant assay kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The acidity of fatty acids of CC, AC, and FAC was determined according to the method described in State Food Administration standard quality centre (2015). Ten grams of the ground samples was added to 50 mL of anhydrous ethanol for 30 min for extraction in a shaker and allowed to stand for 2 min before filtering. After discarding a few drops of the initial filtrate, 25 mL was collected, and 50 mL of carbon dioxide-free distilled water was added, followed by the phenolphthalein indicator. Finally, the KOH standard solution volume was recorded by auto titrator (877Titrino, Metrohm, Switzerland), and the mass of KOH needed to neutralise free fatty acids in a 100 g sample was calculated.

Feed intake and total-tract apparent digestibility

Indigestible NDF in the feed and fecal samples was used as an internal marker, and then, total-tract apparent nutrient digestibility was then estimated by performing calculations according to a previously reported method (Lee and Hristov, 2013). The indigestible NDF content in the TMR, orts, and faeces was evaluated after *in situ* incubation of 288 h, as previously described by Huhtanen et al., 1994. The DM, CP, NDF, ADF, and Starch contents of fecal samples were measured using the same method as the feed samples. The digestibility of DM (%) was calculated using the formula: 1 – (% of indigestible NDF intake/ % of indigestible NDF in faeces)] × 100%. Furthermore, the digestibility of nutrients (%) was calculated from the following equation: nutrient digestibility (%) = {1 – [(% of indigestible NDF intake/ % of indigestible NDF in faeces) × (% of nutrient in faeces/% of nutrient intake)]} × 100%.

Ruminal fermentation

The pH value of ruminal fluid was determined using a pH meter (PB-10; Sartorius Co., Göttingen, Germany). Concentrations of Ammonia-N were determined using the phenol/hypochlorite method (Broderick and Kang, 1980). Concentrations of VFA were determined by a gas chromatography method (CC-8A; Shimadzu Corp., Kyoto, Japan; Stewart and Duncan, 1985).

Lactation performance

Milk protein, lactose, milk fat, milk urea nitrogen concentrations, and somatic cell count of milk samples were measured and analysed at the Heilongjiang Academy of Agricultural Reclamation (Harbin, China) using a 4-channel spectrophotometer (MilkoScan; Foss Electric, Hillerød, Denmark). Moreover, the equation for Energy-corrected milk (**ECM**) was ECM (kg/d) = 0.3273 × milk yield + 12.97 × milk fat yield + 7.21 × milk protein yield (Tyrrell and Reid, 1965). The equation for 4% Fat-corrected milk (**FCM**) was calculated according to NRC (2001) as follows: 4% FCM (kg/d) = 0.4 × milk yield + 15 × fat yield. Considering somatic cell count does not have a normal distribution, somatic cell score (**SCS**) was transformed from somatic cell count using logarithmic transformation (Ali and Shook, 1980) for statistical analysis: SCS = Log_2 (somatic cell count / 100 000) + 3.

Plasma metabolites

The plasma antioxidant capacity, including catalase, malondialdehyde, total antioxidant capacity, and glutathione peroxidase, was measured using an antioxidant assay kit (No. A007-1–1, A003-1–2, A015-1–2, A005-1–2, respectively) provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The plasma immunity parameters, including immunoglobulin A, immunoglobulin G, and immunoglobulin M, were measured using an ELISA kit (No. MM-090502, MM-040302, MM-040202, respectively) provided by SINO-UK Institute of Biotechnology (Beijing, China). Intra-assay CV was 8.02, 6.03, 4.91, 4.28, 5.98, 7.82, and 6.33%, while inter-assay CV was 7.41, 7.16, 2.27, 2.90, 2.58, 7.17, and 3.84% respectively for catalase, malondialdehyde, total antioxidant capacity, glutathione peroxidase, immunoglobulin A, immunoglobulin G, and immunoglobulin M, which were within the range of values declared by the commercial product datasheet. The plasma biochemical parameters, including total protein, triglyceride, total cholesterol, urea nitrogen, and glucose, were measured using an automatic blood biochemical analyzer (HT82-BTS-330; Xihuayi Technology Co. Ltd., Beijing, China).

Bacteria communities

Samples of ruminal fluid were sequenced to determine bacterial community using high-throughput sequencing at Biomarker Technologies Co., Ltd. (Beijing, China). The DNA was extracted from all samples by MN NucleoSpin 96 Soi DNA kit (Gene Company Limited, Beijing, China) following the manufacturers' instructions. According to the method described in (Jiang et al., 2021a), the extracted DNA was subjected to a 2-step polymerase chain reaction and was used to construct a small fragment sequencing library for subsequent sequencing. In the first step, the bacterial 16S rRNA gene spanning V3 to V4 was amplified, using the extracted DNA as a template and 338F, 5′-ACTCCTACGGGAGGCAGCA-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′as primers. A duplicate of the PCR product obtained from the first step was pooled and then used as the template for the second step in the Solexa PCR amplification (Applied Biosystems Inc.). Next, the Solexa PCR products amplified in the previous step were purified and quantified using OMEGA DNA purification column (Gene Company Limited) and Quant-iT PicoGreends DNA Assay Kit (Gene Company Limited), respectively. And then, the amplicons were sequenced on Illumina HiSeq 2500 sequencing platform (Illumina Inc., San Diego, CA; Novaseq 6000; paired-end; 250 bp). The original label data of rumen samples showed 1 440 216 by FLASH software (version 1.2.11; Magoč and Salzberg, 2011). The sequenced raw tags were filtered using Trimmomatic software (version 0.33; Bolger et al., 2014) to obtain clean tag data that did not contain primer sequences (1 436 862 clean tags for the ruminal samples). Final valid tags were obtained (1 049 635 effective tags for the ruminal samples) by identifying and removing chimeric sequences using UCHIME software (version 8.1; Edgar et al., 2011). The sequences obtained from the MiSeq platform were processed using the open-source software pipeline QIIME (version 1.8.0). Operational taxonomic units were clustered using the clustering program USEARCH (version 10.0; Edgar, 2013), demanding a minimum sequence similarity level of 97%. Each operational taxonomic unit obtained was finally taxonomic and assigned. The representative sequences for each operational taxonomic unit were compared to the Silva database, and community composition, including phylum, class, order, family, and genera levels, was obtained. The relative abundances of bacterial taxa at the family and genera level were obtained by QIIME software (version 1.9.1; Kuczynski et al., 2012), and then, the bacterial community composition was compared between different treatments in the rumen. Furthermore, richness and diversity indices such as Chao1, Ace, Shannon, and Simpson were determined by QIIME software (version 1.9.1; Kuczynski et al., 2012).

Statistical analysis

The data on chemical composition and antioxidant enzymatic activity of CC, AC, and FAC samples were subjected to ANOVA using the GLM procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). The carry-over effect of Latin square was first determined using general linear model in the Minitab 17.0 software (Pennsylvania State University, State College, PA): no carry-over effect was present in all data ($P > 0.05$), and then, data on milk production and components, plasma metabolites, nutrient intake and digestibility, rumen fermentation variables, and bacterial communities were analysed in a 3 × 3 Latin square design by PROC MIXED using SAS (version 9.2; SAS Institute Inc., Cary, NC). The following model was used for the analysis: $Y_{ijkm} = \mu + T_m + P_k + C(S)_{ij} + S_i + E_{ijkm}$. Y_{ijkm} was the observation, μ was the overall mean, T_m was the fixed effect of treatment, P_k was the fixed effect of the period, $C(S)_{ij}$ was the random effect of cows within the square, S_i was the random effect of the square, and E_{ijkm} was the residual error. Results were reported as least squares mean. Differences between treatments were determined using a Tukey multiple comparison test. Significant differences were declared at $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

Results

Chemical composition and antioxidant enzymatic activity

Table 2 shows the chemical composition and antioxidant capacity of CC, AC, and FAC samples. The acidity of fatty acids ($P < 0.01$) and malondialdehyde concentration ($P < 0.01$) were higher on AC than on CC. The total antioxidant capacity ($P < 0.01$), superoxide dismutase ($P < 0.01$), and glutathione peroxidase ($P < 0.01$) activity were lower on AC than CC. In addition, the contents of DM ($P < 0.01$), NDF ($P < 0.01$), ADF ($P = 0.01$), acidity of fatty acids ($P < 0.01$), catalase activity ($P < 0.01$) and malondialdehyde concentration ($P < 0.01$) were lower on FAC than AC. The starch content ($P = 0.01$), total antioxidant capacity ($P < 0.01$), superoxide dismutase ($P < 0.01$), and glutathione peroxidase ($P < 0.01$) activity were higher on FAC than on AC.

Feed intake and total-tract apparent digestibility

We observed that the FAC treatment tended to increase the intake of NDF compared with CC ($P = 0.07$, Table 3), but the intake of CP, ADF, and starch did not alter ($P > 0.10$) among treatments. The digestibility of CP ($P = 0.10$) and NDF ($P = 0.09$) tended to be higher in FAC compared with AC. The digestibility of starch was higher on FAC than on AC ($P = 0.01$).

Ruminal fermentation

As shown in Table 4, the FAC treatment increased the concentrations of total VFA ($P = 0.01$) and decreased the rumen pH ($P < 0.01$) relative to CC and AC. The molar proportion of acetate was lower on FAC than on AC ($P = 0.05$). The molar proportion of isovalerate tended to be lower in FAC compared with AC ($P = 0.09$). We did not observe a change ($P > 0.10$) in the concentration of Ammonia-N, the molar proportion of propionate, butyrate, isobutyrate, valerate, and acetate: propionate among treatments.

Bacterial communities

The bacterial community compositions at the family and genera levels in the ruminal fluid among the different treatments are shown in Tables 5 and 6. We did not observe changes ($P > 0.10$)

Table 2
Chemical composition and antioxidant enzymatic activity of conventional corn, aging corn and fermented aging corn in diets offered to lactating Holstein cows.

	Treatment			
Item[1]	CC	AC	FAC	*P*-value
DM, %	87.1 ± 0.57[a]	87.9 ± 0.45[a]	59.0 ± 0.28[b]	< 0.01
CP, DM%	9.60 ± 0.45	9.47 ± 0.27	9.54 ± 0.19	0.88
NDF, DM%	13.2 ± 0.32[a]	12.8 ± 0.30[a]	8.8 ± 0.19[b]	< 0.01
ADF, DM%	3.19 ± 0.14[a]	2.92 ± 0.09[a]	2.09 ± 0.07[b]	0.01
Starch, DM%	71.6 ± 0.21[b]	72.7 ± 0.74[b]	74.2 ± 0.49[a]	0.01
Acidity of fatty acids[1] KOH mg/100 g	53.3 ± 0.34[c]	87.4 ± 0.49[a]	65.7 ± 0.57[b]	< 0.01
T-AOC, U/ml	0.74 ± 0.02[b]	0.40 ± 0.02[c]	0.93 ± 0.03[a]	< 0.01
SOD, U/ml	6.44 ± 0.20[b]	2.77 ± 0.27[c]	8.45 ± 0.34[a]	< 0.01
CAT, U/ml	5.61 ± 0.41[a]	6.24 ± 0.22[a]	2.21 ± 0.22[b]	< 0.01
GSH-PX, U/ml	34.5 ± 1.73[b]	15.6 ± 0.42[c]	47.4 ± 1.16[a]	< 0.01
MDA, nmol/ml	6.60 ± 0.13[b]	10.16 ± 0.37[a]	4.43 ± 0.11[c]	< 0.01

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase; MDA = malondialdehyde.
[1] Acidity of fatty acids: the mass of KOH needed to neutralise free fatty acids in a 100 g sample.
[a–c] Means within a row with different superscripts differ ($P \leq 0.05$).

Table 3
Effects of fermented and unfermented aging corn on intake and total-tract apparent digestibility of nutrients in lactating Holstein cows[1].

	Treatment				
Item	CC	AC	FAC	SEM	*P*-value
Intake, kg/d					
CP	3.62	3.65	3.80	0.13	0.35
NDF	7.12	7.35	7.73	0.23	0.07
ADF	3.77	3.89	3.99	0.18	0.50
Starch	5.04	5.22	5.26	0.18	0.44
Digestibility, %					
DM	71.9	71.6	74.1	1.24	0.12
CP	74.9	74.4	77.2	1.59	0.10
NDF	46.5	46.1	50.0	1.82	0.09
ADF	31.6	31.4	33.2	1.23	0.34
Starch	92.8[ab]	92.4[b]	93.4[a]	0.23	0.01

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn.
[1] Values are least squares means of six replicate cows (n = 6).
[a–b] Means within a row with different superscripts differ ($P \leq 0.05$).

Table 4
Effects of fermented and unfermented aging corn on ruminal fermentation in lactating Holstein cows[1].

	Treatment				
Item	CC	AC	FAC	SEM	*P*-value
pH	6.38[a]	6.37[a]	6.23[b]	0.03	< 0.01
Ammonia-N, mg/dL	16.3	17.2	16.7	0.42	0.17
Total VFA, mmol/L	88.4[b]	90.8[b]	96.9[a]	2.43	0.01
VFA, % total VFA					
Acetate	62.6[ab]	63.9[a]	61.2[b]	0.95	0.05
Propionate	24.2	23.0	24.6	1.20	0.45
Butyrate	9.46	9.28	10.7	0.66	0.12
Isobutyrate	1.02	0.98	0.94	0.06	0.12
Valerate	1.64	1.59	1.53	0.09	0.48
Isovalerate	1.16	1.17	1.07	0.04	0.09
Acetate: propionate	2.61	2.80	2.50	0.17	0.24

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn; VFA = volatile fatty acid.
[1] Values are least squares means of six replicate cows (n = 6).
[a,b] Means within a row with different superscripts differ ($P \leq 0.05$).

in the relative abundance of most bacteria in AC compared with CC, except for increased *Succinivibrionaceae_UCG_002* relative abundance (P = 0.04). At the family level, the relative abundance of *Prevotellaceae* was increased in FAC relative to CC (P = 0.05), but AC did not differ from both. The relative abundance of *Succinivibrionaceae* was decreased by FAC (P < 0.01) and did not differ between CC and AC. Moreover, the relative abundance of *Lachnospiraceae* (P = 0.09) and *Ruminococcaceae* (P = 0.07) tended to be greater in FAC compared with CC. At the genera level, the relative abundance of *Prevotella* (P = 0.02) and *Ruminococcus* (P = 0.05) were higher on FAC than CC and AC, but the relative abundance of *Succinivibrionaceae_UCG_001* was lower on FAC than CC (P = 0.02). Apart from that, we found no changes ($P > 0.10$) in the bacterial community among treatments. As indicated in Table 7, we did not observe a change (P > 0.10) in the Ace index and Chao index among treatments. The Simpson index was lower on FAC than CC and AC (P = 0.03). The Shannon index tended to be higher in FAC compared with AC (P = 0.07).

Table 5
Effects of fermented and unfermented aging corn on the relative abundances (>0.5%) of bacterial families in the ruminal fluids of lactating Holstein cows[1].

Item	Treatment			SEM	*P*-value
	CC	AC	FAC		
Prevotellaceae	0.28[b]	0.29[ab]	0.31[a]	0.011	0.05
Succinivibrionaceae	0.19[a]	0.19[a]	0.17[b]	0.006	< 0.01
Lachnospiraceae	0.10	0.11	0.12	0.008	0.09
Acidaminococcaceae	0.10	0.08	0.09	0.021	0.71
Selenomonadaceae	0.03	0.03	0.03	0.002	0.95
Bacteroidales_RF16_group	0.03	0.03	0.02	0.005	0.58
Rikenellaceae	0.02	0.02	0.03	0.007	0.62
Ruminococcaceae	0.03	0.03	0.04	0.004	0.07
Oscillospiraceae	0.03	0.03	0.03	0.002	0.73
F082	0.02	0.03	0.02	0.006	0.58
Saccharimonadaceae	0.01	0.01	0.01	0.001	0.80
Hungateiclostridiaceae	0.008	0.008	0.01	0.001	0.41
Others	0.13	0.14	0.11	0.020	0.37

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn.
[1] Values are least squares means of six replicate cows (n = 6).
[a,b] Means within a row with different superscripts differ ($P \leq 0.05$).

Table 6
Effects of fermented and unfermented aging corn on the relative abundances (>0.5%) of bacterial genera in the ruminal fluids of lactating Holstein cows[1].

Item	Treatment			SEM	*P*-value
	CC	AC	FAC		
Prevotella	0.20[b]	0.20[b]	0.24[a]	0.012	0.02
Succinivibrionaceae_UCG_001	0.15[a]	0.14[ab]	0.13[b]	0.008	0.02
Succiniclasticum	0.10	0.08	0.09	0.021	0.71
Succinivibrionaceae_UCG_002	0.02[b]	0.03[a]	0.02[b]	0.004	0.04
Selenomonas	0.02	0.02	0.02	0.003	0.93
Lachnospira	0.008	0.008	0.01	0.003	0.76
Rikenellaceae_RC9_gut_group	0.02	0.03	0.02	0.007	0.47
Prevotellaceae_UCG_001	0.02	0.02	0.01	0.002	0.54
Ruminococcus	0.02[b]	0.02[b]	0.03[a]	0.003	0.05
Prevotellaceae_UCG_003	0.01	0.02	0.01	0.003	0.68
Shuttleworthia	0.007	0.007	0.01	0.003	0.12
Pseudobutyrivibrio	0.008	0.007	0.007	0.001	0.47
NK4A214_group	0.02	0.02	0.02	0.006	0.90
Butyrivibrio	0.009	0.009	0.01	0.002	0.50
Others	0.39	0.40	0.37	0.021	0.54

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn.
[1] Values are least squares means of six replicate cows (n = 6).
[a,b] Means within a row with different superscripts differ ($P \leq 0.05$).

Lactation performance

As shown in Table 8, the FAC treatment increased the milk yield ($P = 0.04$), ECM ($P = 0.01$), and milk protein ($P < 0.01$) yields compared with CC and AC. The 4% FCM ($P = 0.02$) and milk fat ($P = 0.04$) yields were higher on FAC than on CC. The lactose yield was higher on FAC than on AC ($P = 0.02$). The SCS was lower on FAC than on CC and AC ($P < 0.01$). We did not observe a change ($P > 0.10$) in DMI, milk urea nitrogen, milk yield/DMI, and ECM/DMI among treatments.

Plasma metabolites

We did not observe a change in the concentrations ($P > 0.10$) of total protein, urea nitrogen, total antioxidant capacity, immunoglobulin A, and immunoglobulin M in plasma among treatments (Table 9). The catalase activity was lower on AC than on FAC ($P = 0.01$). The malondialdehyde concentration was higher on AC than CC and higher on CC than FAC ($P < 0.01$). The triglyceride concentration was lower on FAC than AC ($P = 0.03$). The immunoglobulin G concentration was higher on FAC than on CC and AC ($P = 0.01$). In addition, feeding FAC tended to increase glucose concentration compared with feeding AC ($P = 0.10$).

Discussion

Diet composition

The solid-state fermentation reduced the DM content of AC, which could likely increase spoilage during long-term storage and limit its use. The NDF and ADF components were degraded under the action of LAB in FAC. Meanwhile, fermentation results in a relative reduction in starch content, probably due to the fact that the starch analysis procedure detected some degradation products of the fibre component, and the specific changes in composition need to be further analysed. Aging of corn involves lipolysis, increasing Free Fatty Acid concentration and susceptibility to oxidation, increasing aldehydes, ketones, and other volatile matter, and causing an increase in malondialdehyde which is the lipid peroxidation end-product (Yin et al., 2017; Bailly et al., 2002). Concurrently, we observed that the total antioxidant capacity, superoxide dismutase, and glutathione peroxidase activity of AC were lower than those of CC, which was similar to results obtained in a previous study (Luo et al., 2019). Fermentation inhibits lipolysis and reduces free fatty acids by inhibiting lipase activity, thereby decreasing the acidity of fatty acids (Lee et al., 2008). The result of this study indicates that the total antioxidant capacity, glutathione peroxidase, and superoxide dismutase activity of AC

Table 7
Effects of fermented and unfermented aging corn on the diversity indices of bacterial communities in the ruminal fluids of lactating Holstein cows[1].

	Treatment				
Item	CC	AC	FAC	SEM	*P*-value
OTU	1496.8	1484.5	1467.3	18.4	0.31
Coverage	0.98	0.98	0.98	0.0001	0.43
Shannon index	8.35	8.29	8.54	0.10	0.07
Simpson index	0.91[a]	0.91[a]	0.89[b]	0.006	0.03
Ace index	1516.6	1503.8	1487.0	12.2	0.22
Chao index	1509.4	1489.7	1473.0	16.8	0.18

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn; OTU = operational taxonomic unit.
[1] Values are least squares means of six replicate cows (n = 6).
[a,b] Means within a row with different superscripts differ ($P \leq 0.05$).

Table 8
Effects of fermented and unfermented aging corn on milk performance in lactating Holstein cows[1].

	Treatment				
Item	CC	AC	FAC	SEM	*P*-value
DMI, kg/d	21.2	21.4	22.4	0.66	0.19
Yield, kg/d					
Milk	34.0[b]	33.7[b]	35.2[a]	0.51	0.04
ECM[2]	34.3[b]	34.6[b]	36.1[a]	0.52	0.01
4% FCM[3]	31.7[b]	32.2[ab]	33.3[a]	0.47	0.02
Fat	1.21[b]	1.25[ab]	1.28[a]	0.03	0.04
Protein	1.05[b]	1.03[b]	1.11[a]	0.02	< 0.01
Lactose	1.70[ab]	1.68[b]	1.79[a]	0.03	0.02
Composition					
Fat, %	3.55	3.70	3.65	0.09	0.24
Protein, %	3.08	3.05	3.15	0.04	0.10
Lactose, %	5.00	4.97	5.09	0.05	0.10
MUN, mg/dL	13.7	14.0	14.5	0.65	0.51
SCS[4]	4.42[a]	4.45[a]	4.31[b]	0.03	<0.01
Feed efficiency					
Milk/DMI	1.61	1.59	1.58	0.06	0.86
ECM/DMI	1.62	1.63	1.62	0.07	0.98

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn; DMI = DM intake; ECM = Energy-corrected milk; FCM = Fat-corrected milk; MUN = milk urea nitrogen; SCS = somatic cell score; SCC = somatic cell count.
[1] Values are least squares means of six replicate cows (n = 6).
[2] ECM = 0.3273 × milk yield + 12.97 × milk fat yield + 7.21 × milk protein yield.
[3] 4% FCM = 0.4 × milk yield + 15 × fat yield.
[4] SCS = Log_2 (SCC / 100 000) + 3.
[a,b] Means within a row with different superscripts differ ($P \leq 0.05$).

Table 9
Effects of fermented and unfermented aging corn on plasma metabolites in lactating Holstein cows[1].

	Treatment				
Item[2]	CC	AC	FAC	SEM	*P*-value
Regular blood metabolites					
TP, g/L	68.8	66.5	69.3	1.65	0.24
TC, mmol/L	3.27	3.43	2.95	0.20	0.09
TG, mmol/L	0.17[ab]	0.18[a]	0.15[b]	0.01	0.03
UN, mmol/L	6.68	6.44	6.49	0.24	0.60
Glucose, mmol/L	3.50	3.43	3.81	0.18	0.10
Antioxidant indices					
CAT, U/mL	35.0[ab]	31.6[b]	36.6[a]	1.29	0.01
T-AOC, U/mL	4.43	4.29	4.48	0.27	0.77
GSH-Px, U/mL	383.9	377.7	399.5	9.51	0.10
MDA, nmol/mL	4.12[b]	4.42[a]	3.83[c]	0.12	< 0.01
Immune indices					
IgA, g/L	1.75	1.78	1.84	0.07	0.49
IgM, g/L	1.27	1.33	1.37	0.09	0.54
IgG, g/L	8.29[b]	8.58[b]	9.50[a]	0.30	0.01

Abbreviations: CC = conventional corn; AC = aging corn; FAC = fermented aging corn; TP = Total protein; TC = Total cholesterol; TG = Triglyceride; UN = Urea nitrogen; CAT = catalase; T-AOC = total antioxidant capacity; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; IgA = Immunoglobulin A; IgM = Immunoglobulin M; IgG = Immunoglobulin G.
[1] Values are least squares means of six replicate cows (n = 6).
[a–c] Means within a row with different superscripts differ ($P \leq 0.05$).

increased while malondialdehyde content decreased after fermentation. This might be because *Lactobacillus rhamnosus*, the fermentation inoculum used in this study, regulates the activity of the antioxidant enzymes, thus reducing the risk of reactive oxygen species accumulation (Hou et al., 2019; Kuda et al., 2014). However, the specific mechanism is unclear and needs further studies. Interestingly, unlike other antioxidant enzymes, the fermentation process reduces catalase activity. The possible reason is that the synthesis of the catalase protein may be limited under an acidic environment. A study on alfalfa fermentation had a similar result (Li et al., 2021).

Feed intake and total-tract apparent digestibility

The results in the study differ from those of Zhang et al. (2020a), that feeding fermented TMR to dairy cows decreased nutrient intake and thereby increased feed efficiency. However, Jiang et al. (2021a) observed that feeding fermented corn gluten-wheat bran mixture did not change nutrient intake. The reasons for these differences may be the different fermentation substrates. Cao et al. (2010) observed that feeding fermented TMR in sheep increased the digestibility of CP and NDF. The findings of Jiang et al. (2021a) also show that the fermentation treatment of feed could improve the digestibility of DM and CP in dairy cows. In this study, the starch digestibility was higher in FAC than in AC, and the digestibility of CP and NDF tended to be higher in FAC compared with AC. Although extensive crosslinking of hydrophobic prolamins in endosperm limits the starch obtained by the enzyme (Hoffman et al., 2011), prolamins can be degraded by microbial and acid protease under anaerobic storage, which is conducive to the degradation of starch in the ruminal and total tract (Kung et al., 2014). In addition, feeding FAC increased the relative abundance of *Prevotellaceae*, which may also be a potential reason for the improved nutrient digestibility. As generally known, *Prevotellaceae* is predominantly responsible for metabolising amino acids, protein, hemicellulose, and starch in the rumen and has a positive role in maintaining homeostasis of the rumen environment (Dao et al., 2021; Fondevila and Dehority, 1996).

Ruminal fermentation

Total VFA concentration was greater in FAC compared with CC and AC. The results in this study correspond with that of Miyaji et al. (2017), where it was seen in steers that feeding ensiled rice increased the ruminal VFA compared with dried rice. Jiang et al. (2021a) also observed a linear increase in total VFA concentration with increased fermented feed proportion. One possible reason for the higher total VFA concentration seen in FAC could be the increased energy supply via the increased nutrient digestibility, which might promote the production of fermentation acids. The increase in total VFA concentration in FAC treatment may explain the decrease in rumen pH. Mills et al. (1999) noted that the rate and extent of starch digestion could affect the composition and concentrations of VFA produced by rumen fermentation, and a lower starch digestion rate favours acetate production in the rumen. In this study, the molar proportion of acetate was higher in AC than in FAC, probably due to the starch digestibility of AC being lower than that of FAC. In a previous study, the decline in starch digestibilities of rice, maize, and wheat was reported to be a result of the Maillard reaction, which occurs from prolonged storage time (Rehman, 2006). The molar proportion of isovalerate tended to be greater in AC compared with FAC. Allison et al. (1958) observed that the addition of isoacids could increase the number of fibre-degrading bacteria in the rumen, thereby improving the feed cell wall degradation rate by rumen microorganisms.

Bacterial communities

The clustering results showed that 23 phyla, 46 classes, 111 orders, 213 families, 408 genera, and 557 species of bacteria were identified in the rumen samples. At the family level, bacteria from the *Prevotellaceae*, *Succinivibrionaceae*, *Lachnospiraceae*, and *Acidaminococcaceae* families seem to dominate the core bacterial microbiome in the rumen, regardless of diet. The FAC treatment increased the relative abundance of *Prevotellaceae* compared with CC. Similarly, another study also showed that feeding fermented feed seems to favour the growth of *Prevotellaceae* in the rumen (Jiang et al., 2021a). *Prevotellaceae* has the potential ability to metabolise CP, fibre, starch, and other fermentation substrates in diets (Dao et al., 2021). Feeding FAC tended to increase the relative abundance of *Lachnospiraceae* and *Ruminococcaceae* compared with CC, which may be conducive to the production of volatile fatty acids in the rumen (Rode et al., 1981; Blasco et al., 2020). The competitive relationship among bacterial community structures could be the immediate cause for a low relative abundance of *Succinivibrionaceae* in the FAC treatment. We observed that the most abundant bacterial genera in all ruminal samples were *Prevotella* at the genera level, which is supported by other studies (Callaway et al., 2010; Baldwin et al., 2012). Our study showed an increase in the relative abundance of *Prevotella* in groups fed the FAC. Another study suggested that *Prevotella* could promote the expression of high levels of succinate-CoA synthetase and thus indirectly support propionate production in the rumen (Wang et al., 2020). Wirth et al. (2018) also mentioned that *Prevotella* plays a major role in carbohydrate metabolism as the main functional group in planktonic microbiota. The AC treatment increased the relative abundance of *Succinivibrionaceae_UCG_002*, which produces succinate, a precursor of propionate (Pope et al., 2011); however, there was no increase in the rumen propionate production in this dietary group. The possible reason was that the relative abundance of *Succinivibrionaceae* was close to that of CC treatment. Short-term changes in diet may not allow adequate adaptation of rumen bacteria, and further studies are needed to verify how long-term consumption of aged and fermented corn regulates rumen bacterial communities.

Bacterial diversity

The coverage values in ruminal fluids samples reached more than 0.98, indicating that the ruminal microbiota coverage was high, and the sequencing results reflected the true situation of the bacteria in the rumen. In the case of similar Ace index and Chao index, which represent richness, the Simpson alpha index results suggest that feeding FAC increased the cows' ruminal bacterial diversity. Similarly, Jiang et al. (2021a) observed that feeding fermented corn gluten-wheat bran mixture increased ruminal bacterial diversity. A greater diversity of bacteria is generally perceived as beneficial to host health (Lozupone et al., 2012). Russell and Rychlik (2001) also reported that a high ruminal bacterial diversity enhanced the stability of the microbiota and allowed it to use limited resources more efficiently. Moreover, feeding AC had no modification effects on the Ace, Chao, Shannon, and Simpson index, which seems to suggest that AC does not have anti-microbial activity for the rumen bacteria.

Lactation performance

The AC treatment did not change milk yield and composition, but this may be due to the short period length (21 days) used in this experiment. For example, Belanche et al. (2020) observed that responses to supplementation with an essential oil blend emerged only after 4 weeks. The long-term effect of AC treatment on lacta-

tional performance needs to be further verified. The increased milk yield and lactose production in cows fed FAC are consistent with enhanced digestibility, likely providing more propionate for gluconeogenesis. The FAC treatment decreased the milk SCS and played a positive role in preserving the mammary gland health of cows, which could be related to the *Lactobacillus rhamnosus* in FAC. A study suggested that *Lactobacillus rhamnosus* were involved in the negative feedback regulation of some signalling pathways associated with inflammatory and immune responses, such as MAPK, TNF, and Jak-STAT, thereby having anti-inflammatory and immunomodulatory effects (Hou et al., 2019).

Plasma metabolites

Feeding AC to cows decreased the catalase activity in the plasma, increased the malondialdehyde concentration, and increased the risk of oxidative stress in Holstein cows. Similar results were obtained for feeding aging corn in weaned piglets (Luo et al., 2019). The antioxidant capacity in animals and their diets are closely interlinked, such that the antioxidant capacity in the animal is enhanced when the diets have high antioxidant activity (Khosravi et al., 2018). The FAC treatment had higher antioxidant activity than AC (Table 2), explaining why feeding FAC decreased the plasma malondialdehyde concentration. Moreover, *Lactobacillus rhamnosus* is known to have high antioxidant activity and could reduce related gene expressions of oxidative stress like TNF, EGR2, FBN2, and TNFAIP3 (Hou et al., 2019), which could also be another potential reason for FAC's strong antioxidant capacity. Taken together, fermentation of aging corn can prevent the oxidation of that corn and relieve oxidative stress caused by its consumption. In this study, feeding FAC diets tended to increase the glucose concentration in plasma compared with AC, which could have contributed to the maintenance of the conventional physiological functions of cows and increased milk yield. Feeding FAC increased immunoglobulin G concentration in plasma, which may suggest a higher humoral immune status of the cows. The same conclusions were obtained in a study where fermented feed was fed to laying hen chicks (Zhu et al., 2020), and this could be linked to the production of small peptides during the fermentation process. Short-term feeding may not be sufficient to observe the long-term effect on health, which needs to be studied in more detail.

Conclusion

The AC treatment decreased the plasma antioxidant capacity in dairy cows, but short-term feeding did not affect lactation performance by reducing milk yield and milk quality. On the other hand, the FAC treatment resulted in higher starch digestibility, the total VFA concentration, and changed the bacterial community structure in the rumen. More importantly, feeding FAC increased milk yield, plasma antioxidant and immune capacity while reducing the SCS. In conclusion, our study provides evidence that fermentation of aging corn grain by *L. rhamnosus* increases its nutritional value. The benefits of such fermentation on un-aged corn remain to be explored.

Ethics approval

All procedures of animal handling were performed according to NEAU-[2011]-9 approved by the Animal Care Advisory Committee, Northeast Agricultural University (Harbin, China).

Data and model availability statement

None of the data were deposited in an official repository. The data is available from the authors upon reasonable request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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