Minireview

Shifts in xylanases and the microbial community associated with xylan biodegradation during treatment with rumen fluid

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Summary

Treatment with rumen fluid improves methane production from non-degradable lignocellulosic biomass during subsequent methane fermentation; however, the kinetics of xylanases during treatment with rumen fluid remain unclear. This study aimed to identify key xylanases contributing to xylan degradation and their individual activities during xylan treatment with bovine rumen microorganisms. Xylan was treated with bovine rumen fluid at 37°C for 48 h under anaerobic conditions. Total solids were degraded into volatile fatty acids and gases during the first 24 h. Zymography showed that xylanases of 24, 34, 85, 180, and 200 kDa were highly active during the first 24 h. Therefore, these xylanases are considered to be crucial for xylan degradation during treatment with rumen fluid. Metagenomic analysis revealed that the rumen microbial community’s structure and metabolic function temporally shifted during xylan biodegradation. Although statistical analyses did not reveal significantly positive correlations between xylanase activities and known xylanolytic bacterial genera, they positively correlated with protozoal (e.g., Entodinium, Diplorastra, and Eudioploida) and fungal (e.g., Neocallimastix, Orpinomyces, and Olpidium) genera and unclassified bacteria. Our findings suggest that rumen protozoa, fungi, and unclassified bacteria are associated with key xylanase activities, accelerating xylan biodegradation into volatile fatty acids and gases, during treatment of lignocellulosic biomass with rumen fluid.

Introduction

The rumen is an evolved forestomach and is one of the four stomachs in ruminants, hosting a complex rumen microbial community consisting of 1010 bacterial cells, 106 fungal cells, and 105 protozoal cells per ml (Castillo-González et al., 2014). Ruminants do not produce glycosid hydrolases to degrade ingested lignocellulosic feeds; therefore, they depend on their rumen microbial community to convert ingested lignocellulosic feeds into volatile fatty acids (VFAs) as an energy source. Rumen microbial communities enable ruminants to provide readily accessible animal products (i.e., dairy products, meat, and leather) from human-indigestible lignocellulose. The rumen microbial community produces various fibrolytic enzymes, such as endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), beta-glucosidase (EC 3.2.1.21), xylanase (EC 3.2.1.8), beta-xylosidase (EC 3.2.1.37), alpha-amylase (EC 3.2.1.37), and lignin peroxidase (EC 1.11.1.14), for the efficient digestion of undegradable plant materials into fermentable oligo- and monosaccharides (Wang et al., 2019). In general, Fibrobacter and Ruminococcus are considered the major fibrolytic
bacteria, whereas anaerobic fungi secrete a wide variety of polysaccharide-degrading enzymes and their rhizoidal system can physically penetrate lignified tissue that is not degraded by other microorganisms (Guo et al., 2020). The high fibrolytic activities of these rumen microorganisms have attracted attention recently for the bioproduction of saccharides, VFAs, methane gas, and other valuable products from lignocellulosic biomass (Liang et al., 2020). Lignocellulosic biomass, such as agricultural residues, paper waste, and forest residues, is one of the most abundant organic resources. The global annual production of lignocellulosic biomass is estimated to be approximately $181.5 \times 10^9$ tons, and $4.6 \times 10^9$ tons of lignocellulosic biomass residues are derived from agricultural residues (Dahmen et al., 2018). Lignocellulosic biomass is mainly composed of carbohydrate polymers, such as cellulose, hemicellulose, and lignin. These polymers form a strong structure that resists disintegration and hydrolysis during anaerobic digestion (Sawatdeenarunat et al., 2015). To accelerate the hydrolysis of polymers in lignocellulosic biomass, we previously proposed pretreatment of the biomass with rumen fluid discharged from slaughterhouses (Baba et al., 2013). This pretreatment has been shown to improve methane gas production from rice straw (Zhang et al., 2016), paper sludge (Takizawa et al., 2018), and rapeseed (Baba et al., 2017, 2019) by 1.5–3.4 times. Furthermore, low sodium dodecyl sulfate concentrations improve the efficiency of pretreatment with rumen fluid, enhancing methane production from waste paper (Takizawa et al., 2020a,b,c).

Knowledge of the fibrolytic enzymes of ruminal microorganisms is vital for improving the biodegradation efficiency of lignocellulosic biomass during treatment with rumen fluid. We previously reported endoglucanase activity and the responsible microorganisms (Takizawa et al., 2020a,b,c); however, hemicellulase activity has not yet been clarified. Xylan is the main component of hemicellulose, and the hydrolysis of xylan polymers is one of the rate-limiting steps in anaerobic degradation of lignocellulosic biomass. This study focused on xylanase in the rumen microbial community because xylanase catalyzes the hydrolysis of beta-1,4-glycosidic linkages of xylan polymers, producing xylooligosaccharides and xyloses. Biochemical analyses of crude enzymes have revealed that isolated and/or cultivated ruminal bacteria, protozoa, and fungi produce various xylanases (Wereszka et al., 2006; Novotná et al., 2010; Nyonyo et al., 2014). Recently, culture-independent approaches have played an important role in defining the rumen microbial community. Metatranscriptomic analysis has revealed that two-thirds of the transcripts annotated as putative hemicellulases were produced by members of the characterized genera Ruminococcus, Fibrobacter, and Prevotella, suggesting that these fibrolytic genera are predominant degraders of plant cell-wall polysaccharide in the bovine rumen (Dai et al., 2015; Comtet-Marre et al., 2017). However, these previous studies did not investigate individual xylanases in complex microbial ecosystems.

Additionally, the relationship between individual xylanase activities and the rumen microbial community during treatment with rumen fluid remains obscure. This study aimed to determine individual xylanase activities during treatment with rumen fluid and the rumen microorganisms associated with xylan degradation. We determined the chemical characteristics of xylan degradation, individual xylanase activities, and the rumen microbial community’s structure and metabolic function during xylan treatment with bovine rumen fluid. Additionally, we compared the correlations between key xylanase activities and microbial abundances.

Results

Chemical characteristics of xylan degradation by the rumen microbiota

Xylan was degraded by a bovine rumen microbial community during treatment for 48 h (Fig. 1). The total solids (TS) concentration decreased from 6.7 to 5.1 g reactor$^{-1}$ during 48 h, and 23.4% of the TS was degraded after the treatment (Fig. 1A). The TS degradation rate did not change during the first 24 h; however, it significantly decreased from 24 h to 48 h (Fig. 1B). With xylan degradation, xylan metabolic products increased during treatment. Reducing sugars decreased from 6.4 to 0.7 g l$^{-1}$ during the first 24 h (Fig. 1C). The dominant VFA produced throughout treatment was acetic acid, followed by propionic acid and butyric acid (Fig. 1D). Additionally, lactic acid was detected after 24 h at a concentration comparable to that of butyric acid. The total VFA concentration significantly increased 3.2 times (from 74.4 to 236.6 mmol l$^{-1}$) during the first 24 h. The pH decreased from 7.3 to 4.6 during the first 24 h (Fig. 1E), which reflected the increase in the total VFA concentration. Alongside the increase in total VFAs, the volumes of carbon dioxide gas and methane gas increased 2.3 and 2.2 times, respectively, between 6 h and 24 h (Fig. 1F).

Furthermore, the blank, containing only rumen fluid, did not show remarkable changes in reducing sugars, VFAs, pH, and gas production during 48-h incubation compared with xylan treated with rumen fluid (Fig. S1).

Changes in the rumen microbial community structure

The rumen microbial community structure during the treatment of xylan with rumen fluid was analyzed by metagenomic sequencing. Good’s coverage was above 99.8% in all samples, indicating sufficient sequencing...
depth (Table S1). The numbers of observed phyla and classes did not change throughout the treatment process; however, the numbers of observed orders, families, and genera were decreased at 48 h. Chao1 and ACE did not change significantly throughout the treatment process ($P > 0.05$). The diversity indices (Shannon and Simpson indices) declined during the first 6 h, increased at 12 h, and then declined after 24 h. Principal coordinate analysis indicated that the rumen microbial community structure shifted throughout the treatment process (Fig. 2A), especially from 6 h to 12 h and from 12 h to 24 h.

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To investigate the shift in the rumen microbial community during xylan treatment with the rumen fluid, the taxonomic compositions of domain-, phylum-, and genus-level communities were compared (Table 1 and Fig. S2). Bacteria were the most dominant domains throughout the treatment process, and their relative abundance increased from 64.84% to 94.56% (Fig. S2A). At the phylum-level microbial community, a total of 42 phyla were identified, of which Firmicutes and Bacteroidetes were the most dominant phyla throughout the treatment process (Fig. S2B). The relative abundance of Bacteroidetes increased from 35.62% to 56.67% during the first 6 h, and that of Firmicutes increased from 20.62% to 48.84% between 6 h and 12 h. The relative abundance of unclassified Eukaryota decreased from 27.74% to 0.47% throughout the treatment process. At the genus-level microbial community, 544 genera were identified, 428 of which were classified as bacteria. The bacterial genus Prevotella was the most dominant, followed by the bacterial genera Selenomonas, Lactobacillus, Bacteroides, Clostridium, Ruminococcus, and Butyrivibrio (Table 1). The relative abundance of Prevotella increased by 16.72% (from 26.05% to 42.76%) during the first 6 h. The relative abundances of Selenomonas and Lactobacillus increased at 12 h and 48 h respectively. Entodinium, Diplodiplastron, and Polyplastron were the dominant protozoal genera in the rumen microbial community, and their relative abundances at 0 h were 13.67%, 4.92%, and 4.11% respectively. However, the relative abundances of all protozoal genera significantly decreased to < 1.00% after 24 h. Neocallimastix, Orpinomyces, and Olpidium were the dominant fungal genera in the rumen microbial community; however, their relative abundances were substantially lower than those of the dominant bacterial and protozoal genera. The relative abundances of these fungal genera decreased to 0.00% during the treatment process.

**Functional characteristics of the rumen microbial community**

Metagenomic sequencing revealed the functional characteristics of the rumen microbial community during xylan treatment (Figs 3 and S3). Level-1 Clusters of Orthology Groups (COG) involved in metabolism was 38.561% of total COG at 0 h and did not significantly shift during the treatment (Fig. 3A). Level-2 COG, which is involved in carbohydrate metabolism, significantly decreased by 1.565% (from 26.607% to 25.042%) between 12 h and 48 h (Fig. 3B). Principal coordinate analysis of functional COG revealed that the microbial metabolic function shifted from 6 h to 12 h and from 12 h to 24 h (Fig. 2B). Functional COG involved in beta-galactosidase/beta-glucuronidase (EC: 3.2.1.25) was the most abundant COG (6.406% of total functional COG involved in carbohydrate metabolism), followed by beta-glucosidase-related glycosidases (EC: 3.2.1.52), nucleoside-diphosphate-sugar epimerases (EC: 1.1.1.388), glycosidases (EC: 3.2.1), and phosphoenolpyruvate synthase/pyruvate phosphate dikinase (EC: 2.7.9.2) (Fig. 3). Functional COG annotated as beta-1,4-xylanase (EC: 3.2.1.8) significantly decreased by 0.268% (from 0.656% to 0.388%) of total functional COG involved in
carbohydrate metabolism) during the first 24 h (Fig. 4A). Additionally, functional COG annotated as predicted xylanase slightly increased by 0.032% (from 0.420% to 0.452%) during the first 12 h, and then decreased to 0.360% after 24 h (Fig. 4B). Functional COG annotated as beta-xylanase (EC: 3.2.1.37) significantly increased by 0.496% (from 1.963% to 2.459%) during the first 6 h, and then decreased to 0.16% at 12 h (Fig. 4C). Similar shifts in functional COG associated with xylan degradation were observed in the relative abundance to total abundance of functional COG. Functional COG annotated as beta-1,4-xylanase (EC: 3.2.1.8) significantly decreased by 0.027% (from 0.067% to 0.040% of total COG) during the first 24 h. Moreover, functional COG annotated as predicted xylanase increased by 0.009% (from 0.043% to 0.052% of total COG) during the first 12 h. Functional COG annotated as beta-xylanase (EC: 3.2.1.37) significantly increased by 0.086% (from 0.200% to 0.286% of total COG) during the first 6 h.

### Xylanase activity during xylan treatment

Shifts in xylanase activity during xylan treatment with rumen fluid were determined by zymography (Fig. 5). During xylan treatment with rumen fluid, the zymogram revealed a wide range of xylanases (20–250 kDa) and similar banding patterns during the first 12 h. All xylanase activities were markedly decreased at 24 h and were hardly detected at 48 h. The total peak density significantly decreased by 89.8% and 98.3% at 24 h and 48 h respectively (0 h, 70 115; 24 h, 7177; 48 h, 1166) (Fig. S6). The bands corresponding to 24, 34, 85, 180, and 200 kDa exhibited the highest peak densities throughout the treatment process and represented approximately 70% of the total peak density. Initially, the

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**Table 1. Genus-level microbial community during treatment of xylan with the rumen fluid.**

| Genus              | 0 h (%) | 6 h (%) | 12 h (%) | 24 h (%) | 48 h (%) |
|--------------------|---------|---------|----------|----------|----------|
| **Bacteria**       |         |         |          |          |          |
| Prevotella         | 26.05   | 42.76   | 18.66    | 30.12    | 35.57    |
| Selenomonas        | 0.83    | 1.25    | 13.15    | 10.24    | 6.94     |
| Lactobacillus      | 0.52    | 0.51    | 0.34     | 0.82     | 17.12    |
| Bacteroides        | 3.11    | 3.50    | 2.40     | 4.14     | 5.05     |
| Clostridium        | 3.83    | 3.73    | 5.61     | 1.56     | 1.55     |
| Ruminococcus       | 3.99    | 2.49    | 3.10     | 1.30     | 3.10     |
| Butyrivibrio       | 1.65    | 1.90    | 7.16     | 1.19     | 0.48     |
| Parabacteroides    | 1.43    | 3.34    | 1.72     | 2.08     | 1.37     |
| Porphyromonas      | 1.36    | 1.75    | 1.64     | 2.76     | 1.70     |
| Bilfdobacterium    | 0.02    | 0.01    | 0.13     | 3.90     | 1.84     |
| Eubacterium        | 1.33    | 1.08    | 1.73     | 0.68     | 0.53     |
| Atopobium          | 0.21    | 0.29    | 0.66     | 3.08     | 0.33     |
| Bacteriella        | 0.48    | 1.03    | 0.70     | 0.88     | 0.82     |
| Rikenella          | 0.44    | 0.65    | 0.64     | 1.33     | 0.64     |
| Pseudobutyrivibrio | 0.11    | 0.35    | 3.03     | 0.08     | 0.05     |
| Symbiobacterium    | 0.70    | 0.65    | 1.33     | 0.31     | 0.55     |
| Bacillus           | 0.69    | 0.66    | 1.18     | 0.46     | 0.34     |
| Alistipes          | 0.38    | 0.53    | 0.39     | 0.86     | 0.44     |
| Desulfovomaculum   | 0.52    | 0.49    | 0.59     | 0.31     | 0.64     |
| Capnocytophaga     | 0.38    | 0.45    | 0.50     | 0.85     | 0.54     |
| **Protozoa**       |         |         |          |          |          |
| Entodinium         | 13.67   | 4.25    | 1.16     | 0.91     | 0.21     |
| Diploplastron      | 4.92    | 2.30    | 0.19     | 0.13     | 0.01     |
| Polyploastron      | 4.11    | 0.92    | 0.83     | 0.10     | 0.02     |
| Isotricha          | 1.08    | 0.83    | 3.13     | 0.09     | 0.07     |
| Metadinium         | 2.41    | 0.80    | 0.24     | 0.09     | 0.02     |
| Euplidiophyllum    | 0.61    | 0.74    | 0.70     | 0.18     | 0.12     |
| Ophyrocutis        | 0.49    | 0.00    | 0.00     | 0.00     | 0.00     |
| Dasytricha         | 0.17    | 0.13    | 0.16     | 0.00     | 0.00     |
| Epidinium          | 0.01    | 0.25    | 0.00     | 0.00     | 0.00     |
| Didinium           | 0.07    | 0.01    | 0.00     | 0.00     | 0.00     |
| **Fungi**          |         |         |          |          |          |
| Neocallimastix     | 0.26    | 0.09    | 0.04     | 0.01     | 0.00     |
| Ophidiorhomyces    | 0.11    | 0.04    | 0.05     | 0.00     | 0.00     |
| Olpidium           | 0.06    | 0.02    | 0.00     | 0.01     | 0.00     |

Top 20 bacterial genera, top 10 protozoal genera, and top 3 fungal genera are shown. All data represent the mean ± standard error. Multiple comparisons were performed using the Tukey–Kramer method, and different letters indicate a statistically significant difference (P < 0.05).
band at 85 kDa had the highest density, whereas the band at 180 kDa showed the highest peak density at 12 h. Shifts in peak density during the treatment process differed among the 24-, 34-, 85-, 180-, and 200-kDa bands. The band strength at 24, 34, and 85 kDa decreased throughout the treatment process, especially from 12 h to 24 h. Furthermore, the band at 180 kDa increased in density during the first 12 h, whereas it was not detected after 24 h. Similarly, the band at 200 kDa increased in density during the first 6 h and then decreased. Shifts in protein expression showed a wide range of proteins from 28 kDa to 140 kDa (Fig. S5A). The obvious bands at 85 kDa corresponding to xylanase with the highest peak density were observed during the

Fig. 3. Shifts in functional COG involved in carbohydrate transport and metabolism during xylan treatment with the rumen fluid. Top 20 COG are shown, and other COG are included in the category “other.” All values represent the mean of two reactors.

Fig. 4. Change in functional COG related to xylan degradation during treatment with the rumen fluid.
A. Beta-1,4-xylanase (EC: 3.2.1.8).
B. Predicted xylanase.
C. Beta-xylosidase (EC: 3.2.1.37). Multiple comparisons were performed using the Tukey–Kramer method, and different letters indicate a statistically significant difference ($P < 0.05$).
first 12 h; however, its strength markedly decreased after 24 h. Other bands corresponding to the xylanases were not detected throughout the treatment of xylan.

In the blank (containing only the rumen fluid), xylanases widely ranging from 20 kDa to 250 kDa were observed, and the band patterns remained similar during the first 12 h (Fig. S4). The strength of each band decreased during the incubation. Accordingly, the total band strength decreased throughout the incubation, especially between 12 h and 24 h. The protein expression showed the bands widely ranging from 29 kDa to 68 kDa at 0 h; however, the bands were not obvious after 12 h (Fig. S5B). The bands corresponding to the xylanase with high activity were not detected.

Correlation of xylanase activity with the rumen microbial community

To predict which microorganisms contributed to the xylanase activity, the correlations between xylanase band densities and the relative abundances of ruminal microorganisms were analyzed (Fig. 6). The predominant xylanolytic bacteria, including Prevotella, Clostridium, Ruminococcus, and Butyribrio, did not exhibit positive correlations with xylanase activities ($P > 0.10$). Other xylanolytic genera, including Selenomonas, Lactobacillus, and Bacteroides, were not positively, but negatively correlated with xylanase activities ($r < -0.7$, $P < 0.05$). Besides, the protozoal genera of entodiniomorph and holotrich ciliates exhibited positive correlations with xylanase activities, except for Metadinium and Ophryoscolex. The entodiniomorph genera Entodinium, Polyplastron, and Didinium showed positive correlations with xylanase activity at 24 kDa ($r > 0.90$, $P < 0.05$). Diploplastron had a positive correlation with xylanase activities at 24 kDa ($r = 0.98$, $P < 0.01$) and 85 kDa ($r = 0.89$, $P = 0.05$). Euendodinium exhibited positive correlations with total ($r = 0.95$, $P = 0.01$), 34-kDa ($r = 0.94$, $P = 0.02$), and 180-kDa ($r = 0.88$, $P = 0.05$) xylanase activities. Epidinium showed a positive correlation with xylanase activity at 200 kDa ($r = 0.94$, $P = 0.02$). The holotrich genus Isotricha showed a positive correlation with xylanase activity at 180 kDa ($r = 0.91$, $P = 0.03$). Dasytricha had positive correlations with total ($r = 0.99$, $P < 0.01$), 34-kDa ($r = 0.98$, $P < 0.01$), and 85-kDa ($r = 0.93$, $P = 0.02$) xylanase activities. The predominant fungal genera Necocallimastix, Orpinomyces, and Olpidium had positive correlations with xylanase activities. Neocallimastix and Orpinomyces showed positive correlations with 24-kDa and 85-kDa xylanase activities ($r > 0.89$, $P < 0.05$), and Olpidium exhibited a positive correlation with xylanase activity at 24 kDa ($r = 0.95$, $P = 0.01$). In addition, several unclassified bacteria showed positive correlations with xylanase activities (Fig. S7).

Unclassified, class Deltaproteobacteria exhibited positive correlations with total ($r = 0.97$, $P = 0.01$), 34-kDa ($r = 0.96$, $P = 0.01$), and 85-kDa ($r = 0.91$, $P = 0.03$) xylanase activity. Unclassified, class Epsilonproteobacteria and family Bacillaceae had a positive correlation with xylanase activity at 200 kDa ($r = 0.93$, $P < 0.05$). Unclassified, class Clostridiales exhibited positive correlations with total xylanase activity ($r = 0.92$, $P = 0.03$) and xylanase activities at 34 kDa ($r = 0.91$, $P = 0.03$) and 180 kDa ($r = 0.91$, $P = 0.03$). Unclassified, class Deltaproteobacteria showed positive correlations with total xylanase activity ($r = 0.97$, $P = 0.01$) and xylanase activities at 34 kDa ($r = 0.96$, $P = 0.01$) and 85 kDa ($r = 0.91$, $P = 0.03$). Unclassified, family Methylcoccaceae exhibited positive correlations with total xylanase activity ($r = 0.91$, $P = 0.03$) and xylanase activities at 24 kDa ($r = 0.98$, $P < 0.01$), 34 kDa ($r = 0.93$, $P = 0.02$), and 85 kDa ($r = 0.99$, $P < 0.01$). Unclassified, families Pasturellaceae and Verrucomicrobiaceae showed a positive correlation with xylanase activity at 24 kDa ($r > 0.88$, $P < 0.05$).

Discussion

This study revealed that a bovine rumen microbial community degraded xylan into reducing sugars, VFAs, carbon dioxide gas, and methane gas during the first 24 h of treatment, after which xylan degradation was markedly...
Zhang et al. (2016) treated rice straw with rumen fluid for 72 h and found four degradation phases: an initial exponential phase (0–24 h), a limiting-step phase (24–48 h), a second exponential growth phase (48–72 h), followed by a stationary phase (72–120 h) (Zhang et al., 2016), which are consistent with ours. Our results also showed that the pH decreased from 7.3 to 4.6 during the first 24 h, and xylanase activities of rumen microorganisms markedly decreased after 24 h. Similarly, the alpha diversity of rumen microbial community decreased significantly after 24 h. In a previous investigation of subacute ruminal acidosis, it was reported that the bacterial alpha-diversity was lowered when ruminal pH decreased after cattle transitioned from a high-forage to a high-grain diet (Kim et al., 2018). Primary fibrolytic bacteria such as xylan-degrading bacteria are intolerant

**Fig. 6.** Correlations of xylanase activities with relative abundances of ruminal microorganisms. Square color and size represent the correlation coefficient; blue shading represents a positive correlation, red shading represents a negative correlation, and a larger square represents a stronger correlation, whereas a smaller square represents a weaker correlation. *P < 0.05. Top 15 bacterial genera, top 10 protozoal genera, and top 3 fungal genera are shown.
to the low ruminal pH, and rumen fiber digestion rapidly decreases when the pH drops below 6.0–6.3 (Dijkstra et al., 2012). We assumed that the several genera of rumen microorganisms could not survive and their numbers decreased after 48 h because of the lowered pH, which decreased the alpha diversity of rumen microbial community. In addition, lactic acid production rates increased when the pH drops to 5.0, as lactic acid-producing rumen bacteria are tolerant of low pH compared with other bacteria (Erffle et al., 1982). Therefore, we infer that the decrease in pH during the first 24 h inhibited the xylanolytic microorganisms, but not the VFA-producing microorganisms, resulting in a marked decline in xylan degradation and VFA accumulation after 24 h. These findings suggest that retaining a neutral pH enables xylanolytic microorganisms to degrade xylan throughout the treatment process and yields more efficient xylan biodegradation.

Metagenomic analysis showed that the structure of the rumen microbial community and functional COG shifted throughout the treatment of xylan with rumen fluid, indicating that the microbial community temporally shifted during the xylan biodegradation process. The bacterial phyla Firmicutes and Bacteroidetes were the most dominant and accounted for >50% of the total abundance throughout treatment. Consistent herewith, our previous study reported that Bacteroidetes and Firmicutes were the dominant phyla during the treatment of rapeseed with rumen fluid for 24 h (Baba et al., 2017). In the genus-level bacterial community, the fibrolytic genera Prevotella, Bacteroides, Clostridium, and Ruminococcus were the dominant genera at 0 h. Dai et al. (2015) found that hemicellulase reads in the rumen of Holstein dairy cows fed a corn straw-containing diet were mainly derived from Prevotella, Bacteroides, Fibrobacter, and Clostridium (Dai et al., 2015). Their findings corroborate that the fibrolytic bacterial genera detected in this study play an important role in feed digestion in the rumen fluid collected from Holstein dairy cows. The relative abundances of Selenomonas, Butyryrivibrio, and Pseudobutyryrivibrio increased during the first 12 h. In a previous study, 13 out of 20 Selenomonas ruminantium isolates possessed xylanase activity, while the activities differed among the isolates (Sawanon et al., 2011). Genomic analysis of Butyryrivibrio and Pseudobutyryrivibrio revealed that these genera encoded carbohydrate-active enzymes associated with xylan degradation and suggested that the members of Butyryrivibrio are the predominant xylan degraders (Palevich et al., 2020). Therefore, we infer that Selenomonas, Butyryrivibrio, and Pseudobutyryrivibrio degraded and utilized xylan for growth, which increased the relative abundances of these xylanolytic genera during the first 12 h. The relative abundances of Lactobacillus, Bifidobacterium, and Atopobium increased after 24 h. Several Lactobacillus species produce beta-xylosidase, which hydrolyzes xylooligosaccharides to xylose (Lasrado and Gudipati, 2013; Pontonio et al., 2016) and produces lactic acid from xylose (Okada et al., 1979). Several Bifidobacterium species utilize xylose, xylobiose, xylooligosaccharides, and xylooligosaccharide mixtures mainly composed of xylobiose (Okazaki et al., 1990), and Bifidobacterium bifidum produces lactic acid from xylose (de Vries and Stouthamer, 1968). Atopobium parvulum strain IPP 1126 weakly ferments xylose and produces acid (Copeland et al., 2009). Thus, we speculate that Lactobacillus, Bifidobacterium, and Atopobium utilized xylan metabolites (i.e., xylooligosaccharides and xylose) and produced VFAs, especially lactic acid, after 24 h. The relative abundances of protozoal and fungal genera decreased during treatment. We previously reported that the relative abundances of protozoal and fungal genera significantly decreased during the treatment of carboxymethyl cellulose with rumen fluid for 48 h (Takizawa et al., 2020a,b,c), which was consistent with the present results. Entodinium caudatum, Entodinium exiguum, Epidinium caudatum, and Ophryoscolex purkynei decrease in abundance when the pH decreases below 5.4, whereas they maintain their levels around pH 5.8 (Dehority, 2005). Although Neocallimastix R1 cannot grow in the presence of oxygen, it is sufficiently aerotolerant to survive in the air for a few hours (Trinci et al., 1988). From the present and previous studies, we speculate that the predominant ruminal protozoa and fungi were sensitive to current treatment conditions (i.e., the loss of essential substrates, accumulation of metabolites, low pH, physical shaking, and exposure to oxygen) and therefore declined during xylan treatment with rumen fluid. Taken together, the rumen microbial structure results indicate that the functions of the rumen microbial community temporally shift from xylan degradation to the utilization of xylan metabolites during treatment with rumen fluid. Functional analysis of the rumen microorganisms showed a decrease in xylanase after 24 h and an increase in beta-xylosidase from 12 h to 24 h, which supports the shifts in rumen microbial functions during treatment with rumen fluid.

The individual xylanases in complex rumen microbial ecosystems were visualized by xylanase zymography, which revealed that xylanase activities temporally shifted during the treatment process and significantly decreased after 24 h. We previously treated waste paper with rumen fluid for 48 h and reported that xylanase activity decreased after 24 h (Takizawa et al., 2019), consistently with the current results. In this study, metagenomic analyses showed that the rumen microbial community structure changed, and the relative abundances of xylanases and predicted xylanases declined from 12 to 24 h, indicating that the decrease in xylanolytic
Isotricha 180 kDa No gas production from xylan Takenaka
Eudiplodinium Total, 34, 180 kDa Xylanases of 26, 42, and 54 kDa B
Total, 85, 180 kDa Presence of xylanase activity Takenaka
Dasytricha
Entodinium
Protozoa
Diploplastron 24, 85 kDa Presence of xylanase activity Wereszka
24 kDa Xylanolytic activity has been unclear. DeVillard
200 kDa Xylanolytic activity has been unclear. –
Polyplastron Total, 34, 180 kDa Xylanolytic activity has been unclear. –
Isotrucha 180 kDa Xylanolytic activity has been unclear. –
Methylococccaeae Total, 24, 85 kDa Xylanolytic activity has been unclear. –
Pasteurellaceae 24 kDa Xylanolytic activity has been unclear. –
Verrucicomicrobiaceae 24 kDa Xylanolytic activity has been unclear. –

**Bacteria**

Prevotella Not significant Production of wide range of xylanases Matsui *et al.* (2000)
Bacteroides Negative Transcription of hemicellulases Dai *et al.* (2015)
Clostridium Not significant Transcription of hemicellulases Dai *et al.* (2015)
Ruminococcus Negative Production of several xylanases Saluzzi *et al.* (2001)
Selenomonas Negative Presence of xylanase activity Sawanon *et al.* (2011)
Butyryrivibrio Not significant Predominant xylan degrader Palevich *et al.* (2020)
Pseudoobutyrivibrio Not significant Encode of xylanases Palevich *et al.* (2020)
Bifidobacterium Negative Production of beta-1,4-xylanase Lasrado and Gudipati (2013), Pontonio *et al.* (2016)
Atopobium Not significant Fermentation of xylose Copeland *et al.* (2009)
Fibrobacter Not significant Production of several xylanases Béra-Mailet *et al.* (2004)

**Unclassified bacteria**

Deltaproteobacteria Total, 34, 85 kDa Xylanolytic activity has been unclear. –
Epsilonproteobacteria 200 kDa Xylanolytic activity has been unclear. –
Clostridiales Total, 34, 180 kDa Xylanolytic activity has been unclear. –
Bacilliaceae 200 kDa Xylanolytic activity has been unclear. –
Methylococccaeae Total, 24, 34, 85 kDa Xylanolytic activity has been unclear. –
Pasteurellaceae 24 kDa Xylanolytic activity has been unclear. –
Verrucicomicrobiaceae 24 kDa Xylanolytic activity has been unclear. –

**Protozoa**

Entodinium 24 kDa Presence of xylanase activity Takenaka *et al.* (2004)
Diploplastron 24, 85 kDa Presence of xylanase activity Wereszka *et al.* (2006)
Polyplastron 24 kDa Xylanases of 26, 32, 44, and 50 kDa Devillard *et al.* (1999), Béra-Mailet *et al.* (2005)
Isotrucha 180 kDa No gas production from xylan Takenaka *et al.* (2004)
Eudiplodinium Total, 34, 180 kDa Xylanases of 26, 42, and 54 kDa Béra-Mailet *et al.* (2005)
Dasytricha Total, 34, 180 kDa Presence of xylanase activity Takenaka *et al.* (2004)
Epidinium 200 kDa Presence of xylanase activity Takenaka *et al.* (2004)
Didinium 24 kDa Xylanolytic activity has been unclear. –

**Fungi**

Neocallimastix 24, 85 kDa Production of xylanases Krause *et al.* (2003)
Orpinomyces 24, 85 kDa Production of xylanases Krause *et al.* (2003)
Olpidium 24 kDa Xylanolytic activity has been unclear. –

This study

Positive correlation

Previous study

Reported xylanolytic function

Reference

Table 2. Correlation of dominant microorganisms in this study and their function.

| Genus          | This study   | Previous study                              | Reference               |
|----------------|--------------|---------------------------------------------|-------------------------|
| Prevotella     | Not significant | Production of wide range of xylanases         | Matsui *et al.* (2000)  |
| Bacteroides    | Negative     | Transcription of hemicellulases              | Dai *et al.* (2015)     |
| Clostridium    | Not significant | Transcription of hemicellulases              | Dai *et al.* (2015)     |
| Ruminococcus   | Negative     | Production of several xylanases              | Saluzzi *et al.* (2001) |
| Selenomonas    | Negative     | Presence of xylanase activity                | Sawanon *et al.* (2011) |
| Butyryrivibrio | Not significant | Predominant xylan degrader                  | Palevich *et al.* (2020) |
| Pseudoobutyrivibrio | Not significant | Encode of xylanases                        | Palevich *et al.* (2020) |
| Bifidobacterium| Negative     | Production of beta-1,4-xylanase              | Lasrado and Gudipati (2013), Pontonio *et al.* (2016) |
| Atopobium      | Not significant | Fermentation of xylose                      | Copeland *et al.* (2009) |
| Fibrobacter    | Not significant | Production of several xylanases              | Béra-Mailet *et al.* (2004) |

Microorganisms resulted in low xylanase activity after 24 h. An assessment of protein expression revealed that the protein at 85 kDa was abundant during the first 12 h. Quantification of band densities also showed that xylanases of 24, 34, 85, and 200 kDa were highly active during the period in which xylan was efficiently degraded into VFAs, carbon dioxide gas, and methane gas. Our results indicated that these xylanases, especially 85 kDa of xylanase, contributed to xylan biodegradation during treatment with rumen fluid. As mentioned above, a previous metatranscriptomic analysis revealed that two-thirds of putative hemicellulases were produced by members of the genera *Ruminococcus*, *Prevotella*, and *Fibrobacter* (Dai *et al.*, 2015). *Ruminococcus* produces xylanases of 32, 85, and 210 kDa (Saluzzi *et al.*, 2001). *Prevotella* produces a wide range of xylanases from 60–170 kDa (Matsui *et al.*, 2000), and *Fibrobacter* produces several xylanases between 35 kDa and 130 kDa (Béra-Mailet *et al.*, 2004). Therefore, we hypothesize that these three genera produce high-activity xylanases and contribute to xylan degradation during treatment with rumen fluid. The relative abundances of *Selenomonas*, *Butyryrivibrio*, and *Pseudobutyryrivibrio* increased during xylan degradation, which indicated that these xylanolytic genera also contributed to xylan degradation. However, correlation analyses of xylanase activities with the relative abundances of rumen microorganisms indicated that xylanolytic bacterial genera, namely, *Ruminococcus*, *Prevotella*, *Fibrobacter*, *Butyryrivibrio*, *Selenomonas*, *Butyryrivibrio*, and *Pseudobutyryrivibrio*, did not contribute to xylanase activity (Table 2). Investigation of rumen microorganisms during *in situ* incubation of switchgrass revealed that changes in the microbiome were particularly prevailing within 30 min and after 4 h of rumen incubation (Piao *et al.*, 2014). Therefore, we infer that the xylanolytic bacterial genera produced high-activity xylanases and contributed to xylan degradation during the initial treatment phase (from 0 h to 6 h). Of note, positive correlations between xylanolytic bacterial genera and xylanase activities might have been observed in a more detailed microbial analysis. Further investigation of the temporal dynamics of xylanolytic bacterial genera is needed to elucidate the contribution of bacterial genera.
to xylan degradation during treatment with rumen fluid. Several genera of rumen protozoa and fungi showed significantly positive correlations with xylanase activities. The functional roles of rumen protozoa and fungi in ruminal fermentation and carbohydrate metabolism remain obscure. Rumen protozoa account for a large portion of the biomass in the rumen microbial community. *Dasytricha ruminantium*, *Epidinium caudatum*, *Polyplastron multivesiculatum*, and *Entodinium* spp. have xylanase activity (Takenaka et al., 2004). *Diploplastron affinis* shows the xylanolytic activity and produces at least two xylanases (Werezska et al., 2006). *Polyplastron multivesiculatum* produces xylanases of 26, 32, 38, and 52 kDa (Devillard et al., 1999; Béra-Maillet et al., 2005), and *Eudiplodinium maggi* produces xylanases of 26, 42, and 54 kDa (Béra-Maillet et al., 2005). Rumen fungal species, such as *Neocallimastix frontalis*, *Neocallimastix particiarum*, and *Orpinomyces* sp., produce endoxylanases and degrade lignified tissue that is not degraded by other microorganisms (Krause et al., 2003). Our results suggest that rumen protozoa and fungi contribute to xylanase activity and xylan biodegradation during treatment with rumen fluid. Interestingly, unclassified bacteria positively correlated with xylanase activities. Although the xylanolytic function of unclassified bacteria remains unknown, unclassified bacteria are considered to produce various xylanases and contribute to the degradation of lignocellulosic biomass. Metagenomic analysis revealed that a high proportion of unclassified bacteria were attached to rice straw throughout incubation in the rumen (Cheng et al., 2017). A metatranscriptomic analysis showed that most of the transcripts encoding putative proteins involved in the degradation of plant cell-wall polysaccharides were < 90% similar to known species at the amino acid sequence level (Dai et al., 2015). In this study, unclassified bacteria possibly played an important role in xylan degradation during treatment with rumen fluid. Altogether, our results suggest that rumen protozoa (*Entodinium*, *Diploplastron*, *Polyplastron*, *Isotricha*, *Eudiplodinium*, *Dasytricha*, *Epidinium*, and *Didinium*), fungi (*Neocallimastix*, *Orpinomyces*, and *Olpidium*), and unclassified bacteria (*Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridiales*, *Bacillaceae*, *Methylcoccaceae*, *Pasteurellaceae*, and *Verrucomicrobiaceae*) are associated with xylanase activities of 24, 34, 85, 180, and 200 kDa, which results in xylan biodegradation into VFAs and gas during the treatment with rumen fluid.

In conclusion, the results of this study suggested the relationships among the changes in xylan degradation, xylanase activities, microbial function, and microbial structure (Fig. 7). The chemical analysis revealed that the rumen microorganisms degrade xylan into reducing sugars, VFAs, carbon dioxide gas, and methane gas during the first 24 h, whereas this activity was inhibited at 48 h of xylan treatment with the rumen fluid. Zymography showed that xylanase activities temporally shifted during treatment. Xylanases were active during the first 12 h of treatment; however, their activities considerably decreased after 24 h. The metagenomic analysis indicated a decrease in functional COG annotated as xylanase and a change in the structure of microbial community during 24 h. The relative abundance of several protozoan and fungal genera, as well as unclassified bacteria, showed a positive correlation with the xylanase activities. Taken together, our study suggests that several protozoa (*Entodinium*, *Diploplastron*, *Polyplastron*, *Isotricha*, *Eudiplodinium*, *Dasytricha*, *Epidinium*, and *Didinium*), fungi (*Neocallimastix*, *Orpinomyces*, and *Olpidium*), and unclassified bacteria (*Deltaproteobacteria*, *Epsilonproteobacteria*, *Clostridiales*, *Bacillaceae*, *Methylcoccaceae*, *Pasteurellaceae*, and *Verrucomicrobiaceae*) are associated with the xylanase activities and xylan degradation into sugars, VFAs, and gas during the 24 h treatment with rumen fluid. Although this study focused on the association between the relative abundance of rumen microorganisms and xylanase activities, the absolute abundance of rumen microorganisms and xylanase expression are also important for determining key xylanolytic microorganisms. To identify the key microorganisms contributing to xylan degradation, quantitative and metatranscriptomic analysis are required. Our findings suggest that supplementation and activation.

Fig. 7. Summary of xylanase degradation suggested in this study.
of pivotal microorganisms associated with xylan degradation can effectively boost lignocellulosic biomass bioconversion into bioenergy.

**Experimental procedures**

*Treatment of xylan with rumen fluid*

All experiments were approved and performed in accordance with the regulations of the Institutional Animal Care and Use Committee of Tohoku University. A Holstein cow was fed a diet consisting of 64% timothy grass and 36% concentrate and allowed *ad libitum* access to water. The rumen fluid was collected from the Holstein cow at 2 h post-feeding, using a stomach tube. The collected rumen fluid was transferred to the laboratory within 30 min, and then filtered through a 1 x 1-mm mesh to remove coarse solids. After the filtration, the rumen fluid was immediately used for treatment of xylan.

Treatment with the rumen fluid was conducted according to our previous studies (Takizawa et al., 2018, 2020a,b,c). Xylan powder obtained from corn cores (Tokyo Chemical Industry UK, Tokyo, Japan) was used as a substrate. Four grams of xylan powder were mixed with 200 ml of rumen fluid and purged with nitrogen gas to remove oxygen. Xylan treatment was conducted in a 250-ml reactor on a rotary shaker at 170 r.p.m. at 37°C for 48 h. A blank reactor containing only rumen fluid was incubated under the same conditions. All incubations were conducted in duplicate.

*Chemical analysis*

To evaluate xylan biodegradation rate into metabolic products, TS, reducing sugars, VFAs, and gas production were determined as described previously (Takizawa et al., 2020). Briefly, TS were measured after drying the rumen samples at 105°C overnight. Liquid samples were filtered using a cellulose acetate membrane (0.45 µm pore diameter). Reducing sugars were determined by the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1945) using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). VFA concentrations were determined by high-performance liquid chromatography (Jasco, Tokyo, Japan) using an ion-exchange column (RSpak KC-811; Jasco). The pH was measured with a pH meter (LAQUAtwin; HORIBA, Tokyo, Japan). Gas concentrations (methane and carbon dioxide) were measured by gas chromatography (GC-8A; Shimadzu) with a packed column (Shincarbon-ST; Restek, Bellefonte, PA, USA). A unit equipped with a thermal conductivity detector was connected to an integrator (C-R8A; Shimadzu). Argon gas was used as the carrier gas, and the temperature of injection and detection was set to 100°C and 120°C respectively.

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography*

The rumen fluids were mixed with equal volumes of 2× radioimmunoprecipitation assay buffer (Nacalai Tesque, Kyoto, Japan) containing 2 mM of phenylmethylsulfonyl fluoride and 1× proteinase inhibitor (Sigma-Aldrich, Tokyo, Japan). Proteins were extracted using Lysing Matrix E tubes (MP Biomedicals, Solon, OH, USA). One milliliter of supernatant was mixed with 100 µl of trichloroacetic acid, incubated on ice for 30 min, and centrifuged at 17 000 g for 10 min. After removing the supernatant, 1 ml of acetone at −20°C was mixed with the pellet, and the suspension was centrifuged at 17 000 g for 10 min. This process was repeated, and the pellets were dried at room temperature. The dried pellets were solubilized in 1 ml of 2× sample buffer comprising 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, 0.125 M Tris-HCl (pH 7.4), 10% (w/v) 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and 1× proteinase inhibitor (Sigma-Aldrich). SDS-PAGE and zymography were conducted according to a previous study (Takizawa et al., 2020a,b,c), with some modifications. Briefly, the supernatants were heated at 70°C for 20 min and immediately cooled on ice. Twenty micro-liters of protein extract were separated on an 8% polyacrylamide gel containing 1.0% (w/v) xylan from beechwood (Nacalai Tesque) at 200 V for 60 min. Separated proteins were refolded in 2.5% (v/v) Triton X-100 for 60 min. The gel was soaked in 50 mM of sodium acetate buffer (pH 6.5) for 15 min, followed by incubation in 30 mM of sodium acetate buffer at 37°C for 6 h (observed pH values during treatment were applied for zymography). After incubation, the gel was soaked in distilled water at 4°C and then in 30 mM of sodium acetate buffer (pH 6.5). The gel was stained with 0.1% (w/v) Congo Red and de-stained with 1 M NaCl to visualize endoglucanase activity. Next, the gel was soaked in 0.3% (v/v) acetic acid to enhance the intensity of active bands. Band densities were quantified using the ImageJ software (Schindelin et al., 2012). In addition, SDS-PAGE was performed as mentioned earlier, and the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue to investigate the protein expression during the treatment of xylan.

*Metagenomic analysis*

DNA extraction and metagenomic sequencing were carried out as previously described (Takizawa et al., 2020a,b,c). Briefly, total DNA was extracted from 2 ml of rumen fluid using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer’s instructions. DNA libraries were constructed using the QiAseq FX DNA
Library Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The sizes of the constructed DNA libraries were determined with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) using the Agilent High Sensitivity DNA Kit (Agilent). Paired-end sequencing was conducted on an Illumina HiSeq X instrument (Illumina, San Diego, CA, USA).

Paired-end sequences were uploaded to Metagenome Rapid Annotation using Subsystem Technology server version 4.0.3 (Meyer et al., 2008) for functional and taxonomic annotations. After quality filtering, a total of 137 048 176 sequences (average 13 704 818; minimum 9 942 341; maximum 17 581 176) were obtained from the samples. Hierarchical functional predictions were performed against the COG database (E value of < 10⁻⁶, identity cut-off of > 60%, and alignment length of > 30).

To explore the rumen microbial community structure, rRNA sequences were identified by BLAST searching against the Silva SSU database (E value of < 10⁻⁶, alignment length of > 15 bp) on Metagenome Rapid Annotation using Subsystem Technology. Sequences are available at Metagenome Rapid Annotation using Subsystem Technology under the project accession mgp88122.

Statistical analysis

Pearson’s correlation coefficient and significance levels were evaluated and illustrated using the R package CORRPLOT, version 0.84 (Wei and Simko, 2017), to determine the relationships between endoglucanase band densities and relative abundances of ruminal microorganisms. Good’s coverage was calculated using the R package QsRUTLs, version 0.1.4 (John, 2020). Alpha (choa1, ACE, Shannon, and Simpson) and beta diversity analyses involving principal coordinate analysis based on Bray–Curtis dissimilarity were conducted using the R package VEGAN, version 2.5.6 (Jari et al., 2019). Multiple comparisons were performed according to the Tukey–Kramer method using the R package MULTICOMP, version 1.4.13 (Hothorn et al., 2008), and statistical significance was declared at P < 0.05.

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Conflict of interest

The authors declare no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Changes in chemical characteristics of the rumen fluid in a blank (containing only the rumen fluid). (A) Reducing sugars; (B) VFAs; (C) pH; and (D) carbon dioxide and methane gas production. Multiple comparisons were conducted using the Tukey–Kramer method, and different letters indicate a statistically significant difference ($P < 0.05$).

Fig. S2. Taxonomic composition of the domain-level (A) and phylum-level (B) community. The relative abundances of predominant phyla were shown. All abundances represent the mean of two reactors.

Fig. S3. Changes in the structure of level-1 COG (A), level-2 COG involved in metabolism (B) during the treatment of xylan with the rumen fluid. All values represent the mean of two reactors.

Fig. S4. Xylanase activity in the blank. The pH during zymography of xylan treatment was 7.0 (see Fig. S1). Twenty microliters of protein extract were loaded on an 8% polyacrylamide gel containing 1.0% (w/v) xylan from beech-wood, and incubations for xylanase zymograms were conducted at 37°C for 6 h. Gel images were cropped from different parts of the same gel.

Fig. S5. SDS-PAGE of proteins extracted from rumen microbial community in the treatment of xylan (A) and the blank containing only the rumen fluid (B). Twenty microliters of the protein extract were loaded on 8% polyacrylamide gel and stained with 0.1% Coomassie Brilliant Blue solution.

Fig. S6. Total and five xylanases activities that had a high peak density during the treatment of xylan with the rumen fluid.

Fig. S7. Correlations of xylanase activities with relative abundances of unclassified microorganisms. Square color and size represent each correlation coefficient; blue shading represents a positive correlation, red shading represents a negative correlation, and a larger square represents a stronger correlation, whereas a smaller square represents a weaker correlation. *$P < 0.05$.

Table S1. Alpha diversity during the treatment of xylan with the rumen fluid. All data represent the mean ± standard error. Multiple comparisons were conducted using the Tukey–Kramer method, and different letters indicate a statistically significant difference ($P < 0.05$).