Clarifying Key Cellulase Component Cooperated With Lactic Acid Bacteria for Alfalfa Lignocellulose Degradation to Improve Lactic Acid Production

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Abstract

Clarifying key cellulase component that played synergistic roles with lactic acid bacteria (LAB) in fermenting alfalfa lignocellulose into lactic acid (LA) is valuable in low-temperature seasons. Last cut and low dry matter (DM) alfalfa was ensiled by 9 treatments, combinations of cellulase component genes engineered *Lactoc. lactis* subsp. *lactis* MG1363 strains (HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, separately containing *bgl1*, *cbh2*, and *eg3* gene were mixed at 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1), cellulase (EN), and a combination of *Lactobacillus plantarum* and cellulase (LPEN), and without treatments, as the control, with 4 replicates each. After anaerobic preservation in a silo from late fall through winter (3-20°C) for 140 d, the ensiled alfalfa was sampled and analysed. EN degraded lignocellulose best but the pH was the key limiting factor for lignocellulose saccharification of commercial EN in the simultaneous saccharification and fermentation of LPEN. The optimal combination HT4 caused the fewest disaccharide (1.02 g/kg DM) and the highest conversion of water-soluble carbohydrates (WSC) to LA (170%) and increased LA content to 80.0 g/kg DM maximally since cellobiohydrolase better cooperated with *Lactoc. lactis* host to ferment lignocellulose into LA than endoglucanase and β -glucosidase. Therefore, strong LA production was approached in HT4 by clarifying key cellulase component played synergistic roles with *Lactoc. lactis* host. This study could benefit the development of LA production in fermenting lignocellulosic biomass.

Introduction

The grass is widely distributed renewable lignocellulosic biomass in the world. The lignocellulose is the natural polymers. In grass, lignocellulose mainly contain 29-35% cellulose, 26-32% hemicellulose, and 16-21% lignin (Galkin and Samec 2016). The conversion of lignocellulosic polymers to versatile platform chemicals by anaerobic fermentation is a reliable way to avoid resource competition between food and chemical industries (Liu et al. 2020). Lactic acid (LA) is a versatile chemical and has been widely applied in various industries including food, leather tanning, cosmetics, and pharmaceuticals (Martinez et al. 2013). Approximately 90% of global LA is produced via microbial fermentation (Haag et al. 2016; Alexandri et al. 2019), which requires low production temperature and has low energy requirements and high purity (Wee et al. 2006). Ensiling is an anaerobic fermentation process, which naturally depends on the epiphytic lactic acid bacteria (LAB) fermenting water-soluble carbohydrates (WSC) into LA to decrease pH. Consequently, LA-dominated plants can be well conserved as feedstock (McDonald 1981). In recent years, ensiling has been defined as an eco-friendly, green, and sustainable lignocellulose degradation technique for producing LA from biomass (Haag et al. 2016; Bichot et al. 2018).

To increase the LA in ensiled grass, homo-fermentative LAB, such as *Lactobacillus plantarum* and *Lactococcus lactis*, produce more LA from glucose than hetero-fermentative LAB and are employed in the ensiling yield. Kung (2015) reported that homo-fermentative LAB might have produced more LA during ensiling lignocellulosic biomass compared with the control, but the degree of preventing harmful *Clostridial* fermentation depended on the circumstances, e.g., material and temperature. Alfalfa, containing high protein content, is widely used as ruminant feed and is also a good candidate feedstock for LA production. However, its low contents of WSC and dry matter (DM) and high buffering capacity (BC) lead to difficulty control undesired *Clostridial* fermentation (McDonald et al. 1991). In the cold seasons, weak LA fermentation often occurs (Cao et al. 2011; Wang et al. 2017). Therefore, how to use homo-fermentative LAB maximally to increase LA production and restrain undesired fermentation in ensiling high moisture alfalfa is an interesting research direction.

Most homo-fermentative LAB is not able to secrete the enzymes to degrade lignocellulose, LA production cannot meet the requirement of the industry. Although our group isolated some cellulolytic LAB from the rumen, it did not improve the conversion of WSC into LA (CWL) relative to adding exogenous cellulase in ensiled alfalfa due to the poor ability to produce LA (Liu et al. 2020). Li et al. (2018a) found that cellulolytic LAB derived from rumen degraded the lignocellulose of *Pennisetum sinense* and increased the LA content compared with the control, but stronger LA fermentation and CWL were exhibited by *Lactobacillus plantarum* combined with them. Cellulase consists of endoglucanase (EG), cellobiohydrolase (CBH), and β -glucosidase (BG) and hydrolyzes the β -1,4-D-glycosidic bonds of cellulose. The cellulose is degraded into monosaccharides successfully and completely, which depended on the composing proportion of cellulase. Du et al. (2018) reported that the difference in the composition of cellulase mixture influenced its applicability in different pretreated materials. Optimization of the proportions of individual component in cellulase mixture has been highlighted the importance of lignocellulose hydrolysis. On the other hand, CBH and BG are sensitive to cellobiose and glucose, respectively (Riou et al. 1998; DeCcker et al. 2001; Teugjas and Våljamäe 2013). The BG often plays a rate-limiting role in cellulose degradation. To delete this limitation, simultaneous saccharification and fermentation using combination of cellulase and LAB was applied to ensiling grass. However, some previous studies have described negative effects of the combined treatment of cellulase and LAB on ensiling alfalfa, such as ineffectiveness on decreasing DM loss, neutral detergent fiber (NDF) and acid detergent fiber (ADF), and increasing LA content compared with the control (Kozelov et al. 2007; Lynch et al. 2014). The proposed explanation is that some cellulases cannot degrade lignocellulose of alfalfa (Lynch et al. 2014), some lignocellulose degradation products of cellulase (high-molecular-weight oligosaccharides) cannot be fermented by LAB (Stokes and Chen 1994; Tarraran and Mazzoli 2018), or the timeliness of cellulase and LAB was different (Liu et al. 2019). However, the bottleneck of lignocellulose degradation was that little known about which cellulase

component played synergistic roles with LAB in fermenting alfalfa lignocellulose into lactic acid. Therefore, providing the above information can benefit the development of LA production in ensiling lignocellulosic biomass.

Recently, our group constructed engineered *Lactoc. lactis* subsp. *lactis* MG1363 (MG1363) strains separately containing the *bgl1*, *cbh2*, and *egl3* gene of *Trichoderma reesei*. The combination proportion of engineered MG1363 strains (HT2) containing *bgl1*, *cbh2*, and *egl3* genes at 1:1:1 could secrete cellulase (236 mU/ml FPase activity) (Liu et al. 2019). We hypothesized that optimizing the combinations of engineered MG1363 strains could clarify key cellulase component playing synergistic roles with LAB in fermenting alfalfa lignocellulose to improve LA production. To test the hypothesis, the lignocellulose degradation, sugar profile, fermentation characteristics, and CWL of high-moisture alfalfa was investigated after ensiling in low-temperature seasons for 140 d, using commercial *T. reesei* cellulase (EN), and the combination of *Lactob. plantarum* and commercial cellulase (LPEN) as parallel treatments for the 7 combinations of engineered MG1363 strains treatments.

Materials And Methods

LAB strains, material, and ensiling treatments

The engineered strains of MG1363 containing *bgl1*, *cbh2*, and *egl3* gene of *T. reesei* were constructed in our lab by Liu et al. (2019). After culturing in GM17 broth medium at 30°C for 30 h, engineered strains containing *bgl1*, *cbh2*, and *egl3* gene were mixed at 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1 and were named HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1 separately. The LPEN was a combination of *Lactob. plantarum* and commercial cellulase. *Lactob. plantarum* was isolated from corn silage and stored at -80°C, and incubate in deMan Rogosa and Sharp (MRS) medium at 37°C for 24 h when used. The EN, the activity of cellulase was 50000 U/g FPase, was extracted from the *T. reesei* and purchased from Ruiyang biotechnology company (Wuxi, China). The HT2, HT3, HT4, HT5, E1C1, E1B1, C1B1, LPEN, and EN were used as the nine treatments.

Alfalfa was planted in three fields (100 m²) of Nanjing Agricultural University (Nanjing, China) (Humid subtropical climate, 32°01' N, 118°50'13.63" E, 17 masl) on September 25, 2017, and the last harvest time was November 2, 2018. Forage chopper (Sh-2000, Shanghai Donxe Industrial Co., Ltd., Shanghai, China) was used to chop the fresh alfalfa into 1 to 2 cm long pieces. Before ensiling, DM content, pH value, and BC of alfalfa were 279 g/kg fresh matter (FM), 5.15, and 594 milliequivalents (mEq)/kg DM, respectively. The structural carbohydrate composition: neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose, and hemicellulose were 431, 308, 110, 198, and 123 g/kg DM, respectively. The WSC content was 84.3 g/kg DM, and the fraction of individual soluble carbohydrates was as follows: disaccharides (24.0 g/kg DM), glucose (10.7 g/kg DM), xylose (13.7 g/kg DM), arabinose (0.21 g/kg DM) and fructose (10.5 g/kg DM). The epiphytic LAB [3.40 lg colony-forming units (cfu)/g FM] on alfalfa was lower than that of aerobic bacteria (6.97 lg cfu/g FM) and yeasts (4.98 lg cfu/g FM). The fermentation efficiency (FC) of fresh alfalfa was 29.04.

Alfalfa was harvested from 3 fields (100 m² each). The alfalfa harvested in each field was ensiled with nine treatments (HT2, HT3, HT4, HT5, E1C1, E1B1, C1B1, LPEN, and EN), and without treatment was the control. Each treatment and the control had four replicates. According to McFarland turbidity standards (McFarland, 1907), inoculation dosage (3 mL aqueous solution) of HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1 was at 1×10⁶ cfu/g FM and were sprayed on alfalfa. The EN is added to take the dose (3 mL aqueous solution) at 2g/kg of FM. EN and LP are mixed as LPEN, and LPEN was added at a dose (3 mL aqueous solution) of 2 g/kg + 1×10⁶ cfu/g FM. Three milliliters of distilled water were added to the control. Alfalfa was mixed with different treatments and then filled into the experimental silo. The experimental silo (polyvinyl chloride bottle, 1L) was filled with 720 g of treated alfalfa. The silo was sealed with a lid and tape to create an anaerobic condition. All silos were kept at an ambient temperature ranging from 3 to 20 °C for 140 days of ensiling, and then, the silos were sampled for analysis.

Microbial and chemical analyses

According to the method reported by Liu et al. (2019), the LAB, aerobic bacteria, and clostridia in alfalfa materials were counted after harvesting from the field for 2 h. The BC of alfalfa materials was measured by the method reported by Liu et al. (2018). The DM, NDF, ADF, ADL, and WSC in alfalfa materials were measured by the same method reported by Li et al. (2019). The pH value was determined by mixing 50 g of alfalfa materials with 200 ml of distilled water and stored at 4°C for 18 h. The mixture was filled, and the pH value of filtrate was measure. The FC of alfalfa material was predicted according to the method reported by Addah et al. (2011), as follows: FC = DM% + 8×WSC g/kg DM÷BC mEq/kg DM. FC indicates whether fresh forage is easy or difficult to be ensiled (FC>45 = easy, FC <35 = difficult to ensile). Freeze-dried and ground alfalfa samples were used to determine the contents of monosaccharides (glucose, fructose, arabinose, and xylose) and disaccharides. The sugars in fresh alfalfa were extracted with water and measured by an Agilent HPLC 1260 system equipped with chromatographic column (SP0810 sugar, Shodex, Inc. Japan) and refractive index detector. HPLC grade water was used as the mobile phase at 80°C and the flow rate was 0.75 ml/min. The AOAC-984.13 (2000) method was used to analyze the crude protein of alfalfa materials.

After 140 days of ensiling, the sample (200 g) in the center of the silo was picked up. The DM, crude protein, NDF, ADF, ADL, WSC, sugars, and microbes in the ensiled alfalfa were measured with the same methods as those in the fresh alfalfa materials. According to the formula reported by Porter et al. (1995), DM loss was calculated using the corrected DM content. The pH value, ammonia-N, alcohol, lactic acid, and volatile fatty acids in ensiled alfalfa were determined according to the method reported by Dong et al. (2019).

The oligosaccharide in WSC was analyzed by thin-layer chromatography (TLC) according to the method (Kanaya et al. 1978) and with small changes: using silica gel plate (Size: 100×100 mm; G model; Thickness of coating: 0.20-0.25mm) as chromatoplate, N-butanol: acetic acid: water (2:1:1, V/V) as a solvent system, and ethanol: sulfuric acid (4:1, V/V) as detection.

Statistical analyses

IBM SPSS Statistics software (IBM SPSS 22.0 for Windows) was used for statistical analysis. Using one-way analysis of variance (ANOVA, General Linear Models), the effects of treatments on the degradation of alfalfa lignocellulose degradation, sugar profile, fermentation characteristics, and CWL of alfalfa were evaluated. The means were then compared for significance using Tukey's test at $P < 0.05$. Trends were discussed at $0.05 \leq P \leq 0.10$.

Results And Discussion

Effects of combined cellulase gene engineered MG1363 on lignocellulose degradation

After ensiling for 140 d, treatments influenced most lignocellulosic compositions of alfalfa ($P < 0.05$), except for ADL ($P > 0.05$) (Table 1). It was because the ADL in lignocellulose was the hardest to be degraded during ensiling (Guo et al. 2014). Similar results were reported by Liu et al. (2019) and Li et al. (2018b), EN and LPEN treatments had lower NDF ($P < 0.001$ and $P = 0.011$), ADF ($P < 0.001$ and $P = 0.028$), cellulose ($P < 0.001$ and $P = 0.003$), hemicellulose ($P < 0.001$ and $P = 0.020$), and lignocellulose ($P < 0.001$ and $P = 0.003$) than the control. This was attributed to the efficient ability of cellulase to degrade the lignocellulose and its component. The LPEN had higher NDF ($P = 0.026$), ADF ($P = 0.020$), cellulose ($P = 0.019$), hemicellulose ($P = 0.152$), and lignocellulose ($P = 0.007$) than the EN, which indicated that the synergistic effect of LPEN on lignocellulose degradation did not appear. This result was consistent with the findings reported by Kozelov et al. (2007), but did not agree with the results reported by Li et al. (2018). One reason for the difference in various studies was the DM content and pH value in ensiled alfalfa. Generally, ensiled forage with high-DM content caused higher pH than that with low-DM content (McDonald et al. 1991) and some cellulase, such as commercial *T. reesei* cellulase, had a great activity to degrade lignocellulose at high pH (Lan et al. 2013). Therefore, considering the higher pH in EN than in LPEN (Table 3) ($P = 0.001$), pH adjusted by LP was the key limiting factor for converting lignocellulose into sugar in LPEN. Similar to the LPEN, HT2 had weak lignocellulose degradation than the EN, evidenced by higher NDF ($P = 0.016$), ADF ($P = 0.006$), cellulose ($P < 0.001$), and hemicellulose ($P = 0.225$) and lignocellulose ($P = 0.004$). This might be because the amount and activity of secreted cellulase by HT2 was insufficient and weak like in LPEN. Colombatto et al. (2004) reported that lignocellulose degradation depended on the cellulase level and acidity during ensiling.

Compared with the control, HT3, HT4, and HT5 had lower NDF ($P = 0.022$, $P = 0.011$, and $P < 0.001$), ADF ($P = 0.002$, $P = 0.002$, and $P = 0.001$), cellulose ($P = 0.010$, $P < 0.001$, and $P = 0.004$), and lignocellulose ($P = 0.010$, $P = 0.002$ and $P < 0.001$). These outcomes indicated that these combinations could promote lignocellulose degradation. Interestingly, the degradation degrees of lignocellulose in HT2 differed from that in HT3, HT4, and HT5 due to that the increase of one cellulase component gene engineered MG1363 relative to HT2 altered degradation of the lignocellulosic component. Compared with HT2, HT3 and HT4 had higher ($P = 0.008$ and $P = 0.083$) residual hemicellulose content and had lower cellulose content ($P = 0.182$ and $P = 0.014$). This outcome indicated that the increases of engineered MG1363 containing *egb3* or *cbh2* gene enhanced the cellulose degradation to promote the release of hemicellulose from the intertwined hemicellulose and ADL. The result in HT3 was agreed with the findings reported by Chylenski et al. (2017) due to the optimized hydrolysis in acidic pretreated lignocellulose biomass needed a higher proportion of EG relative to CBH in the cellulase cocktail. As for the outcome in HT4, this was in accordance with what is observed in nature. CBH had the greatest proportion (52-80%) of the total hydrolytic enzymes secreted from *Trichoderma reesei* (Rosgaard et al. 2007). Furthermore, the increase of engineered MG1363 containing the *cbh2* gene in the cocktail had a stronger role in decomposing the cellulose than that containing the *egb3* gene, supported by a greater cellulose-decrease level ($P = 0.047$) in HT3 than HT4 relative to the control. Kim et al. (2015) reported that, for acidic hydrolysis rice straw, the role of CBH was found to be more important than that of EG. In contrast, HT5 had lower contents of hemicellulose ($P = 0.090$), and lignocellulose ($P = 0.017$) than HT2. This was because increased BG promoted the degradation of hemicellulose. Indeed, some BG can degrade hemicellulose composing of xylans (Chaudhary et al. 1982; Zhou et al. 2012).

Compared with the HT2, C1B1 had lower ($P = 0.001$) cellulose content. This indicated that the lack of EG did not influence the cellulose degradation and that disagreed with the traditional view of EG, CBH, and BG synergistically and indispensably in degrading cellulose. The reason was the native bacteria on alfalfa secreting EG to help the engineered MG1363 containing *cbh2* and *bgl1* gene degrade cellulose. Zhang et al. (2019) found that two EGs produced by bacteria derived from ensiled grass, such as *Paenibacillus panacisoli* SDMCC050309,

could degrade carboxymethyl cellulose into celooligosaccharides, in which cellobiose and cellotriose could be used as substrates for the growth of homofermentative LAB. As for the ineffective EG role of native bacteria in HT2 treatment, it was because LAB in HT2 had stronger competitiveness than that in C1B1, supported by more LAB ($P < 0.001$) and less aerobic bacteria ($P = 0.017$), and *Clostridia* spore ($P < 0.001$) in HT2 than C1B1 (Table 3). Compared with HT2, the lack of the engineered MG1363 containing *cbh2* gene markedly influenced the lignocellulose degradation, mainly in hemicellulose, demonstrated by higher contents of hemicellulose ($P < 0.001$) and lignocellulose ($P = 0.001$) in E1B1 treatment. This indicated that the lack of CBH went against the degradation of hemicellulose. It was because that CBH was the main component of cellulase and can degrade hemicellulose (Wei et al. 2017). The lack of engineered MG1363 containing *bg1* gene in E1C1 did not influence the lignocellulosic degradation relative to HT2 treatment, indicated by insignificant differences in NDF, ADF, cellulose, and hemicellulose between E1C1 and HT2 ($P > 0.05$). The reason might be that the MG1363 host could utilize the productions decomposed by EG and CBH. Therefore, CBH played a more key role in lignocellulose degradation than EG and BG during ensiling.

Effects of combined cellulase gene engineered MG1363 on oligosaccharide

As shown in TLC, big molecular-weight oligosaccharides were in the FAM, HT4, HT5, and E1B1 and C1B1 due to the shadow area in the lanes and there were markable small molecular-weight oligosaccharides in all samples (Fig. 1). The former indicated that the broken degree of lignocellulose was greater in HT4, HT5, E1B1, and C1B1 compared with other treatments, while the latter suggested that these small molecular-weight oligosaccharides were not utilized by MG1363 host and native LAB on alfalfa during ensiling. Fewer small molecular-weight oligosaccharides were in the LPEN compared with the EN, HT3, HT4, HT5, and the control due to its light blots. This could be explained by *Lactob. plantarum* had a wider fermentation profile of sugar than MG1363 host and native LAB on alfalfa. Liu et al. (2020) reported that *Lactob. plantarum* could ferment xylose while MG1363 and *E. faecalis* could not. Interestingly, one blot of small molecular-weight oligosaccharides in E1C1, and C1B1 relative to the two blots of small molecular-weight oligosaccharides in the EN, HT2, HT3, HT4, HT5, E1B1, and the control indicated that low sugar diversity in E1C1 and C1B1 due to the consumption of native bacteria or not being cut from lignocellulose. Especially, two blots of small molecular-weight oligosaccharides in the HT4 and HT5 were darker than those in the EN, LPEN, HT2, HT3, and the control. This indicated that more small-molecular oligosaccharides were released from lignocellulose but could not be fermented in treatments of HT4 and HT5.

Effects of combined cellulase gene engineered MG1363 on sugar profile

As shown in Table 2, the control had lower WSC than EN ($P = 0.001$), LPEN ($P = 0.001$), HT2 ($P = 0.067$), and HT3 ($P < 0.001$) after ensiling for 140 d, which inferred that more WSC were released from lignocellulose due to lower lignocellulose in EN ($P < 0.001$), LPEN ($P = 0.005$), and HT2 ($P = 0.010$) (Table 1). In contrast, there was lower WSC content in HT5 ($P < 0.001$) compared with the control, and HT4, C1B1, E1B1, and E1C1 had approximate WSC with the control ($P > 0.05$). The former resulted from that the sugars derived from the lignocellulose degradation could be fermented by MG1363 host on alfalfa during ensiling. This was supported by fewer NDF ($P < 0.001$), ADF ($P = 0.001$), cellulose ($P = 0.004$) and hemicellulose ($P < 0.001$) and more LA ($P < 0.001$) in HT5 than the control (Table 3). Interestingly, the reasons for the low WSC in HT4, C1B1, E1B1, E1C1, and the control were different. Similar to HT5, low WSC content in HT4 was because that WSC released from lignocellulose degradation could be fermented by MG1363 host during ensiling due to the extra engineered MG1363 containing *cbh2* gene. Furthermore, the WSC consumption-ability of HT5 was stronger than HT4. The detailed reason was unknown since there was no difference in the bacteria compositions, and thus which needed further study in the future. Conversely, the low WSC in the control and E1B1 indicated that few fermentable WSC derived from the lignocellulose degradation due to the high lignocellulose composition (Table 1). Consequently, the inherent WSC in alfalfa was consumed in the control and E1B1. The low WSC in the C1B1 and E1C1 was because that the WSC derived from the lignocellulose degradation could be fermented, supported by the lower lignocellulose ($P < 0.001$) and higher LA content ($P = 0.002$ and $P < 0.001$) than the control (Table 3).

Compared with the control, most of treatments had higher disaccharide ($P < 0.05$), except for HT4 ($P > 0.05$). The explanation for the former was that disaccharide was formed from lignocellulose degradation and was not consumed by *Lactoc. lactis* and some native bacteria on the alfalfa. The latter indicated that the HT4 not only strongly converted lignocellulose into disaccharide but also had the strongest ability to consume the disaccharide. The extra CBH secreted by engineered MG1363 strain containing *cbh2* gene in HT4 could attack the non-reducing end of the crystalline cellulose created by EG and release cellobiose (Chylenski et al. 2017), which could be further cut by BG into monosaccharide and was fermented by *Lactoc. lactis* host to produce more LA than other treatments ($P < 0.001$) (Table 3). Amazingly, the lack of engineered MG1363 strain containing the *cbh2* gene in E1B1 resulted in higher disaccharide content than HT4 ($P < 0.001$). It was hard to explain the disaccharide source. The proposed reason might be that the end of the crystalline cellulose created by EG could be digested into disaccharides in an acidic ensiling environment. The higher disaccharide content in EN, HT2, HT3, HT5, and LPEN than in HT4 might be because the consumption level of disaccharide in the above treatments was weaker than in HT4. On the other hand, HT3 had the highest disaccharide content than other treatments. This might result from the extra EG secreted by engineered MG1363 strain containing *eg3* gene in HT3 could produce quite a number of the ends of the cellulose crystallinity to restrain the CBH and BG activity. Some studies reported that cellulase adsorption generally declined as cellulose crystallinity increased (Yang et al. 2011). Crystallinity could greatly impact the adsorption

of Cel7A (CBHI), leading to the decrease of hydrolysis (Jeoh et al. 2007). Cello-oligosaccharide substrates inhibited the activity of BG (Kawai et al. 2004; Bohlin et al. 2013; Teugjas and Valjamae, 2013). Consequently, the channel of cellobiose converting into glucose was blocked by disaccharides which accumulated in HT3. Interestingly, LPEN had higher disaccharide contents than EN ($P < 0.05$). This indicated that the ability of the native LAB on the alfalfa material to ferment disaccharides was weaker than that of *Lactob. plantarum*.

All treatments had higher xylose than the control ($P < 0.05$), except for LPEN (Fig. 2). The reasons for the former were as follows: ① xylose was hardly metabolized by the MG1363 (Erlandson et al. 2000) ② Some native LAB in the control could utilize xylose. Cai found that some isolated native LAB from forage crops, such as *Enteric. casseliflavus* and *Enteric. gallinarum*, and *Enteric. mundtii*, could ferment xylose (Cai, 1999). ③ EN degraded more hemicellulose ($P < 0.001$) into xylose than the control. The reason for the latter was that *Lactob. plantarum* could ferment xylose. Our previous study reported that the MG1363 had the weaker ability to ferment xylose than *Lactob. plantarum* (Liu et al. 2019). Compared with the control, higher arabinose content in HT4 ($P < 0.001$), EN ($P < 0.001$), and LPEN ($P < 0.001$) indicated that hemicellulose was degraded into arabinose due to lower hemicellulose content ($P = 0.384$, $P < 0.001$, and $P < 0.001$) than the control and the extra engineered MG1363 strain containing *cbh2* gene in HT4 relative to HT2, HT3, HT5, C1B1, E1B1, and E1C1 could cut hemicellulose to release arabinose.

Effects of combined cellulase gene engineered MG1363 on the fermentative characteristics and microbial compositions of ensiled alfalfa

As shown in Table 3, compare with the control, all treatments improved the fermentation due to their lower pH values ($P < 0.05$), ammonia-N contents ($P < 0.05$) and DM losses ($P < 0.05$), and higher LA contents ($P < 0.05$). It was because that cellulase derived from the EN and secreted by engineered *Lactoc. laticis* combination could degrade lignocellulose into the sugars to enhance the LA fermentation of *Lactoc. laticis* and native LAB. Interestingly, EN promoted the LA fermentation but did not decrease the acetic acid (AA) content ($P = 0.750$) compared with the control. One reason was that cellulase has an affinity with hemicellulose. The AA and sugars are hemicellulose hydrolyzed production of cellulase (Zheng et al. 2017). Another reason was that EN promoted the hetero-LA fermentation of native LAB on alfalfa, supported by high DM loss and ammonia-N than other treatments. Hetero-LA fermentation was associated with high DM loss, concentrations of AA, and ammonia-N during ensiling compared with homo-LA fermentation (Driehuis et al. 1999).

Compared with EN, DM loss ($P < 0.05$) and AA content ($P < 0.05$) were decreased by the supplementations of HT2, HT3, HT4, HT5, LPEN, E1C1, E1B1, and C1B1, accompanied with higher LA/AA ($P < 0.05$) and lower ammonia-N ($P < 0.05$). This result indicated that the homo-LA fermentation was more vigorous in those treatments than in EN. Liu et al. (2019) demonstrated that engineered homo-fermentative LAB strains containing cellulase gene or the combination of wild-type MG1363 and cellulase had stronger homo-LA fermentation in ensiled alfalfa compared with adding cellulase alone. Tian et al. (2014) and Xing et al. (2009) found that *Lactob. plantarum* combined with cellulase promoted homo-LA fermentation and inhibited the degradation of protein to produce ammonia-N.

Interestingly, compared with the HT2, increasing engineered MG1363 containing one gene of cellulase component altered the LA fermentation. The greatest LA content in the HT4 ($P < 0.05$) and was higher than the HT2 ($P = 0.013$) indicated that increasing engineered MG1363 with the *cbh2* gene had a better effect on promoting LA fermentation. In addition, relative to HT2, increasing engineered MG1363 containing *cbh2* gene was better than increasing engineered MG1363 with *egf3* or *bgf1* gene on increasing LA content ($P < 0.001$), supported by HT4 having higher LA content ($P < 0.05$) and CWL ($P = 0.004$ and $P < 0.001$) than HT3 and HT5 (Fig. 3). This demonstrated that the CBH was the key cellulase component for degrading lignocellulose into fermentable sugar, and followed by MG1363 host converted sugar into more LA, supported by lower cellulose ($P = 0.014$), fewer fermentable disaccharides ($P < 0.001$), and higher LA content ($P = 0.013$) in HT4 than HT2. Therefore, CBH cellobiohydrolase better cooperated with MG1363 to ferment lignocellulose into LA than endoglucanase and β -glucosidase.

Although there were lower LA content ($P < 0.001$) and CWL ($P = 0.038$ and $P < 0.001$) in HT3 and HT5 relative to HT2, the mechanisms of altering LA fermentation were different in HT3 and HT5. Compared with HT2, the channel of fermenting sugar into LA in HT3 was restrained rather than that of converting lignocellulose into sugar since HT3 had approximate residual WSC ($P = 0.385$) and lignocellulose content ($P = 0.788$) and lower LA content ($P < 0.001$) and CWL ($P = 0.038$) relative to HT2. In contrast, the channel of converting lignocellulose into sugar in HT5 was not restrain since HT5 had lower lignocellulose ($P = 0.017$) relative to HT2. Whereas, the channel of fermenting sugar into LA in HT5 was restrained, supported by its lower contents of residual WSC ($P < 0.001$) and LA ($P < 0.001$) and the CWL ($P < 0.001$) relative to HT2 ($P = 0.001$). On the other hand, LPEN had lower LA content ($P = 0.001$), approximate CWL ($P = 0.374$), residual WSC ($P = 0.072$), and lignocellulose ($P = 0.795$) relative to HT2 and had weaker ($P = 0.004$) lignocellulose degradation relative to EN due to the lower pH, which indicated that the pH was the key limiting factor for lignocellulose saccharification of commercial EN in the simultaneous saccharification and fermentation of LPEN. This might be one explanation to clarify the weak or no effect on reducing the contents of NDF and ADF and improving the LA fermentation in ensiled alfalfa treated with the combination of cellulase and LAB in previous studies (Kozelov et al. 2007; Lynch et al. 2014). Therefore, selecting cellulase combined with LAB strain for fermenting lignocellulosic grass to produce LA needs careful consideration.

Accordingly, the lack of any engineered MG1363 containing one gene of cellulase component in C1B1, E1B1, or E1C1 had lower LA production ($P < 0.001$) compared with HT2. This outcome could be explained by followed reasons: ① fewer LAB numbers in C1B1 ($P < 0.001$), E1B1 ($P =$

0.043), and E1C1 ($P < 0.001$) than the HT2; the insufficient unblocked channel of converting lignocellulose into sugar was in E1B1 due to its higher residual lignocellulose ($P = 0.001$) than the HT2; the insufficient unblocked channels of converting sugar into LA were in C1B1 and E1C1 due to their lower LA content ($P < 0.001$) and CWL ($P < 0.001$) than HT2. Therefore, we optimized the cocktail of engineered MG1363 containing cellulase component gene to promote LA fermentation and clarify the key cellulase component playing synergistic role with LAB in fermenting alfalfa lignocellulose into LA, and identified the synergetic disadvantage of cellulase combined with LAB for LA fermentation in ensiled alfalfa feedstock.

Conclusions

HT4 was the optimal combination proportions (at 1:2:1) of engineered MG1363 containing *bgl1*, *cbh2* and *eg3* genes and could improve LA fermentation maximally. The cocktail of cellulase gene engineered MG1363 clarified the CBH played more key in converting lignocellulose into fermentable sugar than EG and BG and cooperated with MG1363 host to produce more LA content. The pH was the key limiting factor for lignocellulose saccharification of commercial EN in the simultaneous saccharification and fermentation of the combination of *Lactob. plantarum* and cellulase in ensiled alfalfa. This study could benefit the development of LA production in fermenting lignocellulosic biomass via optimizing the cocktail of cellulase gene engineered LAB.

Abbreviations

ADF, Acid detergent fiber; ADL, Acid detergent lignin; BC, Buffering capacity; CBH, Cellobiohydrolase; EN, Cellulase; Control, alfalfa ensiled without treatments; CWL, Conversion of WSC into la; cfu, colony-forming units; MRS, Deman rogosa and sharp; DM, Dry matter; EG, Endoglucanase; FAM, fresh alfalfa material; FC, Fermentation efficiency; FM, Fresh matter; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *eg3*, *cbh2*, and *bgl1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately; LA, Lactic acid; LA/AA, the ratio of lactic acid to acetic acid; LAB, Lactic acid bacteria; LPEN, *Lactobacillus plantarum* and cellulase; NDF, Neutral detergent fiber; $\text{NH}_3\text{-N}$, ammonia-N; N, nitrogen; SEM, standard error of the means; TLC, Thin-layer chromatography; WSC, Water-soluble carbohydrates; BG, β -Glucosidase.

Declarations

Authors' contributions

Professor Qinhua Liu and Tao Shao designed the experiment. Dr. Junfeng Li, Master student Qifeng Wu, Cheng Zong, Aili Wu, Dr. Zhihao Dong experimented using the methods in the related references. Dr. Junfeng Li and Professor Qinhua Liu edited the manuscript and Tao Shao provided language service. Professor Qinhua Liu, as the corresponding author, submitted this paper.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Chemical compositions of alfalfa before and after ensiling for 140 d ^a

Items ^b	DM (g/kg FW)	CP (g/kg DM)	NDF	ADF	ADL	Cellulose	Hemicellulose	Lignocellulose
FAM	279	229	431	308	110	198	123	431
CON	236 ^e	237	390 ^a	277 ^a	100	178 ^a	112 ^{ab}	390 ^a
EN	247 ^{de}	227	335 ^c	242 ^d	97.9	144 ^d	93.2 ^d	335 ^d
LPEN	254 ^{bcd}	246	361 ^b	260 ^{bc}	102	158 ^{bc}	101 ^{cd}	361 ^b
HT2	251 ^{cd}	233	363 ^b	264 ^{abc}	94.8	169 ^{ab}	99.4 ^{cd}	363 ^b
HT3	271 ^a	237	364 ^b	251 ^{cd}	90.8	161 ^{bc}	114 ^{ab}	365 ^b
HT4	249 ^{cd}	245	360 ^b	252 ^{cd}	98.5	154 ^{cd}	108 ^{bc}	360 ^b
HT5	250 ^{cd}	244	340 ^{bc}	250 ^{cd}	90.8	159 ^{bc}	90.6 ^d	340 ^{cd}
C1B1	265 ^{ab}	242	357 ^{bc}	257 ^{bcd}	109	148 ^{cd}	100 ^{cd}	357 ^{bc}
E1B1	255 ^{bcd}	235	395 ^a	272 ^{ab}	97.7	174 ^a	123 ^a	394 ^a
E1C1	261 ^{abc}	239	349 ^{bc}	257 ^{bcd}	85.8	171 ^{ab}	92.9 ^d	349 ^{bcd}
SEM	2.5	4.9	7.7	5.2	5.5	4.1	3.5	6.3
1. Value	<0.001	0.179	<0.001	0.002	0.230	<0.001	<0.001	<0.001
2. CON vs All treatment								

^a Before ensiling means that alfalfa material is sampled after harvesting from the field for 2 h, and is not fermented in anaerobic silos; After ensiling means that alfalfa material is fermented in anaerobic silo for 140 d and sampled after silo opened.

^b Means in a column without a common superscript letter differed ($P < 0.05$) as analyzed by one-way ANOVA and the Tukey test. CP, crude protein; DM, dry matter; NDF, neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; ADL, acid detergent lignin; FAM, fresh alfalfa material; Control, alfalfa ensiled without treatments; EN, cellulase; LPEN, the combination of *Lactobacillus plantarum* and cellulase; Lignocellulose, the sum of ADL, cellulose, and hemicellulose; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *egl3*, *cbh2*, and *bg1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately; SEM, standard error of the means.

Table 2 Sugars of alfalfa before and after ensiling for 140 d ^a

Items ^b	Water-soluble carbohydrates (g/kg DM)	Disaccharide (g/kg DM)	Monosaccharide (g/kg DM)			
			Glucose	Xylose	Arabinose	Fructose
FAM	84.3	24.0	10.7	13.7	0.21	10.5
CON	35.9 ^{bc}	0.00 ^f	/	16.8 ^c	0.00 ^c	/
EN	42.8 ^a	7.59 ^c	/	19.1 ^a	0.24 ^a	/
LPEN	42.8 ^a	9.31 ^b	/	17.7 ^{bc}	0.24 ^a	/
HT2	39.4 ^{ab}	8.64 ^{bc}	/	19.2 ^a	0.08 ^{bc}	/
HT3	43.7 ^a	10.9 ^a	/	19.4 ^a	0.02 ^c	0.08
HT4	35.3 ^{bc}	1.02 ^{ef}	/	18.5 ^{ab}	0.20 ^a	/
HT5	26.2 ^d	8.28 ^{bc}	/	18.6 ^{ab}	0.04 ^{bc}	0.06
C1B1	34.4 ^c	4.13 ^d	/	18.9 ^a	0.02 ^c	1.04
E1B1	36.0 ^{bc}	4.52 ^d	/	18.7 ^{ab}	0.08 ^{bc}	/
E1C1	34.9 ^{bc}	2.24 ^e	/	18.6 ^{ab}	0.14 ^c	0.10
SEM	1.42	0.544	/	0.33	0.034	/
P-Value	<0.001	<0.001	/	<0.001	<0.001	/

^a Before ensiling means that alfalfa material is sampled after harvesting from the field for 2 h, and is not fermented in anaerobic silos; After ensiling means that alfalfa material is fermented in anaerobic silo for 140 d and sampled after silo opened.

^b Means in a column without a common superscript letter differed ($P < 0.05$) as analyzed by one-way ANOVA and the Tukey test. FAM, fresh alfalfa material; Control, alfalfa ensiled without treatments; EN, cellulase; LPEN, the combination of *Lactobacillus plantarum* and cellulase; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *egl3*, *cbh2*, and *bgl1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately; SEM, standard error of the means; /, below the detect limitation or without statistical analyses.

Table 3 Fermentative characteristics and microbial compositions of alfalfa before and after ensiling for 140 d ^a

^a Before ensiling means that alfalfa material is sampled after harvesting from the field for 2 h, and is not fermented in anaerobic silos; After ensiling means that alfalfa material is fermented in anaerobic silo for 140 d and sampled after silo opened.

^b Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Tukey test. cfu, colony-forming units; DM, dry matter; LA/AA, the ratio of lactic acid to acetic acid; NH₃-N, ammonia-N; N, nitrogen; LAB, lactic acid bacteria; FAM, fresh alfalfa material; Control, alfalfa ensiled without treatments; EN, cellulase; LPEN, the combination of *Lactobacillus plantarum* and cellulase; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *egl3*, *cbh2*, and *bgl1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately; SEM, standard error of the means; /, no detection.

Figures

Items ^b	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Alcohol	LA/AA	NH ₃ -N	DM loss (%)	Aerobic bacteria	LAB	Clostridia spore
		(g/kg DM)						(g/kg N)		(lg cfu/g FM)		
FAM	6.01	/	/	/	/	/	/	/	/	6.97	3.40	3.54
Control	5.33 ^a	42.4 ^f	15.7 ^a	0.58 ^b	1.11	6.97 ^a	2.70 ^e	150 ^a	2.14 ^a	6.05 ^a	8.69 ^{ab}	5.15 ^{ab}
EN	4.86 ^b	58.3 ^{cd}	13.6 ^{ab}	0.00 ^c	1.18	3.30 ^b	4.79 ^d	120 ^b	1.82 ^b	5.74 ^{ab}	8.24 ^{abc}	5.33 ^a
LPEN	4.61 ^c	61.5 ^c	8.21 ^c	0.00 ^c	1.31	3.00 ^b	7.50 ^{ab}	49.3 ^d	1.56 ^c	5.38 ^{cd}	7.80 ^{bcd}	5.16 ^{ab}
HT2	4.70 ^{bc}	72.2 ^b	10.5 ^{bc}	0.00 ^c	1.73	3.74 ^b	6.91 ^{abc}	75.5 ^c	1.67 ^c	5.18 ^c	8.90 ^a	4.64 ^c
HT3	4.69 ^c	57.2 ^{cd}	8.19 ^c	0.42 ^b	1.24	2.77 ^b	7.01 ^{abc}	58.7 ^{cd}	1.56 ^c	5.56 ^{bc}	7.20 ^d	4.96 ^b
HT4	4.71 ^{bc}	79.9 ^a	10.1 ^{bc}	0.91 ^a	1.64	3.41 ^b	7.98 ^a	75.4 ^c	1.56 ^c	5.64 ^{abc}	7.84 ^{bcd}	5.25 ^{ab}
HT5	4.71 ^{bc}	58.0 ^{cd}	9.11 ^c	0.38 ^b	1.60	3.08 ^b	6.37 ^{bc}	64.6 ^{cd}	1.63 ^c	5.62 ^{abc}	7.46 ^{cd}	5.08 ^{ab}
E1C1	4.72 ^{bc}	53.9 ^{de}	8.59 ^c	0.00 ^c	1.36	2.63 ^b	6.21 ^c	69.3 ^c	1.65 ^c	5.53 ^{bc}	7.70 ^{cd}	5.31 ^a
E1B1	4.74 ^{bc}	49.9 ^e	7.71 ^c	0.10 ^c	1.35	2.67 ^b	6.27 ^{bc}	66.7 ^{cd}	1.61 ^c	5.61 ^{abc}	8.32 ^{abc}	5.27 ^a
C1B1	4.72 ^{bc}	52.5 ^{de}	8.33 ^c	0.00 ^c	1.37	2.67 ^b	6.30 ^{bc}	67.8 ^c	1.62 ^c	5.75 ^{ab}	7.62 ^{cd}	5.32 ^a
SEM	0.035	2.032	0.862	0.072	0.05	0.414	0.239	5.608	0.027	0.098	0.193	0.063
P-Value	<0.001	<0.001	<0.001	<0.001	0.164	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Figure 1

Thin-layer chromatography testing of oligosaccharide after alfalfa ensiled with treatments for 140 d FAM, fresh alfalfa material; Control, alfalfa ensiled without treatments; EN, cellulase; LPEN, the combination of *Lactobacillus plantarum* and cellulase; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *egl3*, *cbh2*, and *bgl1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately.

Figure 2

The effects of treatments on the increasing rate of xylose after ensiling for 140 d. The increasing rate of xylose was calculated by the formula: Increasing rate of xylose = (Xylose ensiled alfalfa – Xylose raw alfalfa material) ÷ Xylose raw alfalfa material × 100%. Bars with different letters (a-c) indicated the difference among the treatments at $P < 0.05$ after being analyzed by one-way ANOVA and the Tukey test. CON, alfalfa ensiled without treatments; EN, cellulase; LPEN, the combination of *Lactobacillus plantarum* and cellulase; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *egl3*, *cbh2*, and *bgl1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately.

Figure 3

The conversions of water-soluble carbohydrates to lactic acid in ensiled alfalfa. Means in a bar without a common letter differ ($P < 0.05$) from each treatment after being analyzed by one-way ANOVA and the Tukey test. CON, alfalfa ensiled without treatments; EN, cellulase; LPEN, the combination of *Lactobacillus plantarum* and cellulase; HT2, HT3, HT4, HT5, E1C1, E1B1, and C1B1, combined engineered *Lactococcus lactis* subsp. *lactis* MG1363 strains containing *egl3*, *cbh2*, and *bgl1* gene mixed at proportions of 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:1:0, 1:0:1, and 0:1:1, separately.

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