Effects of algae derived pure β–Glucan on *In vitro* rumen fermentation

Ekin SUCU[1,a,✉], Füsun (AK) SONAT[2,b]

[1]Department of Animal Science, Bursa Uludağ University Faculty of Agriculture, Bursa 16059, Türkiye; [2]Department of Physiology, Bursa Uludağ University Faculty of Veterinary Medicine, Bursa 16059, Türkiye

[a]ORCID: 0000 0003 1470 2751, [b]ORCID: 0000 0002 3308 0778

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✉Corresponding author
ekins@uludag.edu.tr

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ABSTRACT

The major purpose of this study was to determine how varying doses of algae-derived pure β–glucan affected *in vitro* gas generation, volatile fatty acid (VFA) concentrations, methane production, and protozoa populations. Different doses of β–glucan [i.e., 0, 50, 100, 150, and 200 mg/kg feed (DM basis)] were applied to corn silage as experimental treatments. After 6–96 hours of incubation, the dose of 200 mg/kg of DM β–glucan reduced total gas production compared to control (P<0.01). The concentration of total VFA decreased quadratically (P<0.01) as the amount of β–glucan inclusion decreased (except for 200 mg/kg DM) when compared to the control group. The total VFA concentration was found to be the lowest (P<0.01) at 50, 100, and 150 mg/kg DM β–glucan than the other doses. Propionate and butyrate concentrations increased linearly (P<0.01) in the β–glucan supplemented groups, except for the 50 mg/kg DM dosage. When compared to the control group, all doses of β–glucans lowered acetate and the acetate: propionate ratio linearly and quadratically (P<0.01). The addition of β–glucans reduced the number of protozoa linearly (except at the lowest dose) and reduced the methane generation linearly and quadratically (P<0.01). The concentration of NH_3-N did not differ (Linear, P=0.12; Quadratic, P=0.19) between treatments. The key findings were that β–glucan acted as a rumen modulator, and levels of more than 50 mg/kg of feed DM functioned as a potential methane regulator in the rumen due to reduced acetate and acetate to propionate ratio.

Introduction

Antibiotics have been widely used in animal production as low-dose feed additives with effects on growth and feed conversion efficiency, as well as to reduce infections. Antibiotics have been restricted in the majority of countries due to concerns about the spread of resistant bacteria (1, 11). As a result, nonantibiotic feed additives have emerged as an important research topic. β–glucans are seen as a new generation of promising feed supplements (7, 9, 19, 20, 27). β–glucans contain natural polysaccharides with glucose as their basic elements that are connected by beta-linkages. These polysaccharides not only engage easily with immune cells, but they can effectively bind bacteria, preventing pathogen adhesion and colonization in the intestinal system (27). Plants, yeasts, fungi, algae, protozoa, and bacteria all contain β–glucans (12). Previous research has primarily focused on the impact of nutritional β–glucan administration on poultry and pig growth parameters or immunity (7, 9, 20, 32). Specific findings in studies examining the starter concentrate of β–glucan supplementation revealed immune system stimulation (33), an increase in *Lactobacillus* and fibrolytic bacteria, and a decrease in archaea (16). The addition of yeast β–glucan to milk replacer improved nutrient digestibility, villous height to crypt depth ratio, and immune system performance in calves (19). According to Grove et al. (14), the benefits of barley β–glucans inclusion in beef cattle include altering the rumen bacteria to speed up feed digestion. Ma et al. (19) found that supplementing pre-ruminant calves with yeast β–glucan at 75 mg/kg of feed increased nutrient digestion, strengthened immunity by increasing antibody

concentration and had no negative impacts on metabolism. The addition of yeast glucan to the diet of ewes at a rate of 3 g/kg increased milk production, milk fat percentage, and protein percentage by 13.5–14%, 15–30%, and 11%, respectively (35). A recent study (15) found that feeding yeast β–glucans to lambs improves feed utilization. However, most of the commercially available β–glucans have been derived from yeasts and cereals. β–glucan polysaccharides derived from algae are found in red, green, and brown algae seaweed in the forms of starch and cellulose, laminarin, fibers, and foridean starch, respectively, and are considered superior bioactive molecules. When compared to yeast-derived products, algal-sourced β–glucan has various advantages, including being a more concentrated source with over 95 percent β–glucan, compared to 60-80 percent in purified yeast products. Algal-sourced glucan is predominantly β–1,3 glucan, whereas yeast-based products are a combination of 1,3 and 1,6 glucan, which affects bioavailability. Linear β–1,3 glucan and the small particle size interact more directly with the immune cells (10). The role of pure β–glucan produced from algae in modifying ruminal fermentation has not been adequately investigated. The mechanisms of algae-derived pure β–glucan actions on ruminal metabolic activities, rumen pH, volatile fatty acid (VFA) concentrations, NH_3-N amounts, methane generation, and protozoa counts in cattle are yet unknown. As a result, additional research is required. In this study, we utilized algae-derived pure β–glucan and investigated the effectiveness of different doses on the *in vitro* rumen fermentation pattern and methane production.

Materials and Methods

To monitor rumen fermentation parameters over time, an *in vitro* ruminal fermentation trial was carried out. Rumen fluid was obtained before morning feeding from ruminally cannulated 600 kg Holstein non-lactating dairy cows (n=2). To ensure that the rumen fluid had a balanced cellulolytic and amylolytic activity, the donor cows were fed a 50/50 mix of corn silage and concentrate. Rumen fluid was immediately transferred to the laboratory after being placed in a warm Thermos flask (39 °C). Rumen fluid was squeezed through cheesecloth and placed in an erlenmeyer flask (39 °C). Menke and Steingass (23) method was used to make a buffer combination (comprising micro-and macro-elements, a reducing agent, and a resazurin reduction indicator) for *in vitro* rumen fermentation. Particle-free rumen fluid (15 mL) and buffer medium (25 mL) were mixed in a warmed bottle (39 °C) and gassed with CO_2 on a continual basis. As incubation vessels, glass syringes (Fortuna®, Häberle Labortechnik, Germany) with a calibrated volume of 100 ml were employed. In each syringe, a total of 40 mL of rumen fluid–buffer medium mixture was provided. Approximately 300 mg of dry feed sample was contained in each syringe. Five doses of algae-derived pure β–glucan (Sigma-Aldrich - 89,862–5G-F), i.e. 0, 50, 100, 150, and 200 mg/kg feed (DM basis), were added to corn silage as experimental treatments. Corn silage was used as the rumen fermentation substrate. The nutrient composition of corn silage (Table 1) was performed according to AOAC (2). Metabolizable energy (ME) and net energy of lactation (NEL) values in corn silage were calculated using the equations of Menke and Steingass (22). ME (MJ/kg DM) = 0.136 GP + 0.0057 CP + 0.000286 EE2 + 2.20 and NEL (MJ/kg DM) = 0.096 GP + 0.0038 CP + 0.000173 EE2 + 0.54, where GP is 24-h net gas production (mL 300 mg/ DM), CP and EE, are crude protein and ether extract (% DM), respectively. *In vitro* incubation of the samples was carried out in triplicate. Triplicates of bottles with no substrate were used as blanks. The volume of total gas produced at 3, 6, 12, 24, 48, and 96-hour periods were recorded. The produced gas quantities were calculated according to the model developed by Ørskov and McDonald (25) in the Neway computer program.

Table 1. Nutrient composition of substrate (corn silage) used for incubation (% of DM).

Variable	Corn Silage	SE Mean	StDev
OM	94.38	1.19	2.06
CP	7.18	0.01	0.03
CA	5.64	0.05	0.08
Fat	4.21	0.08	0.14
NDF	40.33	0.36	0.63
ADF	26.31	0.50	0.86
ADL	2.27	0.15	0.25
HSEL	14.02	0.84	1.45
OMD (%)	50.16	2.03	3.51
ME (MJ/kg DM)	7.20	0.31	0.53
NEL (MJ/kg DM)	4.08	0.22	0.37

SEM – standard error of the mean; OM – organic matter; CP, crude protein; CA, crude ash; NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; HSEL –hemicellulose; OMD– organic matter digestibilty; ME – metabolizable energy, NEL – net energy of lactation.

96 hours of gas production were used in 100 mL glass syringes to enable CH_4 measurement and analysis, as described by Kinley et al. (17). After 96 hours of incubation, rumen fluid samples were collected to be analyzed for pH, NH_3-N, VFA, and protozoa. A pH meter (Sartorius PB-20, Germany) was used to measure the pH of the rumen fluid. For NH_3-N and volatile fatty acids (VFA) analysis, each syringe's incubation residue was transferred to a 20-ml centrifugation tube and centrifuged for 15 minutes at 15 000 g at 4°C. Ammonia-N was

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determined according to AOAC (2) using a Kjeltech auto-analyzer (Gerhardt, Bonn, Germany) without a digestion step. The VFA analysis was done in centrifuged media after adding 1 mL supernatant to 0.25 mL meta-phosphoric acid (25 percent, v/v) with an Acclaim 4x250 mm organic acid column using HPLC (ICS 3000, Dionex Corporation, San Francisco, CA). For counting protozoa; one ml of rumen fluid was mixed with 49 ml of rumen protozoa counting solution (2.02 % formalin and 15.15 % glycerol) to determine the counts of rumen protozoa. Diluted ruminal fluid samples were used for counting cells with the help of the Fuchs Rosenthal counting chamber by the method of Boyne et al. (6).

The data were subjected to SAS's GLM analytical processes (Statistical Analysis System, Version 9.1, 2003). To assess the linear and quadratic effects of supplementary algae-derived β–glucan, orthogonal contrast was used. When $P<0.05$, differences between treatments were regarded as significant, and when $0.05<P<0.10$, they were considered tended to be significant.

Results

To evaluate the ruminal fermentation pattern, pH value, and *in vitro* gas production at various hours (Table 2, Figure 1) and quantities of total VFA production and VFA profile, NH_3-N, methane production, and protozoa counts in rumen fluid at 96 h were monitored (Table 3). Rumen pH ranged from 6.82 to 6.93 (Table 2). The highest ($P<0.01$) rumen pH was found at 100 and 200 mg/kg DM β–glucan when compared to the control group. The effects of other doses on rumen pH were found to be insignificant ($P>0.05$). The β–glucan treatment did not affect gas production at 3 hours ($P>0.05$). After 48-96 hours of incubation, the dose of 200 mg/kg DM β–glucan reduced total gas production more than the other doses ($P<0.01$, Table 2, Figure 1). At 6, 24, and 72 hours, 50, 100, and 150 mg/kg DM β–glucan doses did not affect gas production ($P>0.05$) when compared to the control group. However, at 12 and 48 hours, all β–glucan dosages reduced gas production linearly ($P<0.01$). The total VFA concentration decreased quadratically ($P<0.01$) in the β–glucan supplemented groups, while the highest dose (200 mg/kg DM) had no effect. The total VFA concentration at 200 mg/kg DM β–glucan was higher ($P<0.01$) than only 50 mg/kg DM β–glucan. Propionate and butyrate concentrations were linearly elevated ($P<0.01$) in the β–glucan supplemented groups while no effect was observed at the lowest dose (50 mg/kg DM). All doses of β–glucan linearly increased ($P<0.01$) isobutyrate, valerate, and isovalerate concentrations (Table 3). All doses of β–glucans reduced the acetate and the acetate: propionate ratio linearly and quadratically ($P<0.01$) as compared to the control group. The concentration of NH_3-N did not differ (Linear, $P=0.12$; Quadratic, $P=0.19$) between treatments (Table 3). The addition of β–glucans, reduced the number of protozoa linearly (except at the lowest dose) and methane production decreased linearly and quadratically ($P<0.01$, Table 3).

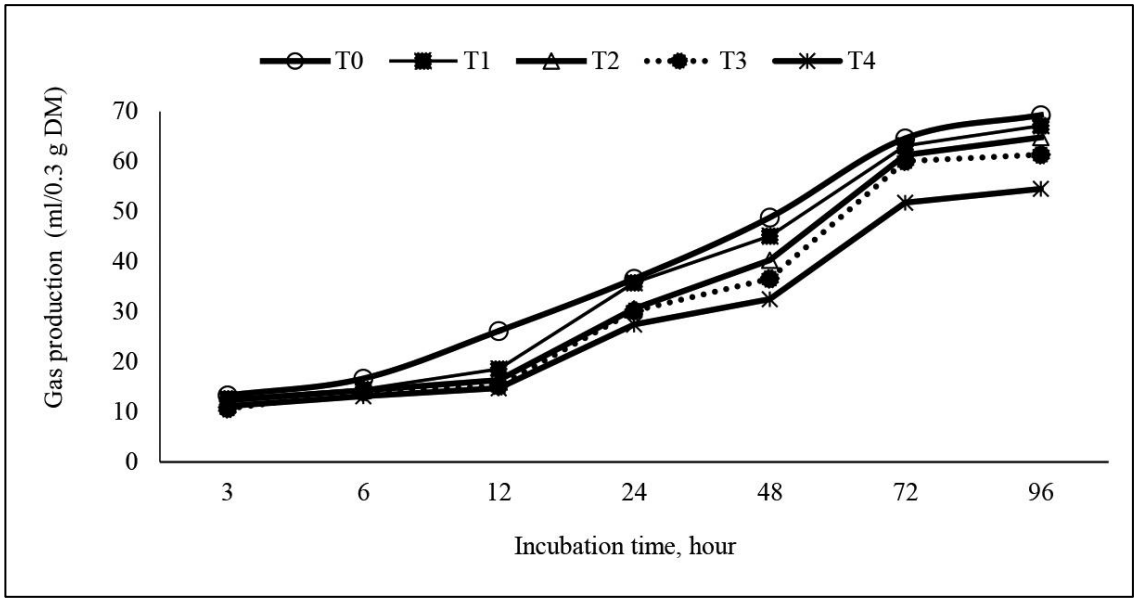

Figure 1. The total gas production (ml/0.3 g DM) from *in vitro* fermentation of corn silage with various β–glucan dosages [T0, T1, T2, T3 and T4 represent the doses of 0, 50, 100, 150, and 200 (mg/kg DM basis), respectively].

Table 2. Effects of different β–glucan levels on *in vitro* ruminal pH and total gas production (ml/0.3 g DM).

							P value	
Variables	T0	T1	T2	T3	T4	StDev	Linear	Quadratic
pH	6.82b	6.85b	6.93a	6.88ab	6.93a	0.03	*	0.30
3 h	13.33	12.67	12.33	10.67	11.17	1.64	0.32	0.77
6 h	16.67a	14.50ab	14.33ab	14.00ab	13.17b	1.10	*	0.26
12 h	26.17a	18.61b	16.34b	15.17b	14.78b	1.58	*	*
24 h	36.60a	35.83a	30.61ab	30.17ab	27.50b	2.63	*	0.81
48 h	48.84a	45.17b	40.35c	36.60d	32.56e	1.09	*	0.89
72 h	64.65a	63.17a	61.33a	60.06a	51.83b	1.98	*	*
96 h	69.30a	67.17a	64.84ab	61.40b	54.56c	1.96	*	*

T0, T1, T2, T3 and T4 represent the doses of 0, 50, 100, 150, and 200 (mg/kg DM basis), respectively.
The differences between the averages indicated with different letters on the same row are significant (P<0.05).

Table 3. Effects of different β–glucan levels on *in vitro* ruminal VFA, NH_3-N concentration, methane production, and protozoa count.

							P value	
Variables	T0	T1	T2	T3	T4	StDev	Linear	Quadratic
Total VFA (mM)	113.74a	105.19c	107.43bc	108.83bc	110.69ab	1.55	0.70	*
Acetate (A, mM)	63.55a	53.53b	51.17bc	52.68bc	50.79c	0.92	*	*
Propionate (P, mM)	35.33c	36.01c	38.99b	38.72b	41.18a	0.80	*	0.89
Butyrate (mM)	9.80c	10.31bc	11.34ab	11.62a	12.42a	0.44	*	0.87
Isobutyrate (mM)	3.24d	3.41c	3.80b	3.72b	4.03a	0.04	*	0.42
Valerate (mM)	0.51d	0.53c	0.59b	0.58b	0.63a	0.01	*	0.42
Isovalerate (mM)	1.32d	1.39c	1.54b	1.51b	1.64a	0.02	*	0.42
A/P	1.80a	1.49b	1.31cd	1.36c	1.23d	0.03	*	*
NH_3-N (mM)	5.37	5.78	5.90	5.89	5.85	0.37	0.12	0.19
Methane (mM)	42.23a	39.00b	39.15b	38.28b	38.12b	0.56	*	*
Protozoa (log 10/mL)	5.65a	5.66a	5.54b	5.56b	5.52c	0.06	*	0.75

T0, T1, T2, T3 and T4 represent the doses of 0, 50, 100, 150, and 200 (mg/kg DM basis), respectively.
The differences between the averages indicated with different letters on the same row are significant (P<0.05).

Discussion and Conclusions

The current study shows that algae-derived β–glucan influences ruminal fermentation, which has previously been demonstrated *in vivo* with yeast-derived β–glucan (8, 16, 19). In this study, the dose level had a substantial impact on gas generation from 3 to 96 hours following the addition of the β–glucan. Although β–glucan doses of 50 and 100 mg/kg did not affect gas generation (except at 12 and 48 hours), the 150 and 200 mg/kg doses significantly reduced overall gas production in the rumen in 12, 48, and 96 hours. Gas release, which results from microbial degradation of the substrate and buffering of acids produced during ruminal fermentation, gives us a brief evaluation of gluconeogenesis (23, 34). The amount of gas produced is proportional to the amount of VFA formed. The majority of the gas is produced during the fermentation of substrate to acetate and butyrate. Propionate production is associated with relatively lower gas production because substrate fermentation to propionate only produces gas from acid buffering (34). Indeed, the current study found that β–glucan supplementation reduced acetate and increased propionate concentrations (Table 3), resulting in a reduction in gas production (Table 2). The increased rate of rumen passage and the kind of VFA produced both contribute to the rate of CH_4 generation by ruminants. Some dietary additives may promote rumen fermentation and digestion while decreasing rumen CH_4 production (5). β–glucans could be predicted to modify and assist rumen microorganisms to expedite fiber digestion and hence increase the generation of volatile fatty acids (14). In contrast to the previous research, the reduction of total VFA production at 50, 100, and 150 mg/kg doses of β–glucan in the current study may be linked to decreased nutrient digestibility and changes in the composition of ruminal microbiota (30). This decrease in total VFA may be unintended or a side effect of feed additives. This warrants further investigation. The primary gases produced during the fermentation inside the

rumen are methane, CO_2, and H_2 (3, 21). The reduction of gas production and decreased proportional CH_4 production in the current study show that β–glucan addition holds promise in reducing CH_4 production and, thus, CH_4 emissions in ruminants. Polyorach et al. (26) discovered that supplementing *Saccharomyces cerevisiae* reduced *in vitro* CH_4 generation, which applies to the present observations. Methanogenic microorganisms are known on the outer layer of rumen ciliate protozoa (31) and as symbionts within the ciliates (13). Methanogens associated with ciliate protozoa were estimated to be responsible for 9 to 25% of methanogenesis in rumen fluid by Newbold et al. (24), and protozoa removal from the rumen (defaunation) has been linked to lower methane generation (29). In the current study, β–glucan supplementation decreased the number of protozoa, which resulted in a decrease in CH_4 production, which agrees with the above statement.

β–glucan doses of 100 and 200 mg/kg DM increased rumen pH, while doses of 100, 150, and 200 mg/kg DM reduced protozoa count to levels acceptable for rumen microbial feed digesting activity but had no effect on ruminal ammonia concentrations (Table 2 and 3). Rumen pH is primarily determined by diet characteristics (chemical and physical) and can range from 5.6 to 7.5 in response to different feedstuffs (18). In contrast to our findings, Cherdthong et al. (8) found that yeast β–glucan supplementation did not affect rumen pH but increased protozoa population at 4 hours of ingestion, with 4.7 g of β–glucan resulting in the largest population. These effects appear to be diet-dependent, with higher responses on high-concentrate diets compared to high-forage diets (28) and differences in derivation source. Similar to our findings, Cherdthong et al. (8) discovered that yeast β–glucan supplementation did not affect ruminal ammonia concentrations.

After 96 hours of *in vitro* rumen fermentation, the addition of algae-derived β–glucan to corn silage significantly impacted total gas production, acetic, propionic, and butyric acid content, the A:P ratio, and CH_4 generation. The addition of β–glucan did not affect the concentration of NH_3-N. The addition of β–glucan dramatically lowered acetate concentrations compared to the control, while significantly increasing propionate, butyrate, isobutyrate, valerate, and isovalerate relative content and lowering the A:P ratio.

The research indicates that β–glucan is performed as a rumen modulator and that levels more than 50 mg/kg feed DM served as a possible methane regulator in the rumen due to lower acetate and acetate to propionate ratios. These findings may also benefit animal performance. Propionate and acetate are in a delicate balance, with butyrate formation playing a key role in methanogenic archaea's H_2 availability. The redirection of metabolic hydrogen to propionate was thought to be a CH_4-blocking strategy (4). However, more research is needed to determine the ideal *in vivo* dose in units of the algae-derived β–glucan, to address the potential impacts on rumen fermentation, animal health, and product quality (milk and meat), and to demonstrate advantages in animal performance and methane emissions.

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Conflict of Interest

The authors are declared that there is no conflict of interest.

Author Contributions

ES conceived and planned the experiments. ES carried out the experiments. FAS helped to identify rumen protozoa. ES and FAS contributed to the interpretation of the results. ES took the lead in writing the manuscript. FAS helped with manuscript editing and proofreading. All authors provided constructive feedback and contributed to the development of the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was carried out after the animal experiment was approved by Bursa Uludağ University Local Ethics Committee (Decision number: 2021-15/04, Approval date: 30.11.2021).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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