Journal of Integrative Agriculture 2015, 14(3): 503–513

Available online at www.sciencedirect.com

ScienceDirect

RESEARCH ARTICLE

The effects of fermentation and adsorption using lactic acid bacteria culture broth on the feed quality of rice straw

LIU Jing-jing[1], LIU Xiao-ping[2], REN Ji-wei[1], ZHAO Hong-yan[3], YUAN Xu-feng[1], WANG Xiao-fen[1], Abdelfattah Z M Salem[4], CUI Zong-jun[1]

[1] *College of Agronomy and Biotechnology/Center of Biomass Engineering, China Agricultural University, Beijing 100193, P.R.China*
[2] *Plants Protecting Station of Agricultural Bureau, Jinxiang 272200, P.R.China*
[3] *Agronomy of Yanbian University, Yanji 133002, P.R.China*
[4] *Faculty of Veterinary Medicine and Animal Science, Autonomous University of the State of Mexico, Toluca P.O. 50000, Mexico*

Abstract

To improve the nutritional value and the palatability of air-dried rice straw, culture broth of the lactic acid bacteria community SFC-2 was used to examine the effects of two different treatments, fermentation and adsorption. Air-dried and chopped rice straw was treated with either fermentation for 30 d after adding 1.5 L nutrient solution (50 mL inocula L^{-1}, 1.2×10^{12} CFU mL^{-1} inocula) kg^{-1} straw dry matter, or spraying a large amount of culture broth (1.5 L kg^{-1} straw dry matter, 1.5×10^{11} CFU mL^{-1} culture broth) on the straw and allowing it to adsorb for 30 min. The feed quality and aerobic stability of the resulting forage were examined. Both treatments improved the feed quality of rice straw, and adsorption was better than fermentation for preserving nutrients and improving digestibility, as evidenced by higher dry matter (DM) and crude protein (CP) concentrations, lower neutral detergent fiber (NDF), acid detergent fiber (ADF) and NH_3-N concentrations, as well as higher lactic acid production and *in vitro* digestibility of DM (IVDMD). The aerobic stability of the adsorbed straw and the fermented straw was 392 and 480 h, respectively. After being exposed to air, chemical components and microbial community of the fermented straw were more stable than the adsorbed straw.

Keywords: adsorption, fermentation, lactic acid bacteria culture broth, rice straw

1. Introduction

Only a small proportion of rice straw is utilized as roughage because of its low crude protein (CP) content, high crude fiber content, low dry-matter digestibility and poor palatability (Khattab *et al*. 2013; Kholif *et al*. 2014). The rice straw output of China amounted to 184 430 400 tons in 2009 (Bi *et al.* 2011). Almost all collectable straw in China is air-dried. Ensilage has been recommended as a way to improve the feed quality of straw (Gado *et al*. 2013). Due to the low water-soluble carbohydrate (WSC) content and small quantity of lactic acid bacteria attached to it, it is difficult to obtain a high-quality fermented product without any additives (Yang *et al.* 2006). Several studies have been conducted to ensile straw with molasses or fresh cheese whey (Lima *et al.* 2010; Cajarville *et al.* 2012) and lactic

Received 19 September, 2013 Accepted 29 April, 2014
LIU Jing-jing, Tel: +86-10-62733437, Fax: +86-10-62731857, E-mail: 151702696@qq.com; Correspondence CUI Zong-jun, Tel: +86-10-62733437, Fax: +86-10-62731857, E-mail: acuizj@cau.edu.cn

doi: 10.1016/S2095-3119(14)60831-5

acid bacteria (Reich and Kung 2010; Tabacco *et al.* 2011; Heinritz *et al.* 2012), and thereby improve the quality of the silage for ruminant feeding. However, it is an indisputable fact that there is considerable loss of dry matter during ensilage. Moreover, the aerobic deterioration of silage as a result of aerobic microbial activity during feed-out is also a significant problem for farm profitability and feed quality throughout the world (Tabacco *et al.* 2011). Even though combining heterolactic acid bacteria with homolactic acid bacteria on corn silage improved its aerobic stability and led to smaller dry matter (DM) losses (Reich and Kung 2010), DM losses and aerobic deterioration are still two undesirable characteristics of ensilage.

Although silage technology is quite mature and has been widely used all over the world, the process consumes valuable space and time. After the harvest, farmers are too busy sowing to ensile. In addition, considering the scattered distribution characteristics of straw resources in China, finding an easy process that is appropriate for small-scale dairy farms would be a breakthrough for the large-scale use of straw as ruminant roughage. In recent years, interest in the effects of "direct-fed microbial" (DFM, primarily *Lactobacillus* species) on animal health and performance has increased (Abdel-Aziz *et al*. 2015; Puniya *et al*. 2015). *Lactobacillus* species, as a primarily bacterial DFM, have been shown to increase daily gain and feed efficiency in feed-lot cattle, enhance milk production in dairy cows and have the potential to decrease ruminal acidosis in feedlot cattle and dairy cows, and improve immune response in stressed calves (Krehbiel *et al.* 2003; Elghandour *et al*. 2015). However, few studies have compared the effects of feeding bacterial DFM directly with feeding lactic acid bacteria inoculated silage. In this study, to improve feed quality and avoid DM losses during rice straw fermentation, we proposed adsorption that is using lactic acid bacteria culture broth as DFM to mix with the straw, and tested the effects of such treatment on the feed quality of straw.

Indicators of aerobic deterioration that are observed commonly include mold development, spontaneous heating, DM loss, increased concentrations of fiber components and reduced nutrient digestibility (Bolsen 1995). In this study, the composition and *in vitro* digestibility of DM (IVDMD) changes as well as microbial dynamics that occurred during air exposure were all assayed. Generally, aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above room temperature (Ranjit *et al.* 2002). However, the nutrient damage related to the aerobic deterioration when most of the silage is fed remains unclear. Thus, the main objects of the present study were to (1) compare the effects of adsorption with those of traditional fermentation (ensilage) and (2) monitor the aerobic deterioration of the fermented straw and the adsorbed straw exposed to air.

2. Results and discussion

2.1. Feed quality and nutrient content

Rice straw after suitable treatment is a valuable raw material for livestock forage. The study presented here was designed to evaluate the efficacy of adsorption treatment using SFC-2 culture broth as a way to shorten fermentation time and lessen DM losses, as well as limit the negative effects of aerobic deterioration on nutritional value.

The lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid and acetic acid, under anaerobic conditions. As a result, the pH decreases and most forage are preserved from attack by spoilage microorganisms (McDonald 1981). In the SFC-2 culture broth, several metabolic products including ethanol, acetic acid, L-lactic acid, D-lactic acid and glycerol were detected (Gao *et al.* 2008). Results of the qualitative analysis of the main volatile products are shown in Fig. 1. There was no difference in the variety of the main volatile products between the two treatments, including ethanol, acetic acid, lactic acid and glycerol. Through quantitative analysis (Table 1), adsorption resulted in more lactic acid and less acetic acid compared with fermentation. Both the straw after adsorbing LAB and after fermenting had a pleasant acid-fragrant smell, indicating that the palatability was improved.

To compare the capacity of different treatments for nutrient preservation, the straw adsorbing nutrient solution was analyzed in this part. The treatments of adsorbing nutrition solution (NS), adsorbing LAB culture broth and fermenting will be referred to as adsorption-nutrition solution, adsorption-LAB and fermentation, respectively. Characteristics of the straw through different treatments are shown in Table 1. The CP content was low (18.9 g kg^{-1} DM). Adding nutrient solution to the straw improved its nutritional value without affecting its palatability. As shown in Table 1, compared with the adsorption-NS, both adsorption-LAB and fermentation increased CP but reduced DM and WSC contents. Furthermore, fermentation increased NH_3-N, neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents, in agreement with previous studies (Gao *et al.* 2008; Reich and Kung 2010). The NH_3-N content was one of the conventional indexs to evaluate the quality of silage. In the very early stages of ensiling, extensive protein breakdown takes place (Kemble 1956), and subsequent amino acid degradation occurs as a result of clostridial growth (Ohshima and McDonald 1978). Identical with the DM loss, this

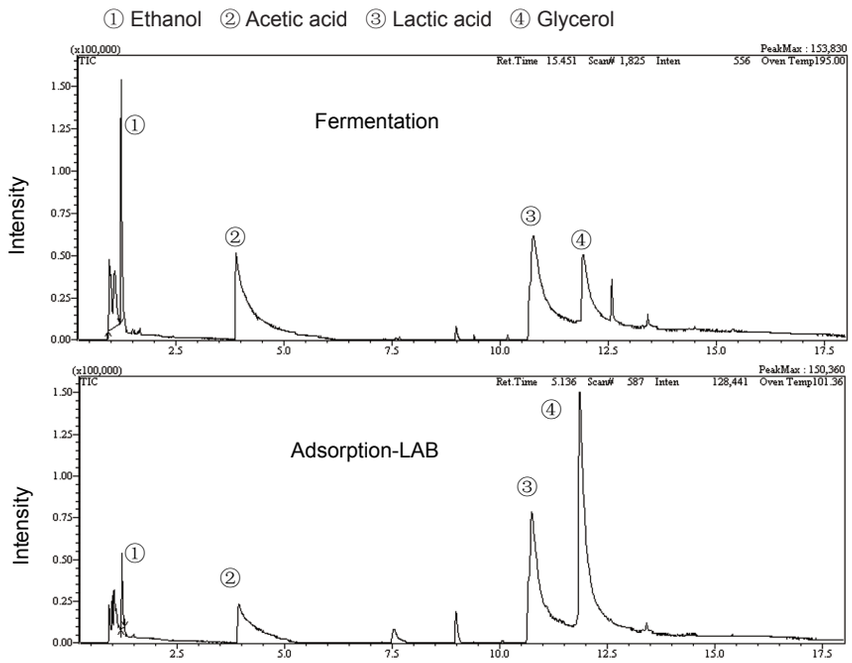

Fig. 1 GC-MS spectrometry maps of volatile products of the fermented straw and the adsorbed straw. Fermentation, fermenting straw after inoculating LB containing lactic acid bacteria (LAB); Adsorption-LAB, adsorbing lactic acid bacteria culture broth to the straw. The same as below.

Table 1 Effect of fermentation and adsorption on characteristics of the straw

Item[1)]	Adsorption-NS[2)]	Adsorption-LAB[3)]	Fermentation[4)]
pH	6.2±0.10 a	4.7±0.10 b	3.8±0.10 c
DM (g kg^{-1} WM)	414.78±3.01 a	402.47±0.82 b	352.31±2.45 c
WSC (g kg^{-1} DM)	68.83±1.54 a	59.59±1.28 b	24.61±2.15 c
CP (g kg^{-1} DM)	35.83±0.98 a	42.06±1.29 b	37.90±0.57 c
NDF (g kg^{-1} DM)	631.51±5.60 a	629.28±2.41 a	642.83±4.23 b
ADF (g kg^{-1} DM)	369.94±2.01 a	367.42±2.49 a	394.56±4.20 b
NH_3-N (g kg^{-1} DM)	2.65±0.77 a	2.71±0.83 a	9.43±1.12 b
Lactic acid (g kg^{-1} WM)	2.32±0.32 a	25.62±0.65 b	22.94±0.353 c
Acetic acid (g kg^{-1} WM)	0.40±0.07 a	3.55±0.14 b	5.49±0.09 c
IVDMD (g kg^{-1})	652.33±3.91 a	671.08±6.53 b	576.72±2.17 c

[1)] DM, dry matter; WM, wet matter; WSC, water soluble carbohydrates; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; IVDMD, *in vitro* DM digestibility.
[2)] Adsorption-NS, adsorbing nutrition solution to the straw.
[3)] Adsorption-LAB, adsorbing lactic acid bacteria culture broth to the straw.
[4)] Fermentation, fermenting straw after inoculating LB containing LAB.
Values are means±standard deviations. Different letters in the same row differ at P<0.05.

proteolysis is also a major disadvantage in preserving grass as silage. Fermentation increased NH_3-N and adsorption made no change, which indicated that adsorption caused no protein loss. The increase in NH_3-N reflects the extent of the proteolysis. In addition, ammonia is rapidly degraded in the rumen leading to poor N utilization (Brown and Valentine 1972). After fermentation, DM was reduced by 15.07%, and WSC decreased by 64.24%, though CP increased by 5.87%. In contrast, adsorption-LAB led to lower DM and WSC losses (2.97 and 13.37%, respectively) and greater CP increase (17.39%). The results indicate that the fermented straw had lower pH, but less DM than the adsorbed straw. Adsorption-LAB had no significant (P<0.05) effect on the NDF, ADF and NH_3-N contents of the forage; however, after fermentation, the concentrations of NDF, ADF and NH_3-N in straw all increased significantly (P<0.05).

In the animal feed industry, attention to the effects of DFM on animal health and performance has increased in recent years. Some studies have shown that DFM products increased cellulolytic bacterial numbers in the rumen and stimulated the production of some fermentation end products (Martin and Nisbet 1992), improved growth and

feed efficiency (Huck *et al.* 1999; Vasconcelos *et al.* 2008; Stephens *et al.* 2010), decreased ruminal acidosis in feedlot cattle and dairy cows (Nocek *et al.* 2002), and improved immune response in stressed calves (Novak *et al.* 2012). Others have shown little influence of DFM on these parameters. Yang *et al.* (2004) reported that addition of DFM had no effect on preventing ruminal acidosis and on fermentation or nutrient digestion. Cull *et al.* (2012) concluded that DFM, bovamine (labeled for 10^6 CFU head^{-1} d^{-1} of *Lactobacillus*), administered alone or in combination with the siderophore receptor and porin proteins-based (SRP) vaccine, does not significantly affect fecal shedding. Vasconcelos *et al.* (2008) reported that the benefits of feeding mixtures of these bacterial species to cattle were dose-dependent. The rational of these studies is that supplementing the rumen with lactic acid producing and/or utilizing bacteria enhances the ability of the rumen ecosystem to moderate excessive lactic acid production (Yang *et al.* 2004). Most bacterial DFM used in cattle for studies contain the lactate-producing *Lactobacillus* species *Lactobacillus acidophilus*. In this study, the lactic acid bacteria community SFC-2 mainly comprised *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus paracacei* and was proved to be an effective silage additive (Gao *et al.* 2007). However, no study has shown its value as DFM.

In contrast to feeding bacterial DFM directly, feeding *Lactobacillus*-inoculated alfalfa silage did not improve milk production and feed intake of dairy cows. Krehbiel *et al.* (2003) assumed that the inoculum may not have survived in the silage, thus few viable cells would have been consumed. In this study, the effects of fermentation and adsorption using lactic acid bacteria culture broth on the feed quality of rice straw were analyzed. The data indicated that adsorption preserved nutrients and improves digestibility better than fermentation. Frizzo *et al.* (2010) evaluated effects of a lactic acid bacterial inoculum composed of *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T. The results indicated that inoculated calves had better growth performance. In this study, IVDMD was used to evaluate the nutrient digestion of cows. The IVDMD of the untreated rice straw was 553.4 g kg^{-1}, this was improved by both treatments, and the effect of adsorption was more significant (P<0.05) than that of fermentation (Table 1). However, adsorption as a new dietary treatment, its effects on the growth performance of ruminant are not clear, and more researches are needed before it can be recommended to dairy or beef producers.

2.2. Aerobic stability and aerobic deterioration

Aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above room temperature (Ranjit *et al.* 2002). Spontaneous heating, DM loss, increased concentrations of fiber components, and reduced nutrient digestibility are observed commonly as indicators of aerobic deterioration (Bolsen 1995). In the present study, to elucidate the nutrient damage related to aerobic deterioration, temperature, pH, residual DM, WSC, CP and NH_3-N contents and IVDMD during the test were monitored.

pH was one of the main factors that affected the extent of fermentation and the silage quality of ensiled forage, as a low pH ensured that the forage was retained in a stable form (Wang *et al.* 2009). The changes of temperature and pH when the forage exposed to open air are shown in Figs. 2 and 3. As shown, the fermented straw had lower pH and higher aerobic stability.

The temperature of the adsorbed straw increased rapidly after 300 h, while the fermented straw remained at about 25°C during the test. The aerobic stability of fermentation and adsorption-LAB were longer than 480 and 392 h, re-

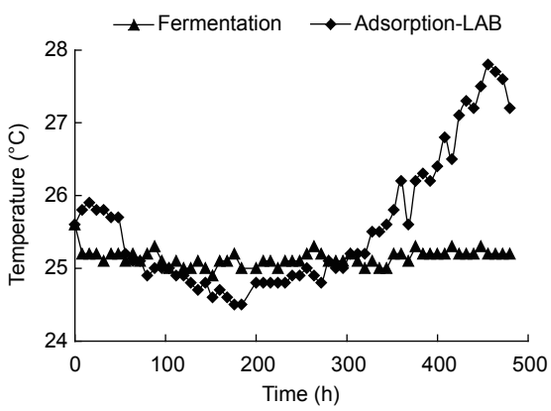

Fig. 2 Changes in temperature of the forages during the aerobic stability trial period.

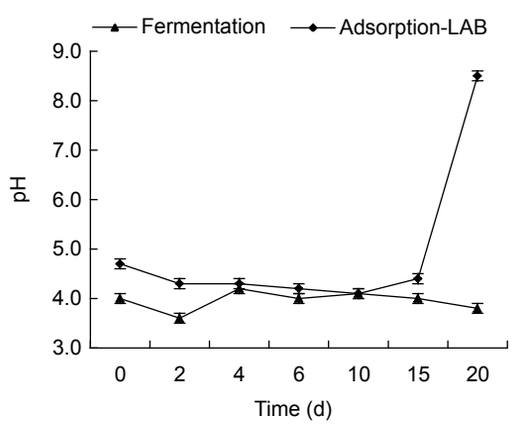

Fig. 3 Changes in pH of the forages during the aerobic stability trial period. Error bars are standard deviation. The same as below.

spectively. Similarly, the fermented straw maintained a pH below 4.2 during the test, while the pH of the adsorbed straw tended to fall (from 4.7 to 4.1) during the first 10 d, and then rose to 8.5 on day 20.

After silos were opened for feeding, air penetrates the silage and promotes the growth of aerobic microorganisms and the oxidation of fermentation products presents in the silage (Danner *et al.* 2003). DM and chemical compositions of the forages during the aerobic stability trial period are shown in Fig. 4. As shown in Fig. 4-A, in the fermented straw, the concentrations of acetic acid and lactic acid increased rapidly up to 7.01 and 102.04 g kg^{-1}, respectively, on the 20th d. In the adsorbed straw, the concentrations of acetic acid and lactic acid increased during the first 10 d and rose to 7.8 and 100.5 g kg^{-1}, respectively, on the 10th d, and then declined; there was no acetic acid detected and lactic acid decreased sharply to 8.0 g kg^{-1} on the 20th d. The WSC (Fig. 4-C) in the adsorbed straw declined to various degrees and was reduced by 58.85% in the first 15 d, which was the same as that of the fermented straw at silo opening, whereas in the fermented straw it was changeless and reduced by only 22.92% by day 20. The main carbon source used by lactic acid bacteria is monosaccharide such as glucose and some oligosaccharides. When the carbon source is limiting, the metabolic activity of the lactic acid bacteria will stop, and so will lactic acid production. Woolford (1990) discovered that yeast played a vital role in the aerobic deterioration of silage. Lactate assimilating yeasts metabolize the lactic acid into carbon dioxide and water, leading to the reduction of lactic acid content and increased pH. Consequently, acid non-tolerant spoilage organisms reproduced massively, which leads to increase in temperature, decrease in CP. The fermented straw had a higher NH_3-N content and a lower CP content compared with the adsorbed straw (Table 1), and thus more deamination occurred during fermentation. After exposure, both CP (Fig. 4-D) and NH_3-N (Fig. 4-E) in the fermented straw increased slightly, yet in the adsorbed straw, CP decreased sharply and NH_3-N increased rapidly. Even after day 10 and day 15, CP was lower and NH_3-N was higher than that in the fermented straw. As shown in Fig. 4-B, the DM content of the fermented straw started to drop on the 6th d of the exposure and continued through day 15, while IVDMD (Fig. 5) declined slightly; yet in the adsorbed straw, DM content decreased significantly to the same quantity as the fermented straw on day 20, IVDMD declined gradually and was lower than that of the fermented straw on the 20th d. These results suggested that adsorbed straw was very stable after exposure to air below 25°C for up to 10 d. This should allow for considerable flexibility with respect to feeding the treated straw within 10 d without significant aerobic deterioration.

Aerobic stability was determined only by the concentration of acetic acid, not the lactic acid content or the final pH (Danner *et al.* 2003). In this study, for the fermented straw, exposure time had no effect on the temperature, pH and NH_3-N content of the fermented straw, indicating that little deterioration occurred. The adsorbed straw had lower pH, lower acetic acid content and higher WSC and lactic acid content, so it deteriorated much more easily than the fermented straw. In the adsorbed straw, the acetic acid concentration was lower than that in the fermented straw, and the aerobic stability was 392 h, which was poorer than that of the fermented straw (480 h).

Silage that has spoiled because of exposure to air is undesirable due to inferior quality, lower digestibility and the risk of negative effects on animal performance (Woolford 1990; Kung *et al.* 1998). Several studies have been conducted to improve the aerobic stability of silages by inoculating them with *Lactobacillus buchneri* (Ranjit *et al.* 2002; Holzer *et al.* 2003; Schmidt *et al.* 2009; Reich and Kung 2010). Though the aerobic stability was improved, spoilage was not fundamentally prevented. One way to fully prevent aerobic spoilage is to have the treated forage eaten on the day of silo opening.

In terms of the nutritional value and digestibility, the adsorbed straw is superior to the fermented straw. Though the adsorbed straw became rancid more rapidly, dry straw can be preserved indefinitely, free from constraints of time and space, and lactic acid bacteria culture broth can be produced continuously, which enables the adsorbed straw to be prepared according to the daily requirements of a feedlot. In this case, aerobic spoilage can be avoided. Furthermore, as shown in Fig. 4, in adsorbed straw over 10 d of exposure to air, the pH tended to drop and the concentrations of lactic acid and acetic acid increased with exposure time, and the WSC and CP contents, as well as IVDMD of the adsorbed straw were all higher than those of the fermented straw. This indicated that even if some nutrients were lost, the feed quality of the adsorbed straw in 10 d of exposure was still superior. According to Graminha *et al.* (2008), *in vitro* data cannot always be directly extrapolated as *in vivo* digestibility, considering the peculiarities of ambient conditions in the rumen. Hence, further researches are needed to reveal the effects of different treatments on rice straw, for example, DM intake, ruminal parameters (fermentation, bacteria) and milk performance. In China, almost all collectable straw is air-dried, adsorption may be a feasible means of improving its feed quality.

2.3. Dynamic change of microorganisms

Different groups of microbes were known to act as spoilage organisms, especially after a silo was opened and the silage was exposed to air. During the aerobic stability trial period,

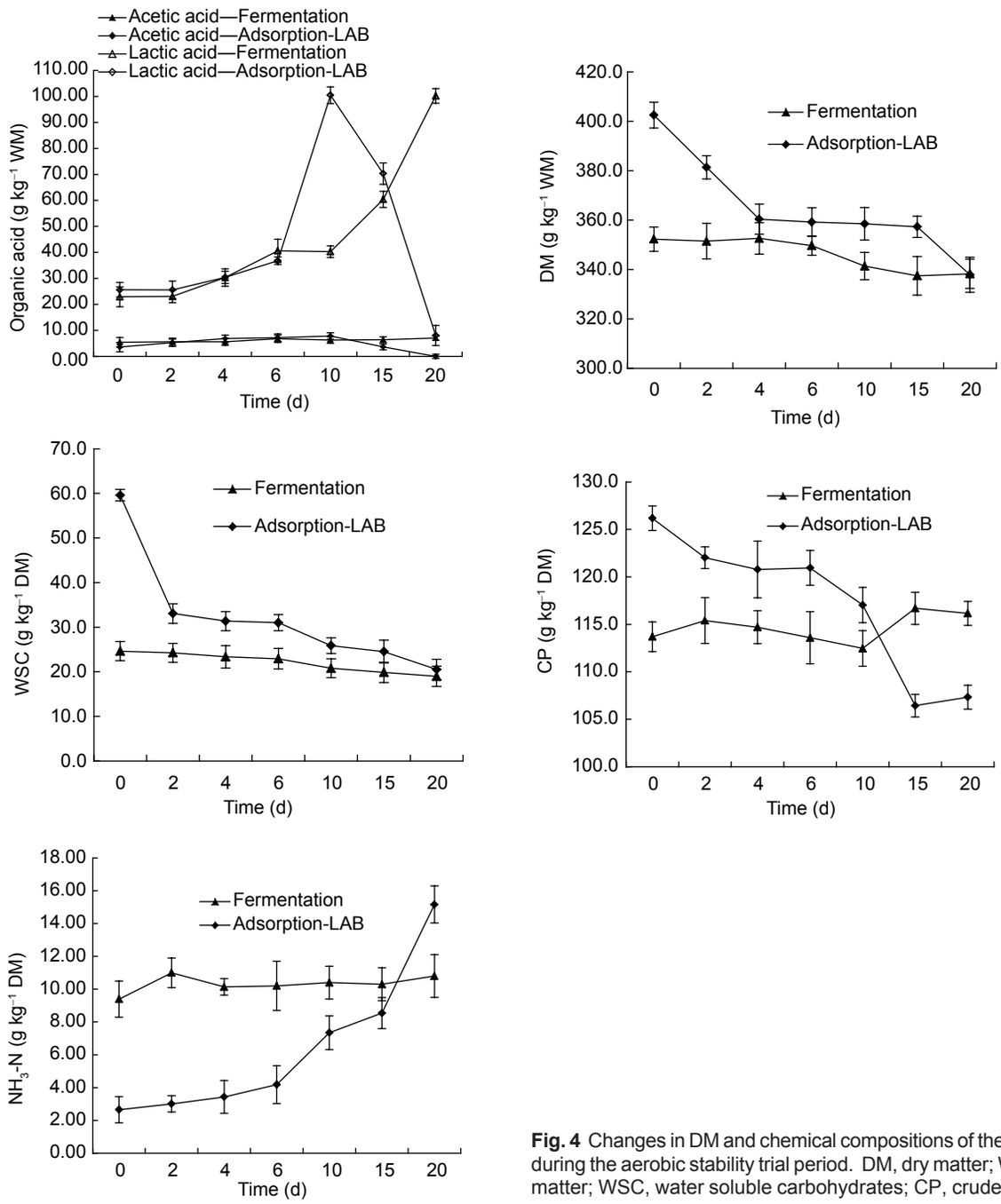

Fig. 4 Changes in DM and chemical compositions of the forages during the aerobic stability trial period. DM, dry matter; WM, wet matter; WSC, water soluble carbohydrates; CP, crude protein.

changes in the population and composition of bacteria and fungi were reflected by changes in the band pattern (Fig. 6). The pattern of the fungi showed that the fungal communities in both treatments were consistent, and did not change with the extension of aerobic exposure time. Sequencing of bands from the denaturing gradient gel electrophoresis (DGGE) profiles of fungi showed that the main closest species of these microorganisms were *Zygosaccharomycespseudorouxii* and *Zygosaccharomycesrouxii*, which played a vital role for the aerobic deterioration of the silage (Woolford 1990). The pattern of the bacteria showed that, 1) species in SFC-2 dominated the microbial community in both treatments; 2) in the adsorbed straw, more microbes (bands) emerged and the bands representing species in SFC-2 became blurred after 10 d, revealing the proliferation of miscellaneous bacteria; 3) the bacterial community in the fermented straw was stable during the trial period. According to previous studies (Gao *et al.* 2007; Ma *et al.* 2008), SFC-2 can inhibit other microorganisms, such as *Enterobactersakazakii*, *Pantoeaagglomerans*, *Enterobacterendosymbiont*, *Pantoeaananatis*, *Enterobacter cloacae*, *Bacillus cereus*, *Klebsiellaoxytoca*, *Citrobacterfreundii*,

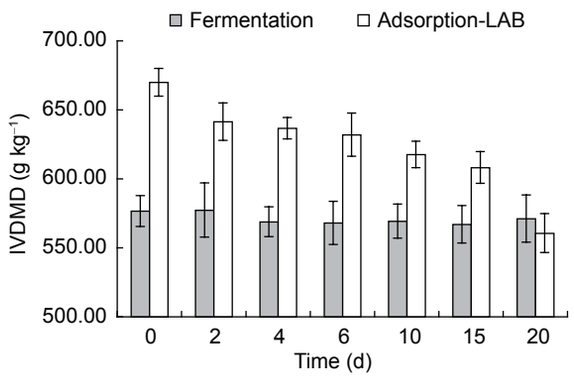

Fig. 5 Changes in *in vitro* dry matter digestion (IVDMD) of the forages during the aerobic stability trial period.

etc., some of which are common pathogens or opportunistic pathogens. This may explain why bacteria other than the SFC-2 community were not detected in the early days of the exposure.

3. Conclusion

The results suggested that adsorption was superior to fermentation for preserving nutrients and improving digestibility. Though the adsorbed straw had poor aerobic stability, it maintained a better quality than the fermented straw over 10 d of exposure to air. In addition, the adsorption treatment consumed fewer nutrients, took less time and space and was easily performed, making it a much easier way to use rice straw at a larger scale in feedlots. Overall, converting air-dried straw into animal fed by adsorbing SFC-2 culture broth is a promising and feasible method, though further researches are still needed.

4. Materials and methods

4.1. Bacteria strains and preparation of culture broth

The lactic acid bacteria community SFC-2 was used as inoculum. It was developed from natural fermentation products of rice straw by continuous enrichment with Reformed Man-Rosa-Sharpe sucrose (MRS-S) broth, and mainly comprises *L. fermentum*, *L. plantarum* and *L. paracacei*, which produce large amounts of lactic acid and acetic acid (Gao *et al.* 2008). The MRS-S medium is a modification of MRS (De Man *et al.* 1960) medium (sucrose instead

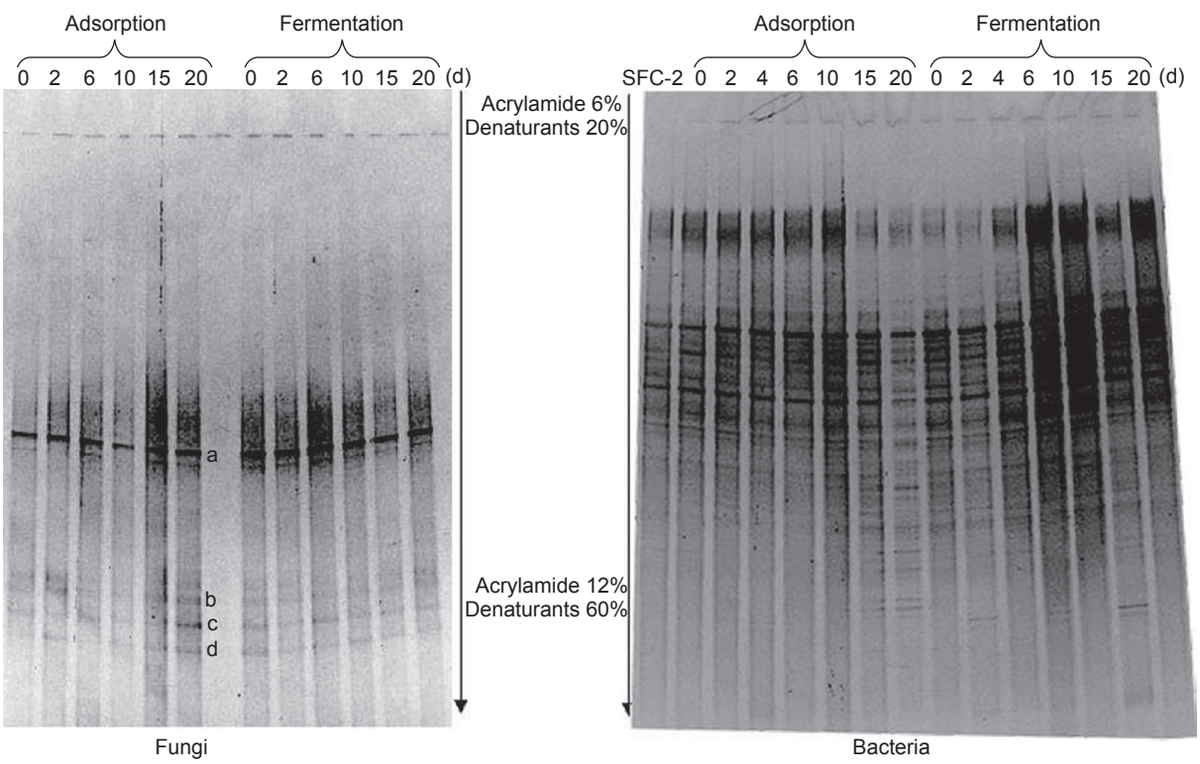

Fig. 6 PCR-denaturing gradient gel electrophoresis (DGGE) profiles of the forages during the aerobic stability trial period. The long arrows represent the direction of the denaturant (35–50%) and the polyacrylamide (6–12%) gradients. Bands: a, *Zygosaccharomycespseudorouxii* (99%, AM947682.1); b, *Zygosaccharomycesrouxii* (97%, KC778596.1); c, *Zygosaccharomycespseudorouxii* (97%, AM94768 2.1); d, *Zygosaccharomycesrouxii* (98%, KC146373.1).

of glucose), which was used for preparation of the seed. Nutrient solution containing sucrose (2%), pepton (1%) and NaCl (0.5%) was used for preparation of the culture broth. After inoculation (seed volume of 50 mL L^{-1}), the medium or nutrient solution was cultured under static conditions at 30°C for 24 h. Microbial inoculants were coated on MRS-S agar plates before hand to confirm their viability, and appropriate amounts of the inoculants were used to achieve the desired application rate. The count of the seed and the culture broth were 1.2×10^{12} and 1.5×10^{11} CFU mL^{-1}, respectively.

4.2. Rice straw and treatments

The air-dried rice straw (90.46% of DM) was collected from the farm of China Agricultural University (Beijing, China), and chopped to a length of approximately 1–2 cm.

Liu *et al.* (2009) reported that the fermentation quality of silage of moderate moisture content (60.24%) was better than that of silage of high (73.68%) or low moisture content (28.88%). Hence, for fermentation, 300-mL nutrient solution containing 15 mL seed culture was sprayed uniformly onto 200 g (at DM) rice straw, to provide lactic acid bacteria, adequate nutrition for the fermentation and a final moisture content of 60%. The wet rice straw was then packed into 500-mL glass bottles, which were compacted and sealed after properly filled, in septuplicate. The bottles were stored in a room at ambient temperature (25–30°C) for 30 d. After the fermentation, bottles were opened and their contents were thoroughly mixed. Samples were randomly obtained for determining the composition of the microbial populations (bacteria and fungi) by use of the polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) and fermentation end-products, and the rest were divided into seven parts. Each part was transferred into a 5-L polystyrene box. For adsorption, 2 100 mL SFC-2 culture broth was sprayed uniformly onto 1 400 g (at DM) rice straw to achieve a final moisture content of 60%. After sampling, approximately 50 g of forage was packed into a nylon-polyethylene bag (1.0 mm aperture, 11.0 cm×14.0 cm), for a total of 18 bags, the rest of each sample was divided into seven parts, and each part together with 3 bags was transferred into a 5-L polystyrene box.

For aerobic stability testing, each box was covered with a polystyrene lid to avoid contamination and drying of the resulting forage, but allow air to infiltrate the forage mass. The boxes were placed at 25°C for 20 d. One box from each treatment was taken for temperature measurement. The rest were designated for sampling after 2, 4, 6, 10, 15 and 20 d of exposure. On each sampling date, one box of each treatment was opened, the contents were thoroughly mixed, and samples were randomly obtained.

4.3. Temperature measurement

Aerobic stability was determined by monitoring temperature increases due to microbial activity in the samples exposed to air, and was measured by placing thermocouple wires at the center of a box. The thermocouple wires were connected to a data logger (YOKOGAWA DX1006, Japan) that recorded the temperature every 30 min for 20 d. Aerobic stability was defined as the time (h) interval before silage temperatures increased by 2°C above ambient temperature (Ranjit *et al.* 2002).

4.4. Chemical analyses

Samples were all split into two respective subsamples. One subsample was dried in a forced-air oven at 105°C for 24 h to determine the DM content, and then ground to pass through a 1-mm screen for chemical analysis and *in vitro* digestibility measurements. The dried samples were analyzed for CP, WSC, NDF, ADF content and IVDMD. NDF and ADF were analyzed according to methods described by van Soest *et al.* (1991). The WSC content was determined by colorimetry after reaction with an anthrone reagent (Thomas 1977). Crude protein was calculated as N×6.25, N was measured using Kjeldahl apparatus (KDY-9830, China) by the Association of Official Agricultural Chemists (AOAC) procedures (Helrich 1990). IVDMD was measured using the method of Tilley and Terry (1963). This is a two-stage *in vitro* technique in which dried silages are milled to pass through a 1-mm screen, then digested by rumen microbes for 48 h, followed by hydrolysis with pepsin-HCl (200 mg of pepsin in 2 L of 0.004 mol L^{-1} HCl, pH 2.4) solution for another 48 h. The rumen liquor was sampled from two rumen ally cannulated cows that were fed a maintenance energy diet of grass silage without concentrates.

The second subsample was stored as a wet sample. A 10 g sample of each fresh forage was homogenized with 90 mL of sterile 25% strength Ringer's solution (Oxoid BR52) on a shaking table (150 r min^{-1}) for 10 min. The macerated samples were filtered through two layers of cheesecloth. Next, 10 mL of filtrate was acidified with HCl and stored at –20°C for NH_3-N analysis. The NH_3-N content was analyzed by the Kjeldahl method (Broderick and Kang 1980). The pH of the filtrate was measured with a compact pH meter (modelB-212, Horiba, Japan), and 1.0 mL of filtrate was centrifuged (4°C, 12 000 r min^{-1}) and filtered (0.22 μm). A 1-μL aliquot of this water extract was analyzed using a gas chromatographic mass spectrometer (model GC-MS-QP2010, Shimadzu, Japan) on line with a CP-Chirasil-Dex CB capillary column (25 m×0.25 mm). The analytical conditions followed Guo *et al.* (2008). The data

were quantitatively analyzed using the National Institute of Standards and Technology (NIST) database. The rest of the filtrate was subpackaged into two sets of 10-mL sterile tubes and was frozen (–80°C) immediately for later extraction of microbial DNA.

4.5. DNA extraction and PCR-DGGE

A 9.0-mL aliquot of water extract was centrifuged at 8 000 r min^{-1} for 10 min, and the supernatant was carefully removed in order to obtain the sediment. Extraction buffer (100 mmol L^{-1} Tris-HCl, 40 mmol L^{-1} EDTA) was used to suspend the sediment. Extraction of total genomic DNA was carried out using the benzyl chloride method (Zhu *et al.* 1993). PCR was performed by using the Gene Amp PCR System (Model 9700, Applied Bio-systems, USA). Bacterial universal primers (Muyzer *et al.* 1993) and fungal universal primers (Florez and Mayo 2006) are shown in Table 2 and were used to amplify the V3 region of the 16S rRNA gene and the D1 region of the 26S rRNA gene, respectively, for DGGE analysis. For the PCR program of V3 region of the 16S rRNA gene, an initial DNA denaturation was performed at 95°C for 10 min, followed by 29 cycles of denaturation at 93°C for 1 min, annealing at 48°C for 1 min, and elongation at 72°C for 1 min and 10 s, with a final elongation step at 72°C for 5 min. For the PCR program of 26S rRNA gene, an initial DNA denaturation was performed at 95°C for 10 min, followed by 29 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 45 s, and elongation at 72°C for 1 min, with a final elongation step at 72°C for 7 min. Each 50-μL PCR contained 1 μL (30 ng) of template DNA, 5 μL of 10× buffer, 4 μL of each dNTP (2.5 mmol L^{-1}), 3 μL $MgCl_2$ (25 mmol L^{-1}), 1 μL of each primer (45 μmol L^{-1}) and 0.25 μL TaKaRa *Taq* (5 U μL^{-1}; TaKaRa, Japan).

PCR products were loaded onto a 6–12% (w/v) polyacrylamide gradient gel in 0.5× TAE electrophoresis buffer (20 mmol L^{-1} Tris-HCl, pH 8.3; 10 mmol L^{-1} acetic acid; 0.5 mmol L^{-1} EDTA), with a 20–60% denaturant gradient (where 100% is defined as 7 mol L^{-1} urea with 40% formamide). Gels were run at a constant voltage of 200 V and temperature of 61°C for 5 h in 0.5× TAE electrophoresis buffer, then stained with SYBR Green I (Molecular Probes, Eugene, OR, USA.) and photographed under UV (302 nm) using an Alpha Imager 2200 Imaging System (Alpha Innotech, USA). The bands were retrieved from the DGGE gel and sequenced as described previously (Gao *et al.* 2008). Sequence similarity searches were performed in the GenBank data library using the BLAST program (http://www.ncbi.nlm.nih. gov/BLAST/).

4.6. Statistical analysis

The raw data were subjected to ANOVA by the general linear model procedure of SPSS software (SPSS STATISTICS V17.0, SPSS, USA). S-N-K test was used to differentiate between means and significance was declared at $P<0.05$.

Table 2 Sequences of primers for PCR-DGGE

Target species	Sequences (5′→3′)	Product size (bp)
Bacteria	Forward: CCTACGGGAGGCAGCAG	160
	Reverse: ATTACCGCGGCTGCTGG	
Fungi	Forward: GCATATCAATAAGCGGAGGAAAAG	200
	Reverse: ATTCCCAAACAACTCGACTC	

Acknowledgements

This work was supported by the National Key Technology Research and Development Program of China during the 12th Five-Year Plan period (2012BAD14B01) and the Special Fund for Agro-Scientific Research in the Public Interest, China (201303080).

References

Abdel-Aziz N A, El-Adawy M, Mariezcurrena-Berasain M A, Salem A Z M, Olivares-Pérez J, Kholif A E, Borhami B E. 2015. Effects of exogenous enzymes, *Lactobacillus acidophilus* or their combination on feed performance response and carcass characteristics of rabbits fed sugarcane bagasse. *Journal of Integrative Agriculture*, **14**, 544–549.

Bi Y Y, Wang H Y, Wang D L, Gao C Y, Wang Y J. 2011. Estimation and utilization of rice straw resources in China. *Chinese Agricultural Science Bulletin*, **15**, 137–143. (in Chinese)

Bolsen K K. 1995. Silage: Basic principles. *Forages*, **5**, 163–176.

Broderick G A, Kang J H. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *Journal of Dairy Science*, **63**, 64–75.

Brown D C, Valentine S C. 1972. Formaldehyde as a silage additive. I. The chemical composition and nutritive value of frozen lucerne, lucerne silage, and formaldehyde-treated lucerne silage. *Crop and Pasture Science*, **23**, 1093–1100.

Cajarville C, Britos A, Garciarena D, Repetto J L. 2012. Temperate forages ensiled with molasses or fresh cheese

whey: Effects on conservation quality, effluent losses and ruminal degradation. *Animal Feed Science and Technology*, **171**, 14–19.

Cull C A, Paddock Z D, Nagaraja T G, Bello N M, Babcock A H, Renter D G. 2012. Efficacy of a vaccine and a direct-fed microbial against fecal shedding of *Escherichia coli* O157: H7 in a randomized pen-level field trial of commercial feedlot cattle. *Vaccine*, **30**, 6210–6215.

Danner H, Holzer M, Mayrhuber E, Braun R. 2003. Acetic acid increases stability of silage under aerobic conditions. *Applied and Environmental Microbiology*, **69**, 562–567.

Elghandour M M Y, Salem A Z M, Martínez Castañeda J S, Camacho L M, Kholif A E, Vázquez Chagoyán J C. 2015. Direct-fed microbes: A tool for improving the utilization of low quality roughages in ruminants. *Journal of Integrative Agriculture*, **14**, 526–533.

Florez A B, Mayo B. 2006. Microbial diversity and succession during the manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by PCR-DGGE. *International Journal of Food Microbiology*, **110**, 165–171.

Frizzo L S, Soto L P, Zbrun M V, Bertozzi E, Sequeira G, Armesto R R, Rosmini M R. 2010. Lactic acid bacteria to improve growth performance in young calves fed milk replacer and spray-dried whey powder. *Animal Feed Science and Technology*, **157**, 159–167.

Gado H M, Salem A Z M, Camacho L M, Elghandour M M Y, Salazar M C. 2013. Influence of exogenous enzyme on *in vitro* ruminal degradation of ensiled rice straw with DDGS. *Animal Nutrition and Feed Technology*, **13**, 569–574.

Gao L, Yang H, Wang X, Huang Z, Ishii M, Igarashi Y, Cui Z. 2008. Rice straw fermentation using lactic acid bacteria. *Bioresource Technology*, **99**, 2742–2748.

Gao L J, Wang X F, Yang H Y, Gao X Z, L Y C, Cui Z J. 2007. Effects of a lactic acid bacteria community SFC-2 treated on rice straw. *Environmental Science*, **28**, 1392–1396.

Graminha E, Goncalves A, Pirota R, Balsalobre M, da Silva R, Gomes E. 2008. Enzyme production by solid-state fermentation: Application to animal nutrition. *Animal Feed Science and Technology*, **144**, 1–22.

Guo P, Wang X, Zhu W, Yang H, Cheng X, Cui Z. 2008. Degradation of corn stalk by the composite microbial system of MC1. *Journal of Environmental Sciences*, **20**, 109–114.

Heinritz S N, Martens S D, Avila P, Hoedtke S. 2012. The effect of inoculant and sucrose addition on the silage quality of tropical forage legumes with varying ensilability. *Animal Feed Science and Technology*, **174**, 201–210.

Helrich K C. 1990. *Official Methods of Analysis of the AOAC*. vol. 2. Association of Official Analytical Chemists, USA.

Holzer M, Mayrhuber E, Danner H, Braun R. 2003. The role of *Lactobacillus buchneri* in forage preservation. *Trends in Biotechnology*, **21**, 282–287.

Huck G L, Kreikemeier K K, Ducharme G A. 1999. Effect of feeding *Lactobacillus acidophilus* BG2FO4 (MicroCell) and *Propionibacterium freudenreichii* P-63 (MicroCell PB) on growth performance of finishing heifers. *Journal of Animal Science*, **77**, 264.

Kemble A R. 1956. Studies on the nitrogen metabolism of the ensilage process. *Journal of the Science of Food and Agriculture*, **7**, 125–130.

Khattab H M, Gado H M, Salem A Z M, Camacho L M, El-Sayed M M, Kholif A M, El-Shewy A A, Kholif A E. 2013. Chemical composition and *in vitro* digestibility of *Pleurotus ostreatus* spent rice straw. *Animal Nutrition and Feed Technology*, **13**, 173–182.

Kholif A E, Khattab H M, El-Shewy A A, Salem A Z M, Kholif A M, El-Sayed M M, Gado H M, Mariezcurrena M D. 2014. Nutrient digestibility, ruminal fermentation activities, serum parameters and milk production and composition of lactating goats fed diets containing rice straw treated with *Pleurotus ostreatus*. *Asian-Australasian Journal of Animal Sciences*, **27**, 357–364.

Krehbiel C R, Rust S R, Zhang G, Gilliland S E. 2003. Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. *Journal of Animal Science*, **81**, E120–E132.

Kung Jr L, Sheperd A C, Smagala A M, Endres K M, Bessett C A, Ranjit N K, Glancey J L. 1998. The effect of preservatives based on propionic acid on the fermentation and aerobic stability of corn silage and a total mixed ration. *Journal of Dairy Science*, **81**, 1322–1330.

Lima R, Lourenco M, Diaz R F, Castro A, Fievez V. 2010. Effect of combined ensiling of sorghum and soybean with or without molasses and lactobacilli on silage quality and *in vitro* rumen fermentation. *Animal Feed Science and Technology*, **155**, 122–131.

Liu Q, Zhang J, Lu X. 2009. The effects of lactic acid bacteria inoculation on the fermentation quality and aerobic stability of king grass silage. *Acta Prataculturae Sinica*, **18**, 131–137. (in Chinese)

Ma J, Wang X, Gao L, Cui Z. 2008. Antibacterial activity of lactic acid bacteria community SFC-2 used for fermentation of air-dried crop straws. *Acta Microbiologica Sinica*, **48**, 1392–1396. (in Chinese)

de Man J C, Rogosa M, Sharpe M E. 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Microbiology*, **23**, 130–135.

Martin S A, Nisbet D J. 1992. Effect of direct-fed microbials on rumen microbial fermentation. *Journal of Dairy Science*, **75**, 1736–1744.

Mcdonald P. 1981. *The Biochemistry of Silage*. John Wiley & Sons, Chichester UK.

Muyzer G, de Waal E C, Uitterlinden A G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695–700.

Nocek J E, Kautz W P, Leedle J, Allman J G. 2002. Ruminal supplementation of direct-fed microbials on diurnal pH variation and *in situ* digestion in dairy cattle. *Journal of Dairy Science*, **85**, 429–433.

Novak K N, Davis E, Wehnes C A, Shields D R, Coalson J A,

Smith A H, Rehberger T G. 2012. Effect of supplementation with an electrolyte containing a *Bacillus*-based direct-fed microbial on immune development in dairy calves. *Research in Veterinary Science*, **92**, 427–434.

Ohshima M, Mcdonald P. 1978. A review of the changes in nitrogenous compounds of herbage during ensilage. *Journal of the Science of Food and Agriculture*, **29**, 497–505.

Puniya A K, Salem A Z M, Kumar S, Dagar S S, Griffith G W, Puniya M, Ravella S R, Kumar N, Dhewa T, Kumar R. 2015. Role of live microbial feed supplements with reference to anaerobic fungi in ruminant productivity: A review. *Journal of Integrative Agriculture*, **14**, 550–560.

Ranjit N K, Taylor C C, Kung Jr L. 2002. Effect of *Lactobacillus buchneri* 40788 on the fermentation, aerobic stability and nutritive value of maize silage. *Grass and Forage Science*, **57**, 73–81.

Reich L J, Kung L. 2010. Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Animal Feed Science and Technology*, **159**, 105–109.

Schmidt R J, Hu W, Mills J A, Kung L. 2009. The development of lactic acid bacteria and *Lactobacillus buchneri* and their effects on the fermentation of alfalfa silage. *Journal of Dairy Science*, **92**, 5005–5010.

Van Soest P V, Robertson J B, Lewis B A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, **74**, 3583–3597.

Stephens T P, Stanford K, Rode L M, Booker C W, Vogstad A R, Schunicht O C, Jim G K, Wildman B K, Perrett T, Mcallister T A. 2010. Effect of a direct-fed microbial on animal performance, carcass characteristics and the shedding of *Escherichia coli* O157 by feedlot cattle. *Animal Feed Science and Technology*, **158**, 65–72.

Tabacco E, Righi F, Quarantelli A, Borreani G. 2011. Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. *Journal of Dairy Science*, **94**, 1409–1419.

Thomas T A. 1977. An automated procedure for the determination of soluble carbohydrates in herbage. *Journal of the Science of Food and Agriculture*, **28**, 639–642.

Tilley J, Terry R A. 1963. A two-stage technique for the *in vitro* digestion of forage crops. *Grass and Forage Science*, **18**, 104–111.

Vasconcelos J T, Elam N A, Brashears M M, Galyean M L. 2008. Effects of increasing dose of live cultures of *Lactobacillus acidophilus* (strain NP 51) combined with a single dose of *Propionibacterium freudenreichii* (strain NP 24) on performance and carcass characteristics of finishing beef steers. *Journal of Animal Science*, **86**, 756–762.

Wang J, Wang J Q, Zhou H, Feng T. 2009. Effects of addition of previously fermented juice prepared from alfalfa on fermentation quality and protein degradation of alfalfa silage. *Animal Feed Science and Technology*, **151**, 280–290.

Woolford M K. 1990. The detrimental effects of air on silage. *Journal of Applied Microbiology*, **68**, 101–116.

Yang H Y, Wang X F, Liu J B, Gao L J, Ishii M, Igarashi Y, Cui Z J. 2006. Effects of water-soluble carbohydrate content on silage fermentation of wheat straw. *Journal of Bioscience and Bioengineering*, **101**, 232–237.

Yang W Z, Beauchemin K A, Vedres D D, Ghorbani G R, Colombatto D, Morgavi D P. 2004. Effects of direct-fed microbial supplementation on ruminal acidosis, digestibility, and bacterial protein synthesis in continuous culture. *Animal Feed Science and Technology*, **114**, 179–193.

Zhu H, Qu F, Zhu L. 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Research*, **21**, 5279.

(Managing editor ZHANG Juan)