Novel and Natural Feed Additive for Ruminants

The Impacts on Behavior, Rumen Fermentation and Microbiome with special reference to Methane Emissions

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AHMED Eslam Khalifa Hassan

Doctoral Program of Animal Science and Agriculture

Graduate School of
Animal and Veterinary Sciences and Agriculture

Obihiro University of Agriculture and Veterinary Medicine

反芻動物用の新規天然飼料添加物がメタン排出量、行動、ルーメン発酵、胃内微生物叢へ及ぼす影響

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帯広畜産大学大学院畜産学研究科
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AHMED Eslam Khalifa Hassan

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General Introduction

The livestock industry, especially in the ruminant sector, plays a crucial role in agricultural sustainability and food security (Reynolds et al., 2015). Ruminants are important for human nutrition, as they are the major source of milk and meat in most countries (Ritchie and Roser, 2019). Globally, they provide 51% of all protein from the livestock industry, of which 67% comes from milk and the other 33% from meat (FAO, 2016). On the other hand, the world population has been increasing rapidly during the last decade, and it is expected to reach approximately 8.6 billion people in 2030 and 9.8 billion in 2050. Thus, the global demand for meat and milk is expected to increase by 73% and 58%, respectively, compared with levels in 2010 (United Nations, 2017). Although ruminants are expected to have a pivotal role in meeting the increasing demand for livestock products, they are blamed to be a significant source of greenhouse gases (GHGs), such as methane (CH_4) and carbon dioxide (CO_2), representing approximately 14–18% of global anthropogenic GHG emissions depending on the accounting approaches by various sources (IPCC, FAO, or others) (Gerber et al., 2013). These GHGs are the major drivers of climate change, which threatens the well-being of the current and future generations. The levels of GHGs are increasing rapidly, and the World Bank has warned that the planet is on track to become 4 °C warmer. This is a dangerous temperature threshold at which scientists have warned that devastating effects, including extreme heatwaves, wildfires, droughts and floods, and social disruptions such as famines and mass migrations would become almost commonplace (Baker, 2012).

Ruminants emit GHGs such as CH_4 and CO_2 as byproducts of their normal digestive process during anaerobic fermentation of feed in a process known as enteric fermentation. Enteric CH_4 is produced by a specific group of rumen microorganisms called methanogenic archaea (methanogens) through reducing CO_2 with hydrogen resulting from digestion of the ingested feed

(Hook et al., 2010). The produced CH_4 is expelled from the animal through eructation, representing a loss up to 15% of the gross energy intake, which could potentially be utilized for animal physiological processes, thus being one of the most important inefficiencies in the ruminant production industry (Opio et al., 2013). Although CH_4 has a shorter atmospheric lifetime than other GHGs, lasting for approximately 10-12 years in the atmosphere, it has a greater global warming effect, as it is 28 times more powerful at trapping the sun's heat than CO_2 (Lashof and Ahuja, 1990). The growing public concern about the adverse effects of enteric CH_4 emissions on the efficiency and sustainability of livestock production and environmental impacts has induced scientific community researchers in the field of animal husbandry to seek management strategies to mitigate GHG emissions from ruminants. These strategies should assist the transition toward more efficient production systems while avoiding negative impacts on the environment, farmer profit, and animal welfare.

Several research groups worldwide have been working to develop strategies to combat CH_4 emissions and to achieve the desired levels of production by optimizing ruminal functions and improving feed conversion efficiency (Grossi et al., 2019). One of the best abatement strategies is dietary intervention using feed additives/supplements. Dietary manipulation has been primarily used to improve animal health and productivity, but recent advances in understanding its impact on rumen microbes have led to the development of various kinds of feed additives as potential candidates to mitigate CH_4 emissions (McAllister et al., 2011; Sun et al., 2021). This approach has a bilateral benefit for efficient livestock production and environmental protection (Min et al., 2020). Several kinds of feed additives have been studied to evaluate their effect on the reduction of CH_4 emissions; however, there are no effective and sustainable candidates to date (Patra et al., 2017; Honan et al., 2021). Another great challenge facing animal husbandry researchers is

achieving the principle of 'clean, green, and ethical' production. This concept implies that livestock products should be produced without the usage of chemical or synthetic compounds in ruminants' feed, with less impact on the environment, and while taking into account animal welfare guidelines.

Currently, there is a global interest in exploiting the bioactive properties of plants and their secondary metabolites as alternative feed additives to synthetic compounds such as antibiotics since the ban of feed-based chemicals in many countries in the European Union in 2006. In addition, they are often inexpensive, environmentally safe, and widely accepted by consumers (Durmic and Blache, 2012). Plant secondary metabolites such as organosulfur compounds, flavonoids, saponins, and tannins have shown the ability to modify animal behavior, modulate rumen fermentation patterns, and inhibit several types of rumen microorganisms (Durmic and Blache, 2012; Martínez-Fernández et al., 2013). Additionally, these plant extracts have showed promising results in inhibiting rumen methanogens (Ku-Vera et al., 2020); however, most of these studies have been performed *in vitro,* with inconsistency when applied *in vivo* (Ugbogu et al., 2019). Furthermore, the dosages that are effective in reducing *in vitro* CH_4 production are usually very high *in vivo* and lead to adverse impacts on feeding behavior, digestion, and fermentation patterns, which negatively affect animal performance (Klevenhusen et al., 2011). As a relatively new approach, researchers are developing combinations of different natural anti-methanogenic compounds with complementary modes of action to achieve effective and persistent CH_4 emission mitigation without adverse effects on animal performance and welfare. Accordingly, a novel combination from plant extracts have been formulated as a mixture known as Mootral.

Mootral (Mootral SA, Rolle, Switzerland) is a new formulation of organosulfur compounds from garlic (*Allium sativum*) and flavonoids extracted from bitter orange (*Citrus aurantium*).

Garlic contains several active metabolites, including sulfur-containing compounds such as alliin, diallyl sulfides, and allicin. These components have been known for their potential to reduce CH_4 along with antimicrobial, antioxidant, and immunomodulatory activities (Kim et al., 2012). However, the CH_4-reduction potential of these compounds reported in the literature is variable and inconsistent, ranging from zero (Panthee et al., 2017) to 38.5% (Pawar et al., 2014). In addition, flavonoids found in most fruits and vegetables have shown the ability to reduce *in vitro* CH_4 production (Oskoueian et al., 2013); however, available information about their role in the fermentation profile is still rare, with growing interest being introduced into ruminant feed, as they are powerful antioxidants with anti-inflammatory and immunity-boosting benefits (Crozier et al., 2006). Therefore, taking advantage of organosulfur compounds and flavonoids in reducing rumen methanogenesis, combining these two plant extracts may lead to persistent and more effective reduction potential.

In this thesis, as an integrated study, the novel natural combination of organosulfur and flavonoids is evaluated for the first time through *in vitro* and *in vivo* trials for its potential to decrease CH_4 production, taking into account its impact on behavior, health status, rumen fermentation characteristics, rumen microbiome, digestibility, and growth performance in ruminants.

References

Baker, J.L., 2012. Climate change, disaster risk, and the urban poor: Cities building resilience for a changing world / Judy L. Baker, editor. World Bank, Washington, D.C.

Crozier, A., Clifford, M.N., Ashihara, H., 2006. Plant secondary metabolites: Occurrence, structure and role in the human diet. Blackwell, Oxford.

Durmic, Z., Blache, D., 2012. Bioactive plants and plant products: Effects on animal function, health and welfare. Anim Feed Sci Technol 176, 150–162. https://doi.org/10.1016/j.anifeedsci.2012.07.018.

FAO, 2016. Reducing enteric methane for improving food security and livelihoods. Food and Agriculture Organization of the United Nations. https://www.fao.org/in-action/enteric-methane/background/theroleofruminants/en/ (accessed 27 October 2021).

Gerber, P.J., Henderson, B., Makkar, H.P.S., 2013. Mitigation of greenhouse gas emissions in livestock production: A review of technical options for non-CO_2 emissions / editors, Pierre J. Gerber, Benjamin Henderson and Harinder P.S. Makkar. Food and Agriculture Organization of the United Nations, Rome.

Grossi, G., Goglio, P., Vitali, A., Williams, A.G., 2019. Livestock and climate change: impact of livestock on climate and mitigation strategies. Anim Front 9, 69–76. https://doi.org/10.1093/af/vfy034.

Honan, M., Feng, X., Tricarico, J.M., Kebreab, E., 2021. Feed additives as a strategic approach to reduce enteric methane production in cattle: modes of action, effectiveness and safety. Anim Prod Sci. https://doi.org/10.1071/AN20295.

Hook, S.E., Wright, A.-D.G., McBride, B.W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010, 945785. https://doi.org/10.1155/2010/945785.

Kim, E.T., Kim, C.-H., Min, K.-S., Lee, S.S., 2012. Effects of plant extracts on microbial population, methane emission and ruminal fermentation characteristics in *in vitro*. Asian-Australas J Anim Sci 25, 806–811. https://doi.org/10.5713/ajas.2011.11447.

Klevenhusen, F., Zeitz, J.O., Duval, S., Kreuzer, M., Soliva, C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Anim Feed Sci Technol 166-167, 356–363. https://doi.org/10.1016/j.anifeedsci.2011.04.071.

Ku-Vera, J.C., Jiménez-Ocampo, R., Valencia-Salazar, S.S., Montoya-Flores, M.D., Molina-Botero, I.C., Arango, J., Gómez-Bravo, C.A., Aguilar-Pérez, C.F., Solorio-Sánchez, F.J., 2020.

Role of secondary plant metabolites on enteric methane mitigation in ruminants. Front Vet Sci 7, 584. https://doi.org/10.3389/fvets.2020.00584.

Lashof, D.A., Ahuja, D.R., 1990. Relative contributions of greenhouse gas emissions to global warming. Nature 344, 529–531. https://doi.org/10.1038/344529a0.

Martínez-Fernández, G., Abecia, L., Martín-García, A.I., Ramos-Morales, E., Hervás, G., Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2013. *In vitro-in vivo* study on the effects of plant compounds on rumen fermentation, microbial abundances and methane emissions in goats. Animal : an international journal of animal bioscience 7, 1925–1934. https://doi.org/10.1017/S1751731113001699.

McAllister, T.A., Beauchemin, K.A., Alazzeh, A.Y., Baah, J., Teather, R.M., Stanford, K., 2011. Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Can J Anim Sci 91, 193–211. https://doi.org/10.4141/cjas10047.

Min, B.R., Solaiman, S., Waldrip, H.M., Parker, D., Todd, R.W., Brauer, D., 2020. Dietary mitigation of enteric methane emissions from ruminants: A review of plant tannin mitigation options. Anim Nutr 6, 231–246. https://doi.org/10.1016/j.aninu.2020.05.002.

Opio, C., Gerber, P., Mottet, A., Falcucci, A., Tempio, G., MacLeod, M., Vellinga, T., Henderson, B., 2013. Greenhouse gas emission from ruminant supply chains. A global life cycle assessment.

Oskoueian, E., Abdullah, N., Oskoueian, A., 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. Biomed Res Int 2013, 349129. https://doi.org/10.1155/2013/349129.

Panthee, A., Matsuno, A., Al-Mamun, M., Sano, H., 2017. Effect of feeding garlic leaves on rumen fermentation, methane emission, plasma glucose kinetics, and nitrogen utilization in sheep. J Anim Sci Technol 59, 14. https://doi.org/10.1186/s40781-017-0139-3.

Patra, A., Park, T., Kim, M., Yu, Z., 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. J Anim Sci Biotechnol 8, 13. https://doi.org/10.1186/s40104-017-0145-9.

Pawar, M.M., Kamra, D.N., Agarwal, N., Chaudhary, L.C., 2014. Effects of essential oils on *in vitro* methanogenesis and feed fermentation with buffalo rumen liquor. Agric Res 3, 67–74. https://doi.org/10.1007/s40003-014-0092-z.

Reynolds, L.P., Wulster-Radcliffe, M.C., Aaron, D.K., Davis, T.A., 2015. Importance of animals in agricultural sustainability and food security. J Nutr 145, 1377–1379. https://doi.org/10.3945/jn.115.212217.

Ritchie, H., Roser, M., 2019. Meat and dairy production. Our world in data. https://ourworldindata.org/meat-production#meat-production-by-animal.

Sun, K., Liu, H., Fan, H., Liu, T., Zheng, C., 2021. Research progress on the application of feed additives in ruminal methane emission reduction: a review. PeerJ 9, e11151. https://doi.org/10.7717/peerj.11151.

Ugbogu, E.A., Elghandour, M.M., Ikpeazu, V.O., Buendía, G.R., Molina, O.M., Arunsi, U.O., Emmanuel, O., Salem, A.Z., 2019. The potential impacts of dietary plant natural products on the sustainable mitigation of methane emission from livestock farming. J Clean Prod 213, 915–925. https://doi.org/10.1016/j.jclepro.2018.12.233.

United Nations, 2017. World Population Prospects: The 2017 revision, key findings and advance tables. Working Paper No. ESA/P/WP/248. United Nations. Department of Economic and Social Affairs. https://esa.un.org/unpd/wpp/publications/files/wpp2017keyfindings.pdf.

Chapter One

Impacts of Mootral on Methane Production, Rumen Fermentation, and Microbial Community in an *in vitro* Study

Abstract

Methane mitigation strategies have a two-side benefit for both environment and efficient livestock production. This preliminary short-term *in vitro* trial using Mootral (garlic and citrus extracts), a novel natural feed supplement, was conducted to evaluate its efficacy on rumen fermentation characteristics, methane production, bacterial and archaeal community. The experiment performed as a batch culture using rumen fluid collected from sheep, and Mootral was supplemented in three concentrations; 0 (Control), 10, and 20% of the substrate (50% Grass: 50% Concentrate). The rumen fermentation data and alpha diversity of microbial community were analayzed by ordinary one-way analysis of variance. The relative abundance and statistical significance of families and OTU among the groups were compared by Kruskal-Wallis H test using Calypso software. After the incubation for 24 hours at 39^{o}C, Mootral in a dose-dependent manner improved the production of total volatile fatty acids and propionate while reduced the acetate proportion and acetate/propionate ratio. The total produced gas was two-times more in Mootral supplemented groups than control ($P < 0.01$), while the proportion of methane in the produced gas reduced by 22 ($P < 0.05$) and 54% ($P < 0.01$) for 10 and 20% Mootral, respectively. Mootral did not change pH, digestibility, and ammonia-nitrogen. Microbial community analyses showed that Mootral effectively changed the ruminal microbiome. The bacterial community showed increase of the relative abundance of propionate-producing family such as *Prevotellaceae* ($P = 0.014$) and *Veillonellaceae* ($P = 0.030$), while there was a decrease in the relative abundance of some hydrogen producing bacteria by Mootral supplementation. In the archaeal community, *Methanobacteriaceae* was decreased by Mootral supplementation comparing with control ($P = 0.032$), while the *Methanomassiliicoccaceae* family increased in a dose-dependent effect ($P = 0.038$). The results of the study showed the efficacy of the new mixture to alter the ruminal

microbial community, produce more propionate and reduce microbial groups associated with methane production, thus suggesting that Mootral is a promising natural mixture for methane reduction from ruminants.

1. Introduction

Researchers are searching for new mixtures by combining many natural anti-methanogenic compounds that could be able to suppress CH_4 production effectively without impairing digestibility and volatile fatty acids (VFA) production (Patra and Yu, 2013). Accordingly, Mootral (Mootral SA, Rolle, Switzerland), a novel combination of garlic (*Allium sativum*) powder and bitter orange (*Citrus aurantium*) extracts, has shown a great efficacy in reduction of CH_4 from ruminant through alteration of the archaeal community without impairing VFA production when used as a feed supplement in *in vitro* trial with 70% hay : 30% concentrate diet using rumen fluid from cows (Eger et al., 2018).

However, there is still limitation to find out its ability in different dietary regimen and in another ruminant species, as well as its impact on not only archaeal but also bacterial community in the rumen. Therefore, in this chapter, a preliminary small-scale *in vitro* study was performed using rumen fluid collected from sheep to evaluate the effect of Mootral supplemented to a 50% hay : 50% concentrate diet on suppressing of CH_4 production, alteration of ruminal microbiome (Bacterial and Archaeal), as well as rumen fermentation profile and digestibility.

2. Materials and Methods

2.1- Rumen fluid collection

Rumen fluid was collected from three Corriedale wether sheep (body weight 63.5±4.4 kg) 4 hours after the morning feeding using stomach tube and vacuum pump. They were fed the basal diet of 50% concentrate and 50% Kleingrass (*Panicum coloratum*) hay at maintenance level for energy requirement. The first amount (100 ml) of the sucked rumen fluid was discarded to prevent contamination of saliva. The second amount was strained by four layers of absorbent gauze into an insulated container and transferred immediately to the laboratory. In the laboratory, the collected samples were mixed together in one beaker under constant stream of CO_2, and kept in a water bath at 39°C prior to adding into the fermentation tubes. Animal management and sampling procedures were approved through the animal care and use committee of Obihiro University of Agriculture and Veterinary Medicine (Approval Number 19-94).

2.2- Experimental design and in vitro incubation

The chemical composition of the feed used in the *in vitro* experiment described in Table 1. The chemical composition of dry matter (DM) (930.15), organic matter (OM) (942.05), crude protein (CP) (984.13) and ether extract (EE) (920.39) were determined according to AOAC (1995). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were measured and expressed inclusive residual ash using an ANKOM^{200} Fiber Analyzer (Ankom Technology Methods 6, 5 and 8, respectively; ANKOM Technology Corp., Macedon, NY, USA). The NDF was measured using sodium sulfite without heat-stable α-amylase. Three groups with three replicates for each were prepared; control (0% Mootral of substrate), 10% (10% Mootral of substrate), and 20% (20% Mootral of substrate). Each group composed of 900 mg of ground feed (Concentrate + Kleingrass hay) as substrate added in a ratio of 50:50 in a sealed nylon bag

(BG1020, Sanshin Industrial Co., Ltd., Kanagawa, Japan) which was placed in a 200 ml fermentation bottle. Feed was ground by a mill to pass through 1-mm sieve. Mootral was used as a feed supplement added directly in the fermentation bottles in either 10% or 20% of the substrate. Mootral composed of a mixture of nine parts garlic powder to one part citrus powder. The chemical composition of Mootral described in Table 2. More information about formulation of Mootral have been published by Eger et al. (2018). Thirty ml of rumen fluid and sixty ml of artificial saliva (McDougall, 1948) were added in the fermentation bottles under continuous flushing with CO_2. Thereafter, tubes were sealed with rubber stoppers and aluminum caps. The incubation was performed for 24 hours at 39^{o}C. At the end of incubation, the produced headspace gas was measured using syringe. Culture fluid was used for measuring pH using pH meter (LAQUA F-72, HORIBA Scientific, Kyoto, Japan), and aliquot was transferred into 1.5 ml tubes and centrifugated at 16,000×g, 4°C for 5 minutes. The supernatant and precipitation were collected and stored at -20°C.

Table 1: Chemical composition of the feed used in this study (g/kg dry matter).

(g/kg dry matter)	Concentrate	Kleingrass hay
Dry Matter (in fresh matter)	837.9	849.6
Organic Matter	942.0	916.2
Crude Ash	58.0	83.8
Crude Protein	229.3	135.8
Ether Extract	40.1	38.1
Neutral Detergent Fiber	281.8	680.0
Acid Detergent Fiber	98.2	329.9
Acid Detergent Lignin	25.2	50.0

Table 2: Chemical composition of Mootral powder (g/100g dry matter).

(g/100g dry matter)	Mootral
Crude Ash	3.9
Crude Protein	22.0
Crude Fat	0.51
Crude Fiber	1.9
Sodium	0.04

2.3- In vitro dry matter digestibility

Bags from each tube were washed by running tap water until the drain drops were clear. After that, they were dried in the oven at 60°C for 48 hours to determine the *in vitro* dry matter digestibility (IVDMD) (Wei et al., 2019).

2.4- Gas composition, Volatile fatty acids and ammonia-nitrogen analysis

The headspace gas samples were analyzed by injection of 1 ml of the gas using Hamilton gastight syringe (Hamilton Company, Reno, Nevada, USA) in a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan) as described previously (Matsui et al., 2013).

VFA were analyzed using high-pressure liquid chromatography (HPLC) (Giang et al., 2016). Briefly, the analytical specifications were as follows; column, Shim-pak SCR-102H (7 mm, i.d. 8.0 mm×300 mm, Shimadzu Corp., Kyoto, Japan); eluent flow rate and mobile phase for organic acid analysis (Shimadzu Corp., Kyoto, Japan) at 0.8 mL/min; column temperature, 40°C; reaction reagent and flow rate, pH buffer for organic acid analysis (Shimadzu Corp., Kyoto, Japan) at 0.8 mL/min; conductivity detector (CDD-10AVP, Shimadzu Corp., Kyoto, Japan). Quantification of VFA concentration was performed using the external standard quantitation method.

For measuring the concentration of ammonia-nitrogen (NH_3-N), samples were diluted 100 times using 0.1 M phosphate buffer (pH 5.5) then analyzed according to the Modified Fujii-Okuda method (Kawasaki et al., 2015) using NH_3 kit (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan).

2.5- DNA extraction, analysis of 16S ribosomal RNA (16S rRNA) and next-generation sequencing

DNA was extracted from rumen fluid samples using repeated beads beating plus column (RBB+C) method and the Maxwell 16 LEV blood DNA kit (Promega, Madison, WI, USA) (Yu

and Morrison, 2004; Warren et al., 2018). The concentration and purity of extracted DNA were measured by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Tokyo, Japan), and then the DNA concentration was adjusted to 5 ng/μL using Tris-EDTA buffer. The variable regions (V3 and V4) of bacterial 16S rRNA gene were amplified from the purified DNA. The primers used in this study consisted of the Illumina overhang adapters and universal primers in the first stage of PCR as follows; the forward overhang adapter and bacterial universal primer; 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and the reverse overhang adapter and bacterial universal primer 5′-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATTACHVGGGTATCTAATCC-3′. For Archaeal 16S rRNA gene, the forward overhang adapter and Arch349F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGYGCASCAGKCGMGAAW-3′) and the reverse overhang adapter and Arch806R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACVSGGGTATCTAAT-3′) were used. In the second PCR, Illumina sequencing adapters and dual index barcodes were added to the amplicons using Nextera® XT Index Kit (Illumina Inc., San Diego, California, USA). The concentration of the second PCR product was quantified using Quantus™ fluorometer (QuantiFluor® dsDNA System, Promega, Madison, Wisconsin, USA) then PCR products from all samples were pooled in one tube in equal amounts. Paired-end sequencing was performed using the Illumina MiSeq (Illumina, San Diego, California, USA). The preparation of 16S rRNA gene amplicon was done as previously described by Pelpolage et al. (2019).

The analysis of raw 16S rRNA gene sequence was done according to the method described before by Warren et al. (2018). Samples with less than 1,000 sequence reads were removed. Sequence reads were clustered into operational taxonomic units (OTUs) with a 97% sequence

identity threshold. The generated biome table was used in Calypso version 8.84 to generate principal-coordinate analysis (PCoA) 3D plot and express relative abundance of bacterial or archaeal taxa among experimental groups. For archaeal community, samples were rarefied to read depth of 1,678 while in bacterial community were 16,246 reads. Nucleotide sequence data reported in this study are available in the DDBJ Sequence Read Archive under the accession numbers DRA011192.

2.6- Statistical analysis

All rumen fermentation profile data and alpha diversity of both bacterial and archaeal community were analyzed statistically using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, California, USA). Data was provided as means ± SEM (Standard Error of the Mean). Ordinary One-way analysis of variance (ANOVA) was performed followed by Tukey's test to find the significance among experimental groups. The relative abundance and statistical significance of families and OTU among the 3 experimental groups were compared using Kruskal-Wallis H test in Calypso version 8.84. Differences were considered statistically significant when *P* value was less than 0.05, and tendency was declared when *P* value was between 0.05 and 0.10.

3. Results

3.1- Effect of Mootral on rumen fermentation characteristics

Mootral exhibited significant changes in the rumen fermentation profile as shown in Table 3. However, pH, IVDMD, and NH_3-N did not show any differences ($P > 0.05$) among experimental groups. The total gas produced was increased ($P < 0.01$) for Mootral supplemented groups compared with control. In contrast, the percentage of the volume of CH_4 in the produced gas was decreased by increasing Mootral dose; it showed a reduction up to 21.98% for 10% Mootral ($P < 0.05$), and 54.25% for 20% Mootral ($P < 0.01$) when compared with control (Figure 1A). However, the volume of the produced CH_4 per day and CH_4/DM were not different between Mootral treated groups and control ($P > 0.05$) but, there was an increase in 10% than 20% ($P < 0.05$) (Table 3). The percentage of the volume of CO_2 in the produced gas was increased by increasing Mootral supplementation. It was higher for 20% when compared with control ($P < 0.01$) and with 10% ($P < 0.05$), also it was higher for 10% comparing with control group ($P < 0.05$) (Figure 1B). Consequently, the total amount of CO_2 produced in 24 hours was higher ($P < 0.01$) by increasing Mootral dosages (69.54 and 76.51 ml for 10% and 20%, respectively) when compared with control (35.01 ml) (Table 3). The CH_4/CO_2 ratio in the total produced gas decreased with increasing Mootral dosage ($P < 0.01$) (Table 3).

Acetic acid ratio showed a reduction among groups ($P < 0.01$) with increasing Mootral supplementation. However, the propionic acid concentration and ratio increased with increasing Mootral supplementation with a significant effect between 20% and control ($P < 0.01$) and between 20% and 10% ($P < 0.05$). Mootral did not affect either the concentration or percentage of butyric acid among groups. The total VFA increased with Mootral supplementation where it reached

significant level between 20% and control group ($P < 0.05$). The acetate/propionate (A/P) ratio decreased with increasing Mootral dosage ($P < 0.01$) (Table 3).

Table 3: Rumen fermentation characteristics of 24 hours *in vitro* incubation.

Parameter	Experimental groups			*P* value
	0%	10%	20%	
pH	6.87±0.04	6.86±0.01	6.80±0.01	0.13
Total gas production (ml)	43.43±1.29^{b}	81.93±2.56^{a}	84.03±2.32^{a}	< 0.01
IVDMD[1] (%)	62.70±4.70	59.10±1.40	59.00±3.30	0.70
CH_4 (ml)	8.42±0.41^{ab}	12.39±0.52^{a}	7.52±1.48^{b}	0.02
CH_4/DM(ml/g)	11.12±0.54^{ab}	16.39±0.68^{a}	9.89±1.95^{b}	0.02
CO_2 (ml)	35.01±1.01^{c}	69.54±2.12^{b}	76.51±0.87^{a}	< 0.01
CH_4/CO_2 ratio	0.241±0.01^{a}	0.178±0.00^{b}	0.098±0.02^{c}	< 0.01
Acetate (mmol/L)	48.22±1.70	48.13±0.26	48.85±0.33	0.86
Propionate (mmol/L)	21.77±1.21^{b}	24.83±0.28^{b}	30.28±0.94^{a}	< 0.01
Butyrate (mmol/L)	11.56±0.86	12.33±0.24	13.03±1.24	0.54
Total VFA[2] (mmol/L)	81.55±3.64^{b}	85.29±0.73^{ab}	92.16±0.68^{a}	0.04
Acetate (%)	59.18±0.79^{a}	56.44±0.27^{b}	53.01±0.08^{c}	< 0.01
Propionate (%)	26.67±0.60^{b}	29.11±0.12^{b}	32.87±1.24^{a}	< 0.01
Butyrate (%)	14.14±0.43	14.45±0.17	14.12±1.26	0.95
A/P ratio[3]	2.22±0.08^{a}	1.94±0.02^{b}	1.62±0.06^{c}	< 0.01
NH_3-N (mg/dL)	11.07±1.38	11.51±0.84	10.33±0.20	0.69

^{a,b,c} Values within the same row with different superscripts are different ($P < 0.05$). [1]IVDMD (*In vitro* dry matter digestibility). [2]VFA (Volatile Fatty Acids). [3]A/P (Acetate/Propionate) ratio. ±: Standard Error of the Mean.

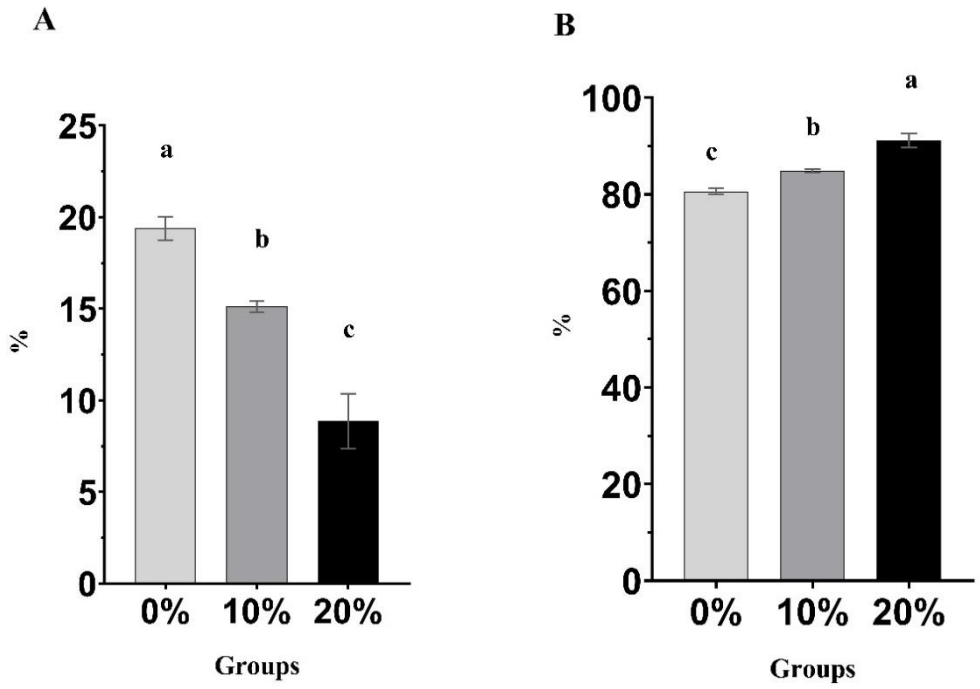


Figure 1: A: Percentage of CH_4 in the produced gas, **B**: Percentage of CO_2 in the produced gas. Bars with different superscripts are different ($P < 0.05$).

3.2- Effect of Mootral on bacterial community diversity and composition

The minimum and maximum sequence reads were 16,246 and 42,030, respectively. The α-diversity indices including Richness, Choa1, Evenness, and Shannon index were not affected by Mootral supplementation ($P > 0.05$) (Table 4). However, based on β-diversity analysis at family level, the control samples were clustered away from Mootral supplemented groups. Also, Mootral treated samples were clustered close to each other (Figure 2A).

Based on family level, Mootral was able to change in the bacterial composition through increasing the relative abundance of family *Prevotellaceae* especially in 20% Mootral (28.07%), this increase was significant when compared with control group (23.16%) ($P < 0.05$) and with 10% Mootral (23.32%) ($P < 0.05$), while there was no difference between 10% Mootral and control ($P = 0.99$) (Figure 3A). Similarly, the relative abundance of family *Veillonellaceae* increased ($P < 0.05$) in 20% Mootral (7.98%) compared with control (6.62%), while the abundance of *Veillonellaceae* in 10% Mootral (7.1%) was not statistically different from that in control ($P = 0.46$) (Figure 3B). Based on OTU level, some bacterial strain belonging to family *Ruminococcaceae* and order *Bacteroidales* significantly decreased between Mootral supplemented groups and control ($P < 0.05$) (Figure 4). The relative abundance of all taxa at phylum and family level of bacteria which were more than 0.1% of total bacteria was described in Table 5.

3.3- Effect of Mootral on archaeal community diversity and composition

The minimum sample sequence read was 1,678 and the maximum was 19,986. Mootral supplementation did not alter the α-diversity indices of archaeal community among experimental groups (Table 4). However, the β-diversity analysis at family level showed shift of the control group samples to be away from Mootral supplemented samples (Figure 2B).

Mootral supplemented groups showed a significant shift in archeal community through decreasing the relative abundance of the major methanogenic group, famliy *Methanobacteriaceae* (94.07 and 92.70% for 10% and 20% Mootral, respectively), when compared with control group (96.42%) ($P < 0.05$) (Figure 5A). In contrast, the relative abundance of family *Methanomassiliicoccaceae* increased with increasing Mootral dosages (5.83 and 7.12% for 10% and 20% Mootral, respectively) when compared with non-supplemented group (3.5%) ($P < 0.05$) (Figure 5B). The relative abundance of all taxa at phylum and family level of archaea which were more than 0.1% of total archaea was described in Table 5.

Table 4: α-Diversity of microbial community based on OTU level.

Parameter	Experimental groups			*P* value
	0%	10%	20%	
		Bacteria[1]		
Richness	992.60±7.63	1004.00±5.27	973.60±9.59	0.08
Chao1	1035.00±0.73	1043.00±0.12	1034.00±4.94	0.14
Evenness	0.825±0.01	0.840±0.01	0.823±0.01	0.17
Shannon index	5.72±0.06	5.84±0.04	5.69±0.05	0.14
		Archaea[2]		
Richness	226.70±20.26	185.90±28.40	232.60±13.37	0.32
Chao1	408.70±7.20	326.70±60.54	406.70±8.60	0.25
Evenness	0.742±0.24	0.731±0.02	0.750±0.01	0.81
Shannon index	4.43±0.17	4.09±0.26	4.45±0.09	0.33

[1]α-Diversity of bacteria of the experimental groups. Samples rarefied to read depth of 16,246. [2]α-Diversity of archaea of the experimental groups. Samples rarefied to read depth of 1,678. ±: Standard Error of the Mean.

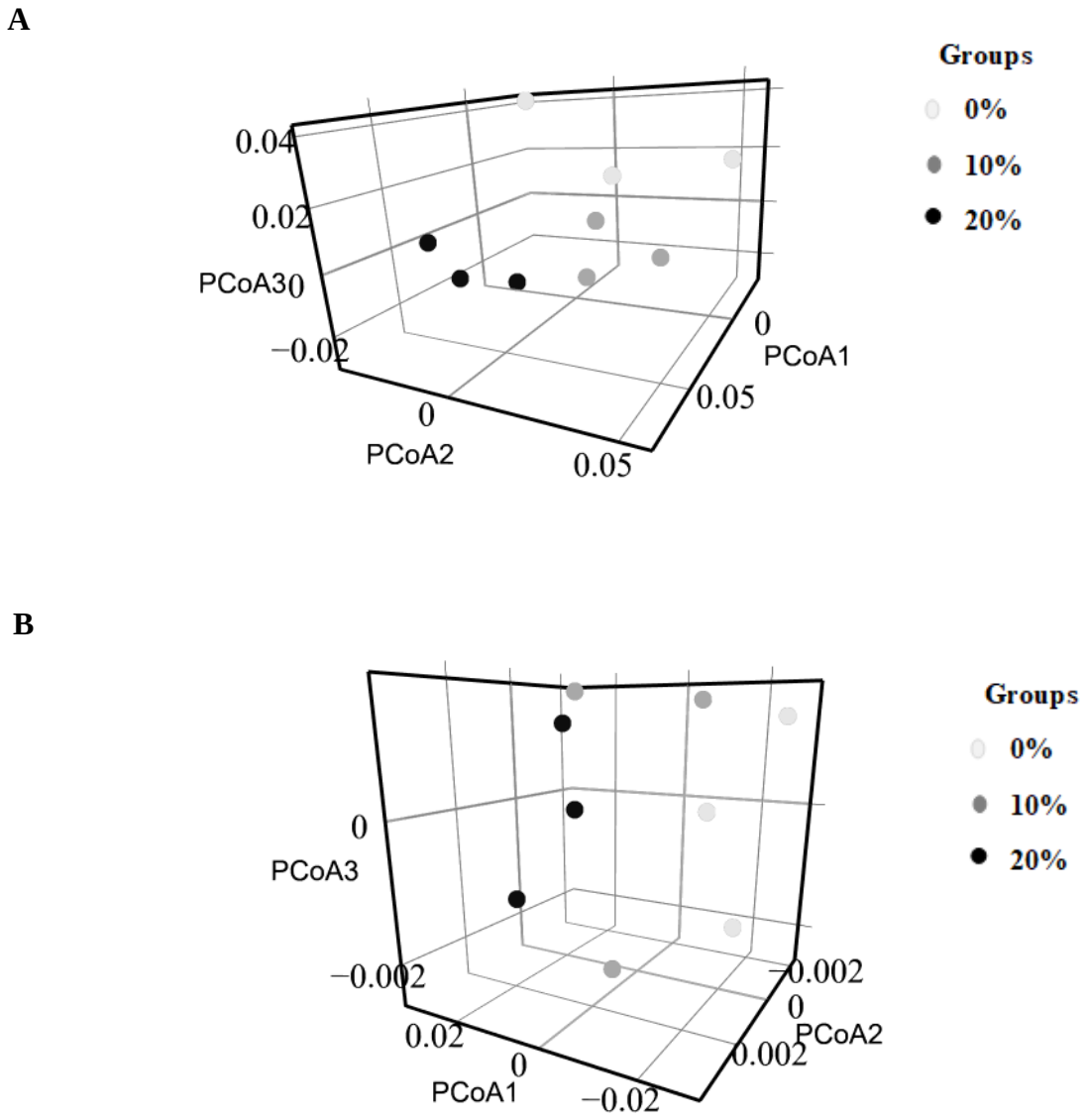
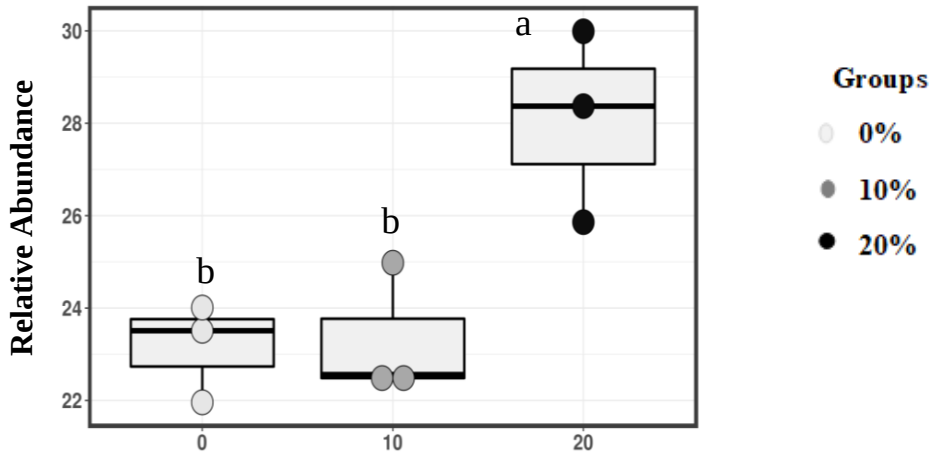


Figure 2: Beta diversity of microbial community analysis by PCoA 3D. **A:** Bacterial community (Family level)**, B:** Archaeal community (Family level).

A



B

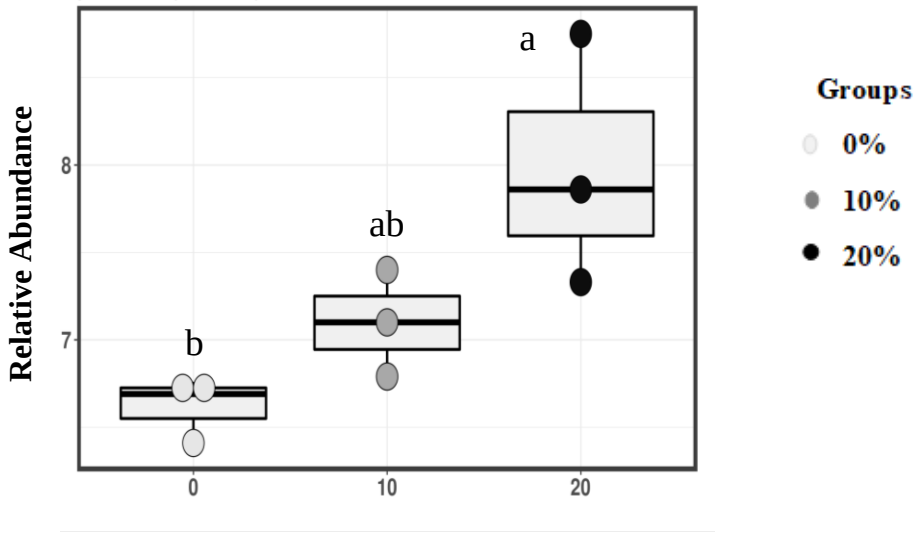


Figure 3: Relative abundance of families**. A:** *Prevotellaceae***,** and **B:** *Veillonellaceae* in total bacterial community. Boxes with different superscripts are different ($P < 0.05$).

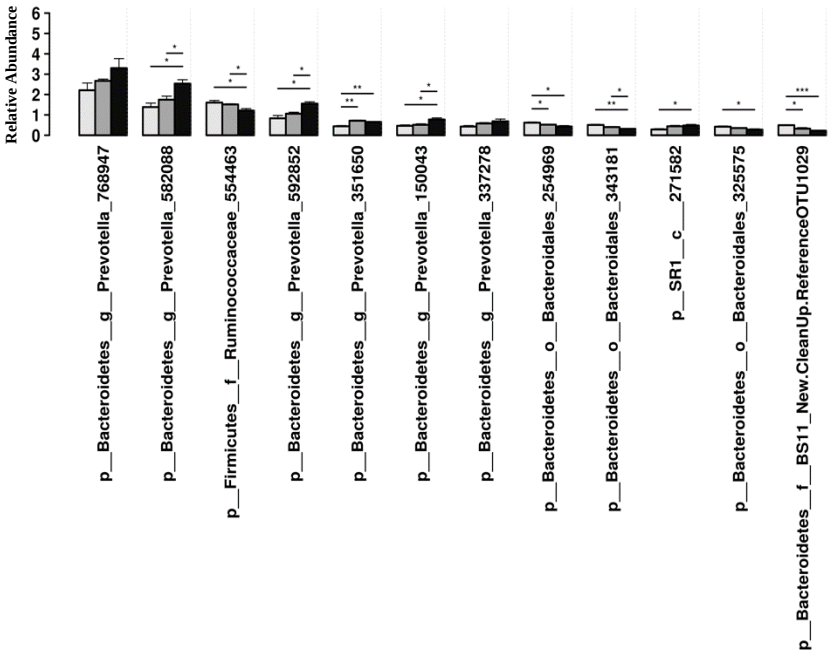


Figure 4: Relative abundance of bacterial community OTU level, (top 20). Asterisks indicate differences ($P < 0.05$).

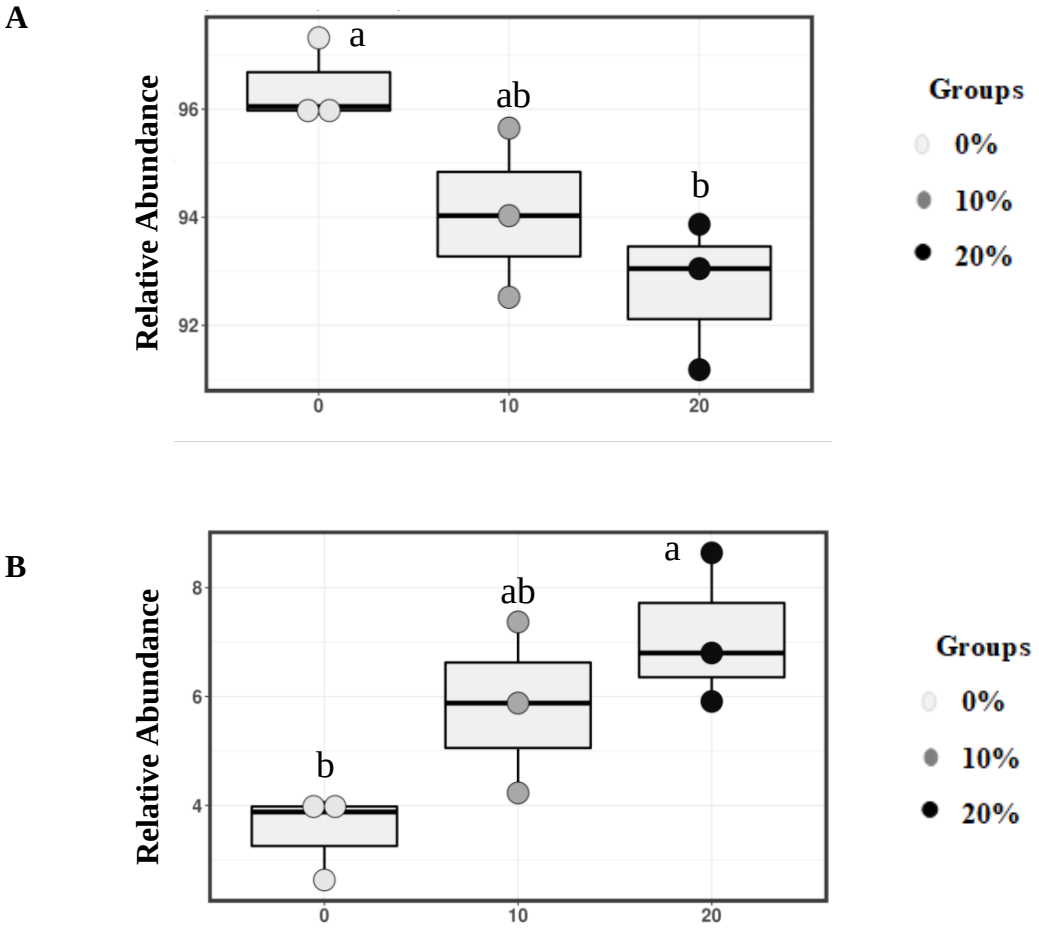


Figure 5: Relative abundance of families**. A:** *Methanobacteriaceae,* and **B:** *Methanomasillicocae* in total archaeal community. Boxes with different superscripts are different ($P < 0.05$).

Table 5: The mean relative abundance of bacteria and archaea based on phylum and family.

Taxa	Experimental groups			*P* value
	0%	10%	20%	
		Bacteria		
Firmicutes	48.33±1.35	49.62±1.65	47.38±0.38	0.49
Unclassified.Clostridiales	18.17± 0.76	17.15± 0.76	18.35±1.31	0.66
Ruminococcaceae	12.00±0.32	14.05±0.53	11.12±1.12	0.08
Lachnospiraceae	6.57±0.7	6.16±0.20	6.30±0.01	0.41
Veillonellaceae	6.62±0.11^{b}	7.10±0.18^{ab}	7.10±0.41^{a}	0.03
Christensenellaceae	0.730±0.14	0.750±0.15	0.358±0.02	0.10
Clostridiaceae	0.757±0.06	0.837±0.07	0.730±0.09	0.59
Mogibacteriaceae	0.817±0.03^{a}	0.787±0.04^{a}	0.613±0.02^{b}	0.01
Erysipelotrichaceae	0.603±0.06^{b}	0.790±0.03^{a}	0.747±0.03^{ab}	0.04
Streptococcaceae	1.95±1.09	1.88±0.39	1.14±0.11	0.66
Dehalobacteriaceae	0.090±0.02	0.087±0.03	0.017±0.01	0.10
Bacteroidetes	42.49±0.95	43.34±1.30	46.29±0.75	0.09
Prevotellaceae	23.16±0.62^{b}	23.32±0.83^{b}	28.07±1.20^{a}	0.01
Unclassified.Bacteroidales	15.40±0.74	15.11±0.45	14.65±0.25	0.61
Paraprevotellaceae	1.50±0.18	1.58±0.03	1.36±0.11	0.50
BS11	1.00±0.04	1.20±0.14	0.903±0.19	0.38
S247	0.457±0.01	0.580±0.11	0.393±0.02	0.19

RF16	0.587±0.14	1.20±0.06	0.643±0.23	0.07
Bacteroidaceae	0.370±0.01[a]	0.327±0.02[ab]	0.253±0.02[b]	0.01
Proteobacteria	3.68±1.28	1.49±0.27	1.35±0.52	0.15
Succinivibrionaceae	3.44±1.32	1.19±0.25	1.03±0.51	0.15
Desulfovibrionaceae	0.217±0.05	0.247±0.02	0.240±0.01	0.79
Spirochaetes	0.760±0.14	0.710±0.07	0.657±0.14	0.83
Spirochaetaceae	0.630±0.15	0.567±0.08	0.563±0.12	0.90
TM7	0.757±0.02[ab]	0.823±0.03[a]	0.703±0.02[b]	0.03
F16	0.757±0.02[ab]	0.823±0.03[a]	0.703±0.02[b]	0.03
SR1	0.627±0.05[b]	0.887±0.07[ab]	0.957±0.08[a]	0.03
Unclassified.SR1	0.627±0.05[b]	0.887±0.07[ab]	0.957±0.09[a]	0.03
Synergistetes	0.863±0.15	0.827±0.00	0.807±0.04	0.90
Dethiosulfovibrionaceae	0.863±0.15	0.827±0.00	0.807±0.04	0.90
Actinobacteria	0.783±0.15	0.717±0.07	0.737±0.04	0.88
Coriobacteriaceae	0.783±0.15	0.717±0.07	0.737±0.04	0.88
Verrucomicrobia	0.553±0.10	0.530±0.04	0.347±0.07	0.18
RFP12	0.347±0.10	0.333±0.02	0.243±0.06	0.53
Chloroflexi	0.263±0.02	0.243±0.01	0.243±0.01	0.53
Anaerolinaceae	0.263±0.02	0.243±0.01	0.243±0.01	0.53
Planctomycetes	0.280±0.03[a]	0.163±0.00[b]	0.147±0.02[b]	0.01
Pirellulaceae	0.280±0.03[a]	0.163±0.00[b]	0.147±0.02[b]	0.01
Tenericutes	0.150±0.04	0.137±0.01	0.097±0.03	0.38
Anaeroplasmataceae	0.100±0.04	0.080±0.01	0.050±0.01	0.35

WPS2	0.137±0.01[a]	0.1067±0.02[ab]	0.063±0.00[b]	0.01
Unclassified.WPS2	0.137±0.013[a]	0.107±0.02[ab]	0.063±0.00[b]	0.01
Lentisphaerae	0.153±0.06	0.207±0.04	0.100±0.02	0.30
Victivallaceae	0.153±0.06	0.207±0.04	0.100±0.02	0.30
		Archaea		
Euryarchaeota	100	100	100	---
Methanobacteriaceae	96.42±0.45[a]	94.07±0.90[ab]	92.70±0.80[b]	0.03
Methanomassiliicoccaceae	3.53±0.45[b]	5.83±0.91[ab]	7.12±0.80[a]	0.04
Unclassified	0.030±0.01[b]	0.093±0.02[ab]	0.163±0.03[a]	0.01

[a,b] Values within the same row with different superscripts are different ($P < 0.05$). ±: Standard Error of the Mean.

4. Discussion

Previous studies have shown the efficacy of garlic compounds and flavonoids on CH_4 suppressing of either *in vivo* or *in vitro* studies (Oskoueian et al., 2013; Ma et al., 2016; Elghandour et al., 2018), however, there still are variations on VFA productions and digestibility (Wanapat et al., 2008; Kongmun et al., 2010; Oskoueian et al., 2013; Hernandez et al., 2017). Combination of these two natural products may have a significant effect on reducing CH_4 without impairing rumen fermentation characteristics. To our knowledge, only two studies were performed using that combination of garlic and citrus (Mootral) to evaluate its effect on CH_4 emission (Roque et al., 2019) and fermentation profile through alterations of archaeal community (Eger et al., 2018), while the bacterial community has not been investigated yet. Further investigations are required to ensure that efficacy in different diet forms and different ruminant species. Therefore, this preliminary *in vitro* trial was performed to evaluate that efficacy in a different feeding regimen (50% Kleingrass hay : 50% Concentrate) and another ruminant species (sheep) through studying its impacts on the rumen fermentation profile and archaeal and bacterial communities.

4.1- Mootral improved rumen fermentation characteristics with reducing the percentage of methane

Most of the anti-methanogenic products studied before, showed negative effects on fermentation profile at high doses to achieve the effective CH_4 reduction (Yang et al., 2007; Patra and Yu, 2013; Patra and Yu, 2015). However, the results of the current study showed an increase in propionic acid and total VFA with increasing Mootral dosages that may be due to stimulation of the of family *Prevotellaceae* which increased in the current study. *Prevotellaceae* is well known as a propionate producing bacteria (Denman et al., 2015). Many researches have proved that fermentation leading to more propionate is strongly associated with decrease in CH_4 production.

For instance, Ungerfeld (2015) reported that reduction of CH_4 in batch cultures lead to redirection of metabolic H_2 toward propionate production. Similarly, Kittelmann et al. (2014) assumed that high propionate was present in low CH_4 emitting cows. The improvement in the production of total VFA by Mootral effect was also observed in the previous study (Eger et al., 2018) and in other studies that either used garlic (Patra et al., 2006) or flavonoids (Balcells et al., 2012). The reduction of acetate and A/P ratios was also reported with inclusion of 300 mg/l garlic oil in continuous culture system (Busquet et al., 2005) and garlic powder (Wanapat et al., 2008). Production of more propionate and less acetate means that H_2 was redirected toward propionate formation as an alternative way other than methanogenesis (Wang et al., 2018). The rumen medium was stabilized in the presence of garlic and citrus, and did not change the pH, IVDMD, and NH_3-N as shown previously (Anassori et al., 2011; Balcells et al., 2012; Seradj et al., 2014) and similarly when the same mixture was used (Eger et al., 2018).

Mootral supplementation showed a strong efficacy to reduce the CH_4 percentage in the produced gas up to 54%. Interestingly, the total gas produced (ml/day) was two-fold more in Mootral supplemented groups than control and that explains why the total CH_4 production/day was not changed between Mootral groups and the control. Trial data of an *in vitro* gas production monitoring for 48 hours from Copenhagen University (Hansen and Nielsen, 2018) showed similar finding to our results. Mootral stimulated fermentation, and the total gas production increased during the first 16 hours. Also, they reported that Mootral reduced the percentage of CH_4 in the produced gas with 58 % from a typical Danish dairy feed ration without affecting IVDMD. The substantial increase in total gas production and CO_2 with reducing CH_4/CO_2 ratio as an important indicators of rumen fermentation profile in the current study might be due to the stimulating effect of Mootral on the activity of some rumen microbes other than methanogens. This interesting

finding has to be proven in further researches using qPCR. Eger et al. (2018) reported that Mootral reduced the percentage and the total production of CH_4 in RUSITEC system as a long-term study (14 and 18 days). As our present study was a batch culture for short-term (24 hours), these differences could contribute to the result's discrepancy.

4.2- Mootral changed the bacterial community composition

Although Mootral was effective to shift the bacterial community toward less H_2 producing bacteria, the α-diversity was not different. By analyzing the bacterial community of ruminants, it has been proven that differences in the bacterial community composition were associated with the level of CH_4 emissions (Kittelmann et al., 2014). The results of the current study revealed that *Prevotellaceae,* the main dominant family in rumen fluid (Ozbayram et al., 2018), was higher with Mootral groups especially the higher dose (20%) as mentioned previously. Genus *Prevotella* is well known to produce propionate through utilizing H_2 via the randomizing (succinate) or non-randomizing (acrylate) pathways through the fermentation of carbohydrates. These pathways were the main ways for consumption of H_2, which accumulated as a consequence of reduced methanogenesis (Denman et al., 2015). Similarly, family *Veillonellaceae* showed a higher abundance in Mootral groups. This might be due to the effect of flavonoids extracted from citrus (Oskoueian et al., 2013). *Megasphaera elsdenii* belonging to the family *Veillonellaceae* is well-known as lactate-utilizing and propionate-producing bacteria (Chen et al., 2019). Moreover, *Quinella* spp., a member of family *Veillonellaceae,* were more numerous in low CH_4 producing cows (Wallace et al., 2015). Tapio et al. (2017) also reported that the lower CH_4 production ruminotype possessed a high relative number of propionate-producing *Quinella ovalis* and succinate-producing bacteria as *Prevotella bryantii.*

Within bacteria, some species belonging to *Ruminococcaceae, Clostridiales,* and *Bacteroidales* are H_2 producers, while *Prevotella* spp. are net H_2 utilizers (Hobson and Stewart, 1997). Denman et al. (2015) attributed that CH_4 emission is depending on the abundance of H_2 producing and consuming bacteria. The results from the current study based on OTU level showed a lower abundance of some OTUs belonging to *Ruminococcaceae and Bacteroidales,* while some OTUs belonging to genus *Prevotella* were higher in Mootral-treated groups compared to control group. Similar findings were observed by Popova et al. (2018) who found that reduction of CH_4 by linseed and nitrate reduced the relative abundance of *Ruminococcaceae* also, linseed supplementation increased the proportion of *Prevotellaceae.* The combination effect of Mootral on the bacterial community is still unclear, but it could have an indirect effect on increasing the relative abundance of H_2 utilizing-bacteria through reducing methanogenesis and stimulate the utilization of accumulated H_2 by those bacteria to produce propionate. A further understanding of the mode of action of Mootral to these bacteria is required to be discovered in upcoming researches.

4.3- Mootral altered the archaeal community composition

Similar to the results in bacterial community, the archaeal α-diversity did not show any changes with Mootral supplementation, which was similar as in previous finding (Eger et al., 2018). The archaeal sequences were assigned to two dominant families; *Methanobacteriaceae* and *Methanomassiliicoccaceae.* These two families were also dominant in other 16S rRNA gene-based studies (Seedorf et al., 2015; Deusch et al., 2017; Difford et al., 2018). Mootral showed significant changes in archaeal community by decreasing the relative abundance of the dominant family group *Methanobacteriaceae.* Family *Methanobacteriaceae* includes the genus *Methanobrevibacter,* which is well known to be one of the major CH_4 producer in the rumen (Danielsson et al., 2012; Henderson et al., 2015). The reduction of family *Methanobacteriaceae* might be related to the

direct effect of organosulfur compounds of garlic in Mootral through interaction with cell membrane and inhibiting certain SH-containing enzymes essential for metabolic activities of methanogenic archaea (Patra and Yu, 2015). The effect of garlic on reduction of archaea has been shown also in previous studies (Calsamiglia et al., 2007; Patra and Yu, 2015). Furthermore, it has been reported that flavonoids may have effect on methanogenic archaea populations (Seradj et al., 2014). Additionally, Oskoueian et al. (2013) reported that flavonoids as naringin and quercetin at the concentration of 4.5% of the substrate suppressed CH_4 production through reduction of total methanogens. However, researchers are still not aware of the mode of action of flavonoids on archaea. Ruminal ciliated protozoa could enhance the methanogenesis as they are major H_2 producer in the rumen, as well as, they act as a host for methanogens. The produced H_2 is utilized by archaea found either inside or on the external surface of the protozoal cells (Finlay et al., 1994). *Methanobacteriaceae* family with its species were found to be associated with protozoa (Janssen and Kirs, 2008; Belanche et al., 2014). It has been shown that flavonoids supplementation reduced the total protozoal number (Oskoueian et al., 2013), which has not been investigated in the current study. So, further researches are required to study the effect of Mootral on protozoa.

Interestingly, family *Methanomassiliicoccaceae* showed a higher abundance in Mootral groups in a dose-dependent pattern. St-Pierre and Wright (2013) reported that its normal abundance within archaea in the rumen is about 5%, which was similar to the results of the current study. Up till now, as a new archaeal group, information on this taxonomy remains limited (Jin et al., 2017). A comprehensive understanding of the community of the family could help to know its function in the rumen. However, Danielsson et al. (2017) also reported that *Methanomassiliicoccaceae* was 1.5-fold more abundant in low CH_4 emitters than that in high CH_4 emitters. Moreover, its abundance was higher in a microbial community with low CH_4 production such as cows

supplemented with nitrate (Veneman et al., 2015), and also in the previous *in vitro* Mootral study (Eger et al., 2018).

5. Conclusion

Mootral supplementation reduced the CH_4 percentage and CH_4/CO_2 ratio in a dose-dependent manner. Mootral was able to shift the fermentation to produce more propionate and less acetate and to increase the production of total VFA without affecting IVDMD. Furthermore, 20% Mootral was effective to increase the abundance of H_2 consuming groups as family *Prevotellaceae* and *Veillonellaceae* and reduce some H_2 producing bacteria. In addition, the archaeal community was altered through reducing the major CH_4 producing family *Methanobacteriaceae* and increasing family *Methanomassiliicoccaceae*. The results of this study suggest that Mootral as a new combination could have the potentiality to be used for reduction of CH_4 for ruminants.

References

Anassori, E., Dalir-Naghadeh, B., Pirmohammadi, R., Taghizadeh, A., Asri-Rezaei, S., Maham, M., Farahmand-Azar, S., Farhoomand, P., 2011. Garlic: A potential alternative for monensin as a rumen modifier. Livest Sci 142, 276–287. https://doi.org/10.1016/j.livsci.2011.08.003.

AOAC, 1995. Official Methods of Analysis. Association of Official Analytical Chemists. Arlington, VA, USA.

Balcells, J., Aris, A., Serrano, A., Seradj, A.R., Crespo, J., Devant, M., 2012. Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. J Anim Sci 90, 4975–4984. https://doi.org/10.2527/jas.2011-4955.

Belanche, A., La Fuente, G. de, Newbold, C.J., 2014. Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiol Ecol 90, 663–677. https://doi.org/10.1111/1574-6941.12423.

Busquet, M., Calsamiglia, S., Ferret, A., Cardozo, P.W., Kamel, C., 2005. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. J Dairy Sci 88, 2508–2516. https://doi.org/10.3168/jds.S0022-0302(05)72928-3.

Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., Ferret, A., 2007. Invited Review: Essential oils as modifiers of rumen microbial fermentation. J Dairy Sci 90, 2580–2595. https://doi.org/10.3168/jds.2006-644.

Chen, L., Shen, Y., Wang, C., Ding, L., Zhao, F., Wang, M., Fu, J., Wang, H., 2019. *Megasphaera elsdenii* lactate degradation pattern shifts in rumen acidosis models. Front Microbiol 10, 162. https://doi.org/10.3389/fmicb.2019.00162.

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Müller, B., Schnürer, A., Bertilsson, J., 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. Front Microbiol 8. https://doi.org/10.3389/fmicb.2017.00226.

Danielsson, R., Schnürer, A., Arthurson, V., Bertilsson, J., 2012. methanogenic population and CH_4 production in Swedish dairy cows fed different levels of forage. Appl Environ Microbiol 78, 6172–6179. https://doi.org/10.1128/AEM.00675-12.

Denman, S.E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., McSweeney, C.S., 2015. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.01087.

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., Seifert, J., 2017. A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. Front Microbiol 8, 1605. https://doi.org/10.3389/fmicb.2017.01605.

Difford, G.F., Plichta, D.R., Løvendahl, P., Lassen, J., Noel, S.J., Højberg, O., Wright, A.-D.G., Zhu, Z., Kristensen, L., Nielsen, H.B., Guldbrandtsen, B., Sahana, G., 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. PLoS Genet 14, e1007580. https://doi.org/10.1371/journal.pgen.1007580.

Eger, M., Graz, M., Riede, S., Breves, G., 2018. Application of Mootral™ reduces methane production by altering the archaea community in the rumen simulation technique. Front Microbiol 9, 2094. https://doi.org/10.3389/fmicb.2018.02094.

Elghandour, M.M.Y., Salem, M.Z.M., Greiner, R., Salem, A.Z.M., 2018. Effects of natural blends of garlic and eucalypt essential oils on biogas production of four fibrous feeds at short-term of incubation in the ruminal anaerobic biosystem. J Sci Food Agric 98, 5313–5321. https://doi.org/10.1002/jsfa.9070.

Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T., Hirt, R.P., 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol Lett 117, 157–161. https://doi.org/10.1111/j.1574-6968.1994.tb06758.x.

Giang, N.T.T., Wanapat, M., Phesatcha, K., Kang, S., 2016. Level of *Leucaena leucocephala* silage feeding on intake, rumen fermentation, and nutrient digestibility in dairy steers. Trop Anim Health Prod 48, 1057–1064. https://doi.org/10.1007/s11250-016-1060-3.

Hansen, H., Nielsen, M., 2018. Impact of Mootral on rumen digestion. University of Copenhagen. https://ivh.ku.dk/nyheder/2018/impact-of-mootral-on-rumen-digestion/ (accessed accessed 31 August 2020).

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P.H., 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci Rep 5. https://doi.org/10.1038/srep14567.

Hernandez, A., Kholif, A.E., Lugo-Coyote, R., Elghandour, M., Cipriano, M., Rodríguez, G.B., Odongo, N.E., Salem, A., 2017. The effect of garlic oil, xylanase enzyme and yeast on biomethane and carbon dioxide production from 60-d old Holstein dairy calves fed a high concentrate diet. J Clean Prod 142, 2384–2392. https://doi.org/10.1016/j.jclepro.2016.11.036.

Hobson, P.N., Stewart, C.S., 1997. The Rumen microbial ecosystem, 2nd ed. Blackie Academic & Professional, London, New York, 719 pp.

Janssen, P.H., Kirs, M., 2008. Structure of the archaeal community of the rumen. AEM 74, 3619–3625. https://doi.org/10.1128/AEM.02812-07.

Jin, W., Cheng, Y., Zhu, W., 2017. The community structure of *Methanomassiliicoccales* in the rumen of Chinese goats and its response to a high-grain diet. J Animal Sci Biotechnol 8. https://doi.org/10.1186/s40104-017-0178-0.

Kawasaki, K., Min, X., Li, X., Hasegawa, E., Sakaguchi, E., 2015. Transfer of blood urea nitrogen to cecal microbial nitrogen is increased by fructo-oligosaccharide feeding in guinea pigs. Anim Sci J 86, 77–82. https://doi.org/10.1111/asj.12238.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., McEwan, J.C., Janssen, P.H., 2014. Two different bacterial community types are linked with the low-methane emission trait in sheep. PLoS ONE 9, e103171. https://doi.org/10.1371/journal.pone.0103171.

Kongmun, P., Wanapat, M., Pakdee, P., Navanukraw, C., 2010. Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique. Livest Sci 127, 38–44. https://doi.org/10.1016/j.livsci.2009.08.008.

Ma, T., Chen, D., Tu, Y., Zhang, N., Si, B., Deng, K., Diao, Q., 2016. Effect of supplementation of allicin on methanogenesis and ruminal microbial flora in Dorper crossbred ewes. J Animal Sci Biotechnol 7. https://doi.org/10.1186/s40104-015-0057-5.

Matsui, H., Wakabayashi, H., Fukushima, N., Ito, K., Nishikawa, A., Yoshimi, R., Ogawa, Y., Yoneda, S., Ban-Tokuda, T., Wakita, M., 2013. Effect of raw rice bran supplementation on rumen methanogen population density and *in vitro* rumen fermentation. Grassl Sci 59, 129–134. https://doi.org/10.1111/grs.12023.

McDougall, E.I., 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem J 43, 99–109. https://doi.org/10.1042/bj0430099.

Oskoueian, E., Abdullah, N., Oskoueian, A., 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. Biomed Res Int 2013, 1–8. https://doi.org/10.1155/2013/349129.

Ozbayram, E., Ince, O., Ince, B., Harms, H., Kleinsteuber, S., 2018. Comparison of rumen and manure microbiomes and implications for the inoculation of anaerobic digesters. Microorganisms 6, 15. https://doi.org/10.3390/microorganisms6010015.

Patra, A.K., Kamra, D.N., Agarwal, N., 2006. Effect of spices on rumen fermentation, methanogenesis and protozoa counts in *in vitro* gas production test. Int Congr Ser 1293, 176–179. https://doi.org/10.1016/j.ics.2006.01.025.

Patra, A.K., Yu, Z., 2013. Effective reduction of enteric methane production by a combination of nitrate and saponin without adverse effect on feed degradability, fermentation, or bacterial and archaeal communities of the rumen. Bioresour Technol 148, 352–360. https://doi.org/10.1016/j.biortech.2013.08.140.

Patra, A.K., Yu, Z., 2015. Effects of adaptation of *in vitro* rumen culture to garlic oil, nitrate, and saponin and their combinations on methanogenesis, fermentation, and abundances and diversity of microbial populations. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.01434.

Pelpolage, S.W., Goto, Y., Nagata, R., Fukuma, N., Furuta, T., Mizu, M., Han, K.-H., Fukushima, M., 2019. Colonic fermentation of water soluble fiber fraction extracted from sugarcane (*Sacchurum officinarum L.*) bagasse in murine models. Food Chem 292, 336–345. https://doi.org/10.1016/j.foodchem.2019.04.063.

Popova, M., Guyader, J., Silberberg, M., Seradj, A.R., Saro, C., Bernard, A., Gérard, C., Martin, C., Morgavi, D.P., 2018. Changes in the rumen microbiota of cows in response to dietary supplementation with nitrate, linseed, and saponin alone or in combination. Appl Environ Microbiol 85, e02657-18. https://doi.org/10.1128/AEM.02657-18.

Roque, B.M., van Lingen, H.J., Vrancken, H., Kebreab, E., 2019. Effect of Mootral—a garlic- and citrus-extract-based feed additive—on enteric methane emissions in feedlot cattle. Transl Anim Sci 3, 1383–1388. https://doi.org/10.1093/tas/txz133.

Seedorf, H., Kittelmann, S., Janssen, P.H., 2015. Few highly abundant operational taxonomic units dominate within rumen methanogenic archaeal species in New Zealand sheep and cattle. Appl Environ Microbiol 81, 986–995. https://doi.org/10.1128/AEM.03018-14.

Seradj, A.R., Abecia, L., Crespo, J., Villalba, D., Fondevila, M., Balcells, J., 2014. The effect of Bioflavex® and its pure flavonoid components on *in vitro* fermentation parameters and methane production in rumen fluid from steers given high concentrate diets. Anim Feed Sci Technol 197, 85–91. https://doi.org/10.1016/j.anifeedsci.2014.08.013.

St-Pierre, B., Wright, A.-D.G., 2013. Diversity of gut methanogens in herbivorous animals. Animal 7, 49–56. https://doi.org/10.1017/S1751731112000912.

Tapio, I., Snelling, T.J., Strozzi, F., Wallace, R.J., 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. J Animal Sci Biotechnol 8. https://doi.org/10.1186/s40104-017-0141-0.

Ungerfeld, E.M., 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.00037.

Veneman, J.B., Muetzel, S., Hart, K.J., Faulkner, C.L., Moorby, J.M., Perdok, H.B., Newbold, C.J., 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS ONE 10, e0140282. https://doi.org/10.1371/journal.pone.0140282.

Wallace, R.J., Rooke, J.A., McKain, N., Duthie, C.-A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M., Roehe, R., 2015. The rumen microbial metagenome associated with high methane production in cattle. BMC Genomics 16. https://doi.org/10.1186/s12864-015-2032-0.

Wanapat, M., Khejornsart, P., Pakdee, P., Wanapat, S., 2008. Effect of supplementation of garlic powder on rumen ecology and digestibility of nutrients in ruminants. J Sci Food Agric 88, 2231–2237. https://doi.org/10.1002/jsfa.3333.

Wang, K., Nan, X., Chu, K., Tong, J., Yang, L., Zheng, S., Zhao, G., Jiang, L., Xiong, B., 2018. Shifts of hydrogen metabolism from methanogenesis to propionate production in response to replacement of forage fiber with non-forage fiber sources in diets *in vitro*. Front Microbiol 9, 2764. https://doi.org/10.3389/fmicb.2018.02764.

Warren, F.J., Fukuma, N.M., Mikkelsen, D., Flanagan, B.M., Williams, B.A., Lisle, A.T., Ó Cuív, P., Morrison, M., Gidley, M.J., 2018. Food starch structure impacts gut microbiome composition. mSphere 3, e00086-18. https://doi.org/10.1128/mSphere.00086-18.

Wei, C., Guyader, J., Collazos, L., Beauchemin, K.A., Zhao, G.Y., 2019. Effects of gallic acid on *in vitro* rumen fermentation and methane production using rumen simulation (Rusitec) and batch-culture techniques. Anim Prod Sci 59, 277. https://doi.org/10.1071/AN17365.

Yang, W.Z., Benchaar, C., Ametaj, B.N., Chaves, A.V., He, M.L., McAllister, T.A., 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. J Dairy Sci 90, 5671–5681. https://doi.org/10.3168/jds.2007-0369.

Yu, Z., Morrison, M., 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques 36, 808–812. https://doi.org/10.2144/04365ST04.

Chapter Two

Mootral: Impacts on Behavior, Feed Intake, Rumen Fermentation, and Digestibility in Sheep

Abstract

There is global interest in decreasing CH_4 emissions from ruminants due to their negative impacts on the efficiency of the livestock industry and the environment. The present study was conducted to evaluate the effects of a natural combination of garlic and citrus, Mootral, as a novel feed supplement on behavior, feed intake (FI), rumen fermentation, digestibility, growth performance, and CH_4 reduction in sheep. Four Corriedale wether sheep, kept in individual metabolic cages and used in a 4 × 4 Latin square design, were fed a 50 : 50 grass hay : concentrate ratio diet at 55 g DN/kg $BW^{0.75}$/day. Mootral was supplemented at four concentrations: 0 (control), 1, 5, and 10 g/kg on a DM basis. The experiment was performed over four 21-day periods, each consisting of 14 days for the adaptation period and 7 days for sample collection. Health status was checked daily, while body weight was recorded weekly. Refusal feed, fecal, and urine samples were collected for 5 days. Behavioral observations were performed for 2 continuous days. CH_4 and CO_2 emissions were quantified by an open-circuit respiratory system on days 19 and 20, while rumen fluid was collected three times, both before (0 h) and after (3 and 6 h) morning feeding on day 21. The results of the current study showed that animals supplemented with Mootral dedicated more time to eating ($P < 0.05$) and had a higher frequency of lying around feeding time ($P < 0.001$) than non-supplemented animals. Continuous observation revealed that Mootral supplementation decreased the incidence of oral non-nutritive behaviors ($P < 0.001$) and abnormal behavior ($P = 0.016$) of the sheep around feeding times compared with the control group. Moreover, rumination activity was observed to be higher for supplemented animals through instantaneous scan sampling data ($P < 0.001$). Mootral supplementation did not affect total FI or any rumen fermentation parameters, digestibility, or growth performance. Compared with the control group, 5 and 10 g Mootral/kg DM decreased the CH_4 emission yield per digestible DM intake up to 7% ($P < 0.05$) and 12.8% ($P < 0.01$), respectively, while no effect on CO_2 emissions was observed. In conclusion,

Mootral, a combination of garlic and citrus, is a promising natural feed supplement that can be used to reduce CH_4 emissions from ruminants without causing any negative impact on FI, rumen fermentation, or digestibility, thereby leading to improved animal welfare.

1. Introduction

Our previous *in vitro* study presented in Chapter 1, using Mootral supplementation demonstrated alterations in the microbial community (bacteria and archaea) in a dose-dependent manner without impairing rumen fermentation. This *in vitro* study was performed using rumen fluid collected from sheep, and Mootral was supplemented at 10% and 20% of the substrate (50% grass hay : 50% concentrate). Therefore, in this chapter, an *in vivo* study using the same feeding ratio was conducted to investigate the optimal effective dose by evaluating increasing Mootral dosage levels for sheep to reduce CH_4 emissions considering its impact on behavior, rumen fermentation characteristics, digestibility, and growth performance.

2. Materials and Methods

This study was conducted from December 2019 to March 2020. All the experimental procedures and animal management were approved by the Animal Ethics and Care Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval number 19-49). The feed supplement Mootral, a mixture of garlic powder and bitter orange extracts, was provided by the Swiss company (Mootral SA, Rolle, Switzerland).

2.1- Animals, treatments, and experimental procedure

Four Corriedale wether sheep approximately 4 years old with an average body weight of 84.1 ± 2.76 kg at the beginning of the experiment were used in a 4 × 4 Latin square design. The experiment was carried out over a period of 84 days with four 21-day periods, with each period consisting of 14 days for adaptation and 7 days for sampling. Each animal was kept in an individual metabolic cage equipped with a ventilated respiratory collection hood where the space was enough for animals to stand and lie down easily. The sheep were fed twice a day at 8:30 and 16:30 h on a basal diet at 55 g DM/kg $BW^{0.75}$/day (Pen et al., 2007). The diet was composed of Kleingrass (*Panicum coloratum*) hay and a commercial concentrate mixture (Alpha-Kotan, Chubu Shiryo Co., Ltd., Aichi, Japan) at a ratio of (50 : 50). Animals had free access to clean drinking water and a mineral block (KOEN® E250 TZ, Nippon Zenyaku Kogyo Co., Fukushima, Japan). The dietary treatments were 0 (basal diet only), 1 (basal diet + 1 g Mootral/kg DM), 5 (basal diet + 5 g Mootral/kg DM) and 10 (basal diet + 10 g Mootral/kg DM). The required amount of Mootral per day was added in the morning feeding and mixed thoroughly with concentrates. The Mootral mixture was composed of 900 g/kg garlic granules and 100 g/kg of citrus extract powder. The garlic powder used for Mootral preparation was sourced from cultivated and carefully processed and dried non-GMO garlic of Chinese origin. The dried garlic granules were standardized to

contain 1% (w/w) allicin potential (S-Prop-2-en-1-yl prop-2-ene-1-sulfinothioate). The allicin concentration was determined by HPLC as described in detail by Eger et al. (2018). The citrus components for the Mootral mixture (naringin, naringenin, neohesperidin, rhoifolin, and neoeriocitrin) were developed from commercially available citrus extracts (Khush Ingredients, Oxford, United Kingdom) mainly extracted from bitter oranges. The total polyphenol content of the citrus extract was standardized to 45% (w/w) by the Folin-Ciocalteu method (Kaur and Kapoor, 2002). Flavonoid concentrations were analyzed by HPLC using standards from Sigma-Aldrich Ltd. (Dorset, United Kingdom). Further information on the Mootral preparation was described in detail by Eger et al. (2018). The proximate analysis of Mootral is described in Table 1. Animals were kept in the same room where the temperature was controlled and maintained at 18 ± 1 ˚C with light from 8:00 to 17:00 h. Aliquots of feed, orts, feces, and urine were collected during the collection period and analyzed for nutrient content following standard procedures.

2.2- Chemical analyses and apparent total-tract digestibility assay

During the collection period, all refusal feed, feces, and urine were collected for 5 days (days 15 to 19), their weights were recorded, and samples were stored at -20 °C until further analysis. At the end of the period, fecal samples from each treatment were thawed by keeping them at room temperature for 24 h and then pooled into a 200 g subsample, which was dried at 60 °C for 48 h in a forced-air oven and ground to pass through a 1-mm sieve for later laboratory analysis to determine apparent total tract digestibility. Total urine was collected daily in buckets containing 100 mL of 10% (v/v) H_2SO_4 to maintain a pH below 3. Approximately 150 mL of the total urine collected was stored daily at -20 °C for subsequent nitrogen (N) analysis.

According to the procedures of AOAC International (1995), samples of offered and refusal feed, Mootral, and feces were analyzed in triplicate for each treatment and period where DM content was measured by drying samples in a forced-air oven at 135 °C for 2 h (method 930.15). OM and ash were measured by placing samples into a muffle furnace at 500 °C for 3 h (method 942.05). The EE was determined according to method 920.39, while the N content of samples and urine was measured according to the method of Kjeldahl (method 984.13) using an electrical heating digester (DK 20, VELP Scientifica, Usmate (MB), Italy) and an automatic distillation apparatus (UDK 129 VELP Scientifica, Usmate (MB), Italy). Then CP was estimated as N × 6.25. The NDF, ADF, and ADL were measured using an ANKOM^{200} fiber analyzer (Ankom Technology Methods 6, 5 and 8, respectively; ANKOM Technology Corp., Macedon, NY, USA). The NDF and ADF were measured and expressed inclusive of residual ash. The NDF was measured using sodium sulfite without heat-stable α-amylase. The GE content of the grass hay, concentrate, and Mootral was analyzed by Japan Scientific Feeds Association, Tokyo, Japan, using a bomb calorimeter (C1, IKA® Werke GmbH & Co., KG, Staufen, Germany). The chemical composition of the ration is described in Table 1.

Table 1: Chemical composition of the feed and experimental diets (g/kg dry matter).

	Diet ingredients (g/kg DM)			Levels of Mootral (g/kg DM)			
	Kleingrass hay	**Concentrate**	**Mootral**	**0**	**1**	**5**	**10**
Dry Matter (in fresh matter)	923.3	880.7	871.5	902.0	902.0	901.8	901.7
Organic Matter	917.0	928.8	955.5	922.9	922.9	923.0	923.2
Crude Protein	118.8	227.9	210.7	173.4	173.4	173.6	173.7
Ether Extract	32.3	38.2	17.1	35.3	35.3	35.2	35.1
Neutral Detergent Fiber	635.2	265.1	35.5	450.2	449.7	448.1	446.0
Acid Detergent Fiber	315.1	109.4	33.7	212.2	212.0	211.3	210.4
Acid Detergent Lignin	55.6	32.8	1.8	16.4	16.4	16.3	16.2
Non-Fiber Carbohydrate	197.8	469.8	692.2	264.1	264.5	264.9	268.4
Gross Energy (MJ/kg DM)	18.8	19.2	19.2	19.0	19.0	19.0	19.0

2.3- Health status and growth performance

The health status of the animals was monitored every day by recording the incidence of cough, diarrhea, and nasal and ocular discharges. Body weight and average daily gain (ADG) were recorded weekly, while FI was recorded daily.

2.4- Behavioral observation

Sheep behavior was recorded continuously for a 48-h period on days 16 and 17 using an automated camera recording system installed in the experimental room. Eight cameras of 1080P full HD (2 cameras for each animal) were used and connected to Network Digital Video Recorder (DVR) (ZOSI Technology Co., Ltd, Hong Kong) with a 2-terabyte hard disk for recording and saving digital video recordings. One camera was fitted in front of the animal, and the other camera was fitted on the opposite side to capture the back side of each animal to ensure a wider field of vision and recording of all activities. Cameras were preset to ensure good quality recording even during the night using the night-vision infrared function. Data were backed up from DVR by USB and analyzed on a computer according to the ethogram described in Table 2. Behavioral data analysis was conducted using two methods (Altmann, 1974). The first method was a continuous focal sampling technique to determine the total time spent (duration), average of bout (duration), and frequency of each behavioral activity performed before, during, and after feeding times. For this method, animals were continuously observed for a total of 5 h each day. This comprised 30 minutes of observation before feeding (from 7:30 – 8:00 h and 15:30 – 16:00 h) and 2 h observation starting from the feeding time in both the morning and evening. The second method was the instantaneous scan sampling technique, which was recorded for 10 seconds at 15 minute-intervals

throughout the day regardless of the 5 h of continuous sampling. This method was used to estimate the frequency of any behavior exhibited by animals at fixed time intervals (Colgan, 1978).

Table 2: Behavioral ethogram to identify the behavioral activities performed by sheep.

Behavior	Description	Behavioral class
Eating	Animal's head in or over the feeder and engaged in chewing or swallowing food.	Ingestive
Drinking	Standing with muzzle within the water bucket and swallowing the water.	Ingestive
Licking mineral block	Standing with muzzle inside mineral bucket and licking the block.	Ingestive
Rumination	Irregular and repetitive mastication of the bolus without discernible food in the mouth.	Rumination
Standing	Animal stands in an upright position on all four legs with or without eating and ruminating.	Body posture
Lying	Full contact of body on floor of the cage and body not supported by all 4 legs, independently of any activity the animal might perform.	Body posture
Grooming	Scratching his head with the hindleg, licking or biting his leg, and rubbing his body or face on the edge of feeder or against the fixtures of the cage.	Body maintenance (Self grooming)
Oral non-nutritive	Licking or biting fixtures with non-nutritive finality.	Abnormal behavior
Abnormal	Number of times sheep's leg paw or dig on the floor of the cage.	Abnormal behavior

2.5- Gas emission measurement and calculations

The measurements of CH_4 and CO_2 were performed for two days (days 19 and 20) in open-circuit respiration chambers. Individual gas collection chambers were designed to retain the sheep's head inside the chamber for systematic collection of gas emissions. Emissions of CH_4 and CO_2 from each chamber were measured with an infrared gas analyzer (Shimadzu, URA-207, Kyoto, Japan). The analyzer was connected to the computer, programmed for data collection from each individual animal as described previously (Takahashi et al., 1999). Prior to gaseous exchange measurement, the gas analyzer was calibrated during each period on day 19 in the morning, just before starting the measurements using a span gas containing 2.04% CO_2 and 907 ppm CH_4 (Taiyo Nippon Sanso Corporation, Tokyo, Japan). The CH_4 energy was calculated from the gas volume obtained from the respiratory chamber according to the following calculation: CH_4 energy (kJ) = CH_4 (L) × 39.57 (Brouwer, 1965).

2.6- Rumen fluid sampling and rumen fermentation parameters analysis

On day 21, rumen fluid was collected at three different times: 1 h before morning feeding (0 h), followed by 3 h and 6 h after morning feeding. Rumen fluid samples were obtained by inserting a rubber stomach tube (Fujihira Industry Co., Ltd., Tokyo, Japan), and the fluid was sucked by a dry vacuum pump (DAP-15, Ulvac Kiko, Inc., Miyazaki, Japan). The first portion of rumen fluid (100 mL) was discarded, and the second portion was immediately strained through four layers of medical gauze. Approximately 35 mL of the rumen fluid from each animal was collected into 50-mL sterile tubes. The pH was measured immediately using a pH meter (LAQUA F-72, HORIBA Scientific, Kyoto, Japan). In the laboratory, 3 mL from each tube was further transferred into two 2-mL Eppendorf tubes® (Eppendorf AG, Hamburg, Germany). For NH_3-N analysis, samples were diluted 200 times with 0.1 M phosphate buffer (pH 5.5). Further preparation and analysis of

samples for NH_3-N and VFA were performed as previously described by Ahmed et al. (2021). Rumen samples were analyzed in duplicate for each animal and period.

2.7- Statistical analysis

All data were analyzed with a replicated 4 × 4 Latin square design using the MIXED model procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The model considered treatment and period as fixed effects and animal as a random effect. For CH_4, CO_2, and behavior data, 2 days were included as a fixed effect. For the rumen fermentation data, treatment, time, and treatment × time interactions were also included in the model as fixed effects. The orthogonal polynomial contrasts were used for variables associated with CH_4 to determine the linear and quadratic responses to increasing levels of Mootral in the diet. When significant differences were found, multiple comparisons were performed to check differences between groups using Tukey's method, and differences were accepted if $P < 0.05$ with tendencies detected at $0.05 < P < 0.10$. The results are presented as the least square means with the SEM. The correlation coefficients of variables were calculated using PROC CORR in SAS with Pearson's method, and significance was determined by a two-tailed test.

3. Results

Animals supplemented with or without Mootral did not exhibit any disease symptoms throughout the experiment. Continuous observations around feeding times for five h (Table 3) revealed that animals supplemented with higher doses of Mootral had an increase in eating times when compared with the control diet. Although the eating rate (g DM intake (DMI)/min) was lower in the 5-g group than in the 1-g Mootral/kg DM ($P = 0.04$), all animals supplemented with Mootral showed no significant difference in eating rate when compared with animals in the control group ($P > 0.10$). The duration, frequency of rumination, and rumination bout did not differ among the experimental groups ($P > 0.10$). The frequency of lying down showed increase for the Mootral-supplemented groups when compared with the control group ($P < 0.01$). While, the frequencies of licking mineral blocks and grooming did not differ among the experimental groups. Abnormal and oral non-nutritive behaviors were lower at all Mootral dosages when compared with the control group ($P < 0.05$). The results from the instantaneous scan sampling technique (Table 4) showed that rumination frequency was higher for 5 g ($P < 0.01$) than for 1 g ($P < 0.05$), yet the 10-g dosage was not significantly different ($P > 0.10$) when compared with 0 g Mootral/kg DM.

Mootral supplementation did not affect the total FI (fresh matter basis) or nutrient intake ($P > 0.10$, Table 5). Mootral supplementation to the sheep's diet also had no significant effect on any fermentation characteristics when compared with the control ($P > 0.10$). Butyrate concentration (mmol/L), however, in the 10-g Mootral/kg DM group was significantly lower ($P = 0.04$) than in the 5-g Mootral/kg DM group and tended ($P = 0.09$) to be lower than the 1-g Mootral/kg DM group but comparable to the 0-g Mootral/kg DM group ($P = 0.87$) (Table 6). The different collection times had a significant effect on all fermentation parameters except NH_3-N and molar proportions of propionate and butyrate. No significant interactions were found between treatment

and sampling time for any of the fermentation parameters reported (Table 6). Consequently, supplementation of sheep with different dosages of Mootral had no significant effect on ADG and apparent total tract digestibility of nutrients, although 5 g Mootral/kg DM showed a tendency toward lower digestibility of DM and NDF compared to 0 and 1 g Mootral/kg DM (Table 7).

Table 3: Effect of Mootral supplementation on behavioral activities (duration[1] and frequency[2]) of continuous observation for five hours in sheep.

Behavior[4]	Treatments[3]				SEM	*P* value
	0	1	5	10		
Eating (Duration, min)	89.84^{b}	87.37^{b}	106.21^{a}	93.58^{ab}	3.04	0.013
Eating rate (g of DMI/min)	17.33^{ab}	17.83^{a}	15.34^{b}	16.85^{ab}	2.25	0.045
Rumination (Duration, min)	28.84	22.67	31.83	27.78	2.11	0.333
Rumination (Frequency)	30.50	24.50	33.75	28.13	2.12	0.279
Rumination bout (Average, min)	0.65	0.72	0.74	0.83	0.053	0.569
Drinking (Duration, min)	3.98^{ab}	3.11^{b}	3.40^{ab}	4.38^{a}	0.268	0.047
Drinking (Frequency)	12.50^{ab}	10.00^{b}	12.00^{ab}	13.88^{a}	0.645	0.032
Standing (Duration, min)	206.77^{a}	172.09^{b}	192.75^{ab}	186.50^{ab}	7.30	0.044
Standing (Frequency)	5.13^{b}	4.88^{b}	5.50^{b}	7.25^{a}	0.452	<0.001
Lying (Duration, min)	89.83^{b}	124.14^{a}	103.19^{ab}	109.42^{ab}	7.16	0.063
Lying (Frequency)	3.88^{c}	4.88^{b}	4.88^{b}	6.75^{a}	0.493	<0.001
Licking mineral block (Duration, min)	8.00	4.74	6.57	5.23	0.518	0.093
Licking mineral block (Frequency)	13.38	11.13	13.00	10.88	0.868	0.642
Grooming (Frequency)	13.50	16.00	18.88	13.38	1.26	0.252
Oral non-nutritive (Frequency)	12.75^{a}	6.25^{bc}	3.75^{c}	7.75^{b}	1.19	<0.001
Abnormal (Frequency)	2.13^{a}	1.00^{ab}	0.00^{b}	0.50^{ab}	0.427	0.016

[1] Duration: the total length of time the animal consumed in such behavior in (min) minutes. **[2]** Frequency: total number of times such behavior was done by animal. **[3]** 0, 1, 5, 10 g Mootral/kg of dry matter. **[4]** DMI: dry matter intake, Rumination bout: the average time for a single rumination cycle occurrence in min. **[a,b,c]** Means with different superscripts within a row are significantly different ($P < 0.05$) by Tukey test. SEM: standard error of the mean.

Table 4: Effect of Mootral supplementation on the frequency[1] of behavioral activities using instantaneous scan sampling technique in sheep.

Behavior	Treatments[2]				SEM	*P* value
	0	1	5	10		
Eating	0.38[ab]	0.13[b]	0.88[ab]	1.13[a]	0.147	0.040
Rumination	20.63[c]	24.00[ab]	25.75[a]	21.50[cb]	0.610	<0.001
Drinking	--	--	0.25	--	0	--
Licking mineral block	0.88	0.38	1.13	0.63	0.191	0.588
Standing	22.50[a]	17.38[b]	24.13[a]	26.75[a]	1.55	<0.001
Lying	50.75[a]	55.88[b]	49.50[a]	48.0[a]	1.58	<0.001
Grooming	1.13	1.25	0.50	1.13	0.207	0.592
Oral non-nutritive	0.38	0.88	0.88	1.75	0.24	0.072
Abnormal	--	--	--	--	--	--

[1] Frequency: total number of times such behavior was done by animal. [2] 0, 1, 5, 10 g Mootral/kg of dry matter. [a,b,c] Means with different superscripts within a row are significantly different ($P < 0.05$) by Tukey test. (--): not observed. SEM: standard error of the mean.

Table 5: Effect of Mootral supplementation on nutrient intake in sheep (g/day).

Variable[2]	Treatments[1]				SEM	*P* value
	0	1	5	10		
FI (Fresh matter)	1705.94	1715.10	1715.76	1726.09	17.41	0.296
DM	1537.85	1545.44	1545.95	1556.22	16.34	0.335
OM	1419.67	1426.10	1426.69	1436.06	15.40	0.378
CP	266.68	267.83	267.78	269.17	3.22	0.259
NDF	688.89	694.71	695.00	699.30	6.62	0.337
ADF	323.54	326.73	326.58	329.14	5.700	0.300
GE (MJ/day)	29.22	29.37	29.38	29.58	0.311	0.317

[1] 0, 1, 5, 10 g Mootral/kg of dry matter. [2] FI: feed intake, DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber, GE: gross energy, SEM: standard error of the mean.

Table 6: Effect of Mootral supplementation on rumen fermentation profile at different times in sheep.

Variable[2]	Treatments[1] (g/kg)				Time (h)			SEM	*P* value		
	0	1	5	10	0	3	6		Treatment	Time	Treatment × Time
pH	7.00	6.91	6.94	7.01	7.16^{x}	6.78^{y}	6.96^{z}	0.047	0.484	<0.001	0.352
NH_3-N (mg/dL)	33.27	33.60	28.95	28.89	30.76	35.45	27.31	1.80	0.459	0.071	0.440
TVFA (mmol/L)	102.84	114.54	113.97	100.89	95.46^{y}	119.14^{x}	109.59^{x}	3.48	0.062	<0.001	0.253
Acetate (mmol/L)	74.01	81.99	81.66	72.36	69.02^{y}	84.91^{x}	78.58^{x}	2.50	0.072	0.001	0.256
Propionate (mmol/L)	19.35	21.79	21.31	19.52	17.47^{y}	23.37^{x}	20.64^{x}	0.731	0.185	<0.001	0.364
Butyrate (mmol/L)	9.69^{ab}	10.76^{ab}	11.00^{a}	9.01^{b}	8.97^{y}	11.00^{x}	10.37^{xy}	0.36	0.029	0.008	0.558
Acetate (mol/100 mol)	71.76	71.65	71.65	71.80	72.25^{x}	71.21^{y}	71.68^{xy}	0.20	0.963	0.008	0.806
Propionate (mol/100 mol)	18.61	18.96	18.66	19.29	18.37	19.46	18.82	0.226	0.601	0.095	0.973
Butyrate (mol/100 mol)	9.63	9.39	9.68	8.91	9.38	9.33	9.46	0.180	0.312	0.912	0.100
A/P ratio	3.90	3.80	3.86	3.75	3.96^{x}	3.69^{y}	3.83^{xy}	0.052	0.590	0.048	0.944

[1] 0, 1, 5, 10 g Mootral/kg of dry matter. **[2]** TVFA: total volatile fatty acids, A/P ratio: acetate/propionate ratio. **[a,b]** Means with different superscripts within a row are significantly different ($P < 0.05$) for treatment effect by Tukey test. **[x,y,z]** Means with different superscripts within a row are significantly different ($P < 0.05$) for time effect (hour) by Tukey test. SEM: standard error of the mean.

Table 7: Effect of Mootral supplementation on apparent total tract digestibility of nutrients (g/kg), CP retention (g/day), and average daily gain (ADG) (g/day) in sheep.

Variable[2]	Treatments[1]				SEM	*P* value
	0	1	5	10		
DM	745.94	740.59	734.79	746.48	3.96	0.047
OM	743.15	740.93	731.00	743.13	4.20	0.157
CP	768.62	772.96	755.24	763.88	4.39	0.120
CP retention (g/day)	41.65	40.96	32.07	59.51	4. 85	0.155
NDF	677.59	661.72	655.92	677.71	8.15	0.038
ADF	572.54	555.17	551.40	569.20	9.75	0.129
ADG	200.19	238.41	212.72	198.93	15.33	0.164

1 0, 1, 5, 10 g Mootral/kg of dry matter. **2** DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber, ADG: average daily gain. SEM: standard error of the mean.

Compared with the control group, Mootral supplementation decreased CH_4 emissions in a dose-dependent manner (linear effect, $P < 0.01$). The dose of 1 g Mootral/kg DM did not decrease CH_4 emissions when expressed as g/day nor g/kg DMI ($P > 0.10$), while 5 g Mootral/kg DM decreased CH_4 emissions up to 8% ($P < 0.05$, Table 8) when expressed as g/day and g/kg DMI. In addition, 10 g Mootral/kg DM decreased CH_4/DMI (g/kg) up to 11.6% compared with the control group ($P < 0.01$, Table 8). A similar effect was observed in terms of CH_4/OM intake (OMI) (g/kg) ($P < 0.01$). The CH_4 yield per digestible DMI and OMI decreased linearly ($P < 0.01$) by approximately 7% and 12.8% for 5 g and 10 g Mootral/kg DM, respectively. Additionally, the CH_4 intensity (g/kg ADG) was reduced in a linear manner ($P < 0.01$) by 9.3% and 12.8% for 5 g and 10 g Mootral/kg DM, respectively, when compared with the control. Furthermore, the lost energy in the form of CH_4 and the ratio of CH_4 energy loss to GE intake were lower in the 5- and 10-g Mootral/kg DM groups than in the control group ($P < 0.01$, Table 8). However, CO_2 was not affected by different Mootral dosages ($P > 0.10$, Table 9).

Table 8: Effect of Mootral supplementation on methane (CH_4) emissions in sheep.

CH_4 emission[2]	Treatments[1]				SEM	Contrast *P* value	
	0	1	5	10		Linear	Quadratic
ml/min/MBW	0.878^{a}	0.827^{ab}	0.806^{b}	0.781^{b}	0.024	0.002	0.252
L/day	37.29^{a}	34.95^{ab}	34.24^{b}	33.52^{b}	0.987	0.002	0.234
g/day	26.63^{a}	24.97^{ab}	24.46^{b}	23.94^{b}	0.705	0.002	0.234
L/kg DMI	24.23^{a}	22.77^{ab}	22.21^{b}	21.41^{b}	0.618	0.001	0.257
g/kg DMI	17.31^{a}	16.27^{ab}	15.86^{b}	15.30^{b}	0.441	0.001	0.257
L/kg OMI	26.88^{a}	25.02^{ab}	24.41^{b}	23.84^{b}	0.689	0.002	0.121
g/kg OMI	19.20^{a}	17.87^{ab}	17.44^{b}	17.03^{b}	0.492	0.002	0.121
g/kg DDMI	23.17^{a}	21.79^{ab}	21.55^{b}	20.20^{b}	0.575	0.001	0.755
g/kg DOMI	25.26^{a}	23.64^{ab}	23.45^{b}	22.05^{b}	0.608	0.001	0.677
CH_4 intensity (g/kg ADG)	82.25^{a}	76.15^{b}	74.64^{b}	71.72^{b}	2.14	0.001	0.200
CH_4 energy (MJ/day)	1.48^{a}	1.38^{ab}	1.36^{b}	1.33^{b}	0.039	0.002	0.234
CH_4 energy/GEI (MJ/MJ)	0.052^{a}	0.049^{ab}	0.048^{b}	0.047^{b}	0.001	0.002	0.126

[1] 0, 1, 5, 10 g Mootral/kg of dry matter. **[2]** MBW: metabolic body weight, DMI: dry matter intake, OMI: organic matter intake, DDMI: digestible dry matter intake, DOMI: digestible organic matter intake, ADG: average daily gain, GEI: gross energy intake. **[a,b]** Means with different superscripts within a row are significantly different ($P < 0.05$) by Tukey test. SEM: standard error of the mean.

Table 9: Effect of Mootral supplementation on Carbon dioxide (CO_2) emissions in sheep.

CO_2 emission[2]	Treatments[1]				SEM	*P* value
	0	1	5	10		
ml/min/MBW	11.24	11.25	11.32	10.88	0.239	0.609
L/day	477.38	477.90	481.42	466.44	10.55	0.575
g/day	937.72	938.74	945.65	916.22	20.73	0.575
L/kg DMI	309.27	310.33	312.16	304.28	5.59	0.613
g/kg DMI	607.50	609.57	613.18	597.69	10.98	0.613

[1] 0, 1, 5, 10 g Mootral/kg of dry matter. [2] MBW: metabolic body weight, DMI: dry matter intake. SEM: standard error of the mean.

Table 10 shows the correlation coefficients between different measured variables. The DMI was negatively correlated with non-nutritive behavior (r = -0.47, *P* = 0.07) and abnormal behavior (r = -0.51, *P* = 0.04). Similarly, eating behavior showed a negative correlation with non-nutritive behavior (r = -0.45, *P* = 0.08) but positive correlations with total VFA (r = 0.56, *P* = 0.02), NH_3-N (r = 0.57, *P* = 0.02) and ADG (r = 0.44, *P* = 0.09). The production of total VFA had strong positive correlations with NH_3-N (r = 0.66, *P* < 0.01) and ADG (r = 0.56, *P* = 0.02) but negative correlations with non-nutritive behavior (r = -0.57, *P* = 0.02) and abnormal behavior (r = -0.55, *P* = 0.03). The ADG showed a positive correlation with eating behavior, duration of rumination (r = 0.52, *P* = 0.04), and total VFA. Moreover, the current study showed that the production of CH_4 was correlated with several variables measured in this experiment (Table 11). For instance, CH_4/OMI (g/kg) had a positive correlation with abnormal behavior (r = 0.52, *P* = 0.02), nutrient digestibility (r = 0.60, *P* < 0.05 for OM, and *P* < 0.1 for DM and ADF), acetate ratio (r = 0.50, *P* = 0.03), and acetate/propionate (A/P) ratio (r = 0.70, *P* < 0.01), while its correlation was negative only with propionate ratio (r = -0.67, *P* < 0.01) (Table 11). Similarly, CH_4 production in terms of g/day and digestible OMI showed almost the same correlations with the aforementioned variables as well as a positive correlation with NH_3-N (r = 0.60, r = 0.53, *P* < 0.05, respectively, Table 11).

Table 10: Correlation coefficients between different variables in sheep.

Variable[1]	Variable[1]					
	DMI (kg)	**Eating behavior (min)**	**TVFA (mmol/L)**	**DM digestibility (g/kg)**	**OM digestibility (g/kg)**	**ADG (g)**
Eating behavior (min)	0.10	---	0.56**	0.01	0.18	0.44*
Non-nutritive behavior (frequency)	-0.47*	-0.45*	-0.57**	0.43*	0.34	-0.03
Abnormal behavior (frequency)	-0.51**	-0.20	-0.55**	0.67***	0.47*	-0.28
OMI (kg)	1.00***	0.06	0.38	-0.39	-0.24	0.33
Rumination (min)	-0.01	0.12	0.11	0.10	0.26	0.52**
Acetate (mol/100 mol)	-0.19	0.29	0.23	0.40	0.71***	0.46*
Propionate (mol/100 mol)	0.25	-0.34	0.01	-0.50**	-0.49*	-0.17
Butyrate (mol/100 mol)	-0.11	0.11	-0.23	0.19	-0.14	-0.27
A/P ratio	-0.26	0.34	0.03	0.52**	0.58**	0.22
TVFA (mmol/L)	0.39	0.56**	---	-0.52**	-0.08	0.56**
NH_3-N (mg/dL)	0.40	0.57**	0.66***	-0.12	0.12	0.35
NDF digestibility (g/kg)	-0.32	0.04	-0.52**	0.98***	0.78***	0.03
ADF digestibility (g/kg)	-0.47*	0.13	-0.21	0.83***	0.90***	0.33
ADG (g)	0.38	0.44*	0.56**	0.02	0.31	---

[1] DMI: dry matter intake, OMI: organic matter intake, NDF: neutral detergent fiber, ADF: acid detergent fiber, A/P ratio: acetate/propionate ratio, TVFA: total volatile fatty acids, ADG: average daily gain. * ($P < 0.1$), ** ($P < 0.05$), *** ($P < 0.01$) by Pearson correlation.

Table 11: Correlation coefficients between methane emission and different variables in sheep

Variable[1]	Methane		
	g/day	g/kg OMI	g/kg digestible OMI
DMI (kg)	0.26	-0.03	-0.03
OMI (kg)	0.26	-0.03	0.01
Rumination (min)	-0.11	-0.10	-0.16
Abnormal behavior (frequency)	0.34	0.52**	0.59*
Digestible DMI (kg)	0.42	0.18	0.18
Digestible OMI (kg)	0.51**	0.27	0.26
DM digestibility	0.30	0.43*	0.34
OM digestibility	0.51**	0.60**	0.50**
NDF digestibility (g/kg)	0.28	0.40	0.31
ADF digestibility (g/kg)	0.31	0.47*	0.37
Acetate (mol/100 mol)	0.42	0.50**	0.43*
Propionate (mol/100 mol)	-0.57**	-0.67***	-0.65***
Butyrate (mol/100 mol)	0.25	0.29	0.34
A/P ratio	0.61**	0.71***	0.68***
TVFA (mmol/L)	0.32	0.21	0.25
NH_3-N (mg/dL)	0.60**	0.49*	0.53**
ADG (g)	0.31	0.22	0.20

[1] DMI: dry matter intake, OMI: organic matter intake, NDF: neutral detergent fiber, ADF: acid detergent fiber, A/P ratio: acetate/propionate ratio, TVFA: total volatile fatty acids, ADG: average daily gain. * ($P < 0.1$), ** ($P < 0.05$), *** ($P < 0.01$) by Pearson correlation.

4. Discussion

A large number of synthetic and natural compounds have shown promising *in vitro* CH_4 reduction effects (Cattani et al., 2016; Chagas et al., 2019; Jayanegara et al., 2020). However, *in vivo* experiments have shown that most of these anti-methanogenic compounds often exhibit inconsistent efficacy and lead to negative impacts on FI, digestibility, and total VFA production when fed at high doses to achieve effective CH_4 reduction (Patra et al., 2017). The combination of different anti-methanogenic compounds is a relatively new approach, and few *in vivo* trials have been reported. Our *in vitro* trial in Chapter 1, using a natural feed supplement combination, Mootral, with the same feeding style (50 : 50) as the current study demonstrated supplement efficacy to reduce the abundance of family *Methanobacteriaceae* and reduce the proportion of CH_4 in the produced gas without impairing fermentation. Therefore, this study was conducted to evaluate the efficacy of Mootral supplementation in reducing CH_4 emissions with an emphasis on behavior, FI, fermentation characteristics, and digestibility in sheep.

It has been reported that diet has the potential to modulate animal behavior through the communication network between the gastrointestinal system, microbiota, and central nervous system (Kraimi et al., 2019). The findings of the current study showed that Mootral supplementation to the diet of sheep led to significant changes in their behavior. With increasing the Mootral's supplementation level, animals dedicated more time to eating which may be attributed to the reduction in the gene expression of bitter taste receptors in the gastrointestinal tract, such as taste 2 receptor (TAS2R). Similarly, Paniagua et al. (2019) demonstrated that dietary supplementation with 0.04% bitter orange extract to bulls led to an increase in eating. The authors attributed the observed results to the reduction in the gene expression of TAS2R in the rumen epithelium, caused by the effect of naringin, which degraded in the rumen to naringenin acting as

an important bitter masking molecule (Ley et al., 2014). Da Silva et al. (2017) found that administration of garlic bulb extract did not have any effect on eating time in sheep fed with 50 : 50 concentrate : hay grass; thus, we can hypothesize that modulation of eating might be related to the role of flavonoids extracted from citrus. Interestingly, Mootral supplementation at all doses reduced the abnormal behaviors in sheep, as these animals were more devoted to eating and therefore had less time to exhibit abnormal behaviors, such as pawing the floor and licking cage bars. The observed correlation in the present study further supports the aforementioned findings, as DMI and eating behavior were negatively correlated with the incidence of abnormal behaviors. It is well known that abnormal behaviors and oral non-nutritive behaviors are indicators of poor welfare, and performance in farm animals with gut dysfunction has been suggested to be one of the possible reasons (Mason and Rushen, 2006). The aforementioned is also confirmed in the correlation analysis of this study, where total VFA production as the main energy source for ruminants was negatively correlated with the reported abnormal behaviors. It has been reported previously that flavonoid supplementation reduced the gene expression of some proteins related to inflammation in the rumen epithelium and that inflammation is involved in serotonin release (Paniagua et al., 2019). Serotonin has been associated with a reduction in aggressive and abnormal behaviors (Haagensen et al., 2014), which might explain the reduction in such incidences in the current study but needs further investigation. Furthermore, the increase in the duration and frequency of lying down around feeding times in Mootral-supplemented animals might be attributable to the role of serotonin in mood regulation and sleep induction (Bacqué-Cazenave et al., 2020). Durmic and Blache (2012) have also reported that secondary compounds in plants may affect the central and peripheral nervous systems and have an impact on psychological responses such as emotions, stress-related disorders, and calming effects on animals. The results of scan

sampling conducted throughout the day revealed that rumination activity was lower for the control group than Mootral-supplemented animals, which may explain the higher incidence of oral non-nutritive behaviors in the control group as a result of digestive dysfunction (Devant et al., 2016; Paniagua et al., 2019). Paniagua et al. (2018) reported that bulls supplemented with *Citrus aurantium* displayed improved rumen health, as they had lighter and reduced baldness areas in the rumen walls compared with the control group, which exhibited more oral non-nutritive behaviors. Additionally, Manchope et al. (2017) reported that naringenin could act as a potent antioxidant and has anti-inflammatory effects. Studies on the influence of feed supplements and the microbiota gut-brain axis on the behavior of farm animals are still few, suggesting that it could have large consequences on animal welfare. This is the first study reporting the impact of Mootral on behavioral activities in ruminants. More research considering gene expression in the gut is strongly needed to better understand the mechanism by which this novel feed supplement modulates animal behavior.

Garlic and citrus are characterized by their bitter taste, which may interfere with FI. It has been reported that sheep's acceptability of garlic varies with the rate of its inclusion in the diet (Strickland et al., 2009). Nonetheless, the results of the current study showed that Mootral supplementation even at the highest dose (10 g/kg DM), did not cause any negative impact on the eating rate (g DMI/min) or, consequently, on the total FI. The reason could be that proper mixing of the Mootral supplement with concentrates prevented any possible alterations in diet palatability. Our finding is similar to the results of Patra et al. (2011), who fed sheep *Allium sativum* bulbs at a rate of 10 g/kg DM, and Kim et al. (2018), who found no change in DMI when rams were supplemented with garlic powder at 5 g/kg concentrate.

The results of the current study showed that the different Mootral dosages had no negative effect on rumen fermentation parameters, suggesting that the studied dosages might not have been high enough to cause obvious alterations in the fermentation profile. Mootral supplementation at higher dosages of 10% and 20% substrate in the rumen-simulation technique increased the production of total VFA and the butyrate ratio but decreased the propionate ratio (Eger et al., 2018), while Mootral supplementation at 20% substrate in a batch-culture technique for 24 h led to an increase in total VFA and the propionate ratio with a decrease in the A/P ratio (Chapter 1). Reports on the effect of garlic on total VFA production have been inconsistent, with some studies reporting no effect (Patra et al., 2007; Klevenhusen et al., 2011; Panthee et al., 2017), while others have reported an increase in total VFA with inconsistency for VFA concentrations (Busquet et al., 2005; Yang et al., 2007). In addition, Wanapat et al. (2008) reported that the addition of garlic powder to the diet of steers resulted in a reduction in total VFA concentrations. The abovementioned discrepancies among the results may be related to the different diets and dosages used.

Similar to the rumen fermentation profile, Mootral supplementation did not affect apparent total tract digestibility of nutrients and consequently had no negative effect on ADG, which is consistent with previous studies performed using garlic (Chaves et al., 2008) or flavonoids (Oskoueian et al., 2013) supplementation (individually) or when Mootral was added to the total mixed ration of steers (Roque et al., 2019). Although ADG did not differ among the experimental groups, the current study showed that all animals on the experimental diets gained significant weight (average: 212.5 g/daily) during the experimental period (84 days), which might be attributable to the high-quality diet offered and consumed by the animals (173.5 CP, 35 EE, and

265 NFC, g/kg DM, and 19 MJ/kg DM, GE). Along these lines, ADG showed a strong positive correlation with OMI and the production of total VFA.

Although flavonoids showed effective CH_4 reduction through *in vitro* experiments, they have not been extensively evaluated with respect to rumen methanogenesis *in vivo*. Besides, reports on the effect of garlic on CH_4 reduction *in vivo* have been inconsistent, where some found effective reduction (Zhu et al., 2012; Ma et al., 2016) with others reporting no such effect (Klevenhusen et al., 2011). The current study showed that the combination of allicin from garlic and flavonoids had a synergistic effect in reducing CH_4 production in a dose-dependent manner. *In vitro* experiments with Mootral supplementation have reported a direct inhibitory effect on ruminal archaea through decreasing the relative abundance of the major CH_4-producing group, the *Methanobacteriaceae* family (Eger et al., 2018; Chapter 1). Organosulfur compounds such as allicin, which is also a main component in Mootral have been found to reduce methanogenesis through inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A, an enzyme important for different metabolic activities in archaea (Busquet et al., 2005). It is well established that ciliated protozoa can enhance methanogenesis as they are major H_2 producers and act as symbiotic hosts for methanogens (Belanche et al., 2014). Therefore, in the current study, indirect inhibition of methanogenesis might have occurred due to the effect of flavonoids such as naringin in reducing the total number of protozoa (Oskoueian et al., 2013), but this needs confirmation in upcoming studies.

The current study showed a reduction in CH_4 without any change in propionate, suggesting that the accumulated H_2 resulting from inhibition of methanogenesis was redirected into a metabolic pathway other than propionogenesis. In a meta-analysis study, Ungerfeld (2015) reported that inhibition of methanogens could stimulate amino acid and ruminal formate synthesis and consequently increase microbial biomass as a H_2 sink. Alternative H_2 sinks for nitrate/nitrite-

reducing bacteria and sulfate-reducing bacteria in the rumen have been reported previously (Beauchemin et al., 2020). A study by Martinez-Fernandez et al. (2016) showed that inhibition of CH_4 production was accompanied by expulsion of H_2 as a gas. The authors measured H_2 and CH_4 production from steers fed a roughage hay diet and 60% roughage hay : 40% concentrate diet supplemented with an anti-methanogenic compound (chloroform). The authors found that increasing levels of chloroform resulted in an increase in H_2 expulsion as CH_4 production decreased without any effect on DMI. Similar findings were observed by Mitsumori et al. (2012) when a diet with 50% grass and 50% concentrate was supplemented with bromochloromethane to Japanese goats. Similar findings as above were observed without an effect on FI and digestibility when studying 3-nitrooxypropanol (Veneman et al., 2015; Vyas et al., 2018), linseed oil, and nitrate (Hristov et al., 2015). Moreover, using Mootral, Eger et al. (2018) observed that the reduction of CH_4 was accompanied by an increase in H_2 production without an increase in propionate, revealing that another H_2 sink has been stimulated, which would be of interest for further research.

The correlation analysis between CH_4 production and other measured variables confirmed what was mentioned previously. For instance, in the current study, CH_4 production was highly correlated with acetate and negatively correlated with propionate, which was also reported in previous studies (Johnson and Johnson, 1995; Moss et al., 2000), because the fermentation pathways for producing more acetate are associated with H_2 production utilized for CH_4 formation, while propionate pathways are alternative sinks for H_2. Moreover, CH_4 production was positively correlated with nutrient digestibility: As the more substrate fermented, the more H_2 there was available for methanogens. This finding has been confirmed previously in several studies (Wang et al., 2007; Patra, 2010; Cabezas-Garcia et al., 2017). Wang et al. (2007) and Wang et al. (2009)

observed a positive correlation between CH_4 and NH_3-N in sheep, which was also confirmed in the current study. Interestingly, this study showed a positive correlation between the production of CH_4 and the incidence of abnormal behavior around the feeding time. To the best of our knowledge, this finding has not been corroborated previously and requires further research for confirmation.

To our knowledge, this is the first study reporting the impact of Mootral supplementation on CH_4 emissions in sheep while considering behavior, FI, rumen fermentation, and digestibility at the same time. Further research using higher doses of Mootral at different forage-to-concentrate ratios is required to investigate the supplement's potential in effective CH_4 reduction, taking into account the impacts on rumen fermentation, digestibility, and behavior with gene analysis.

5. Conclusion

Dietary supplementation of Mootral to sheep improved their behavior and welfare by reducing the incidence of abnormal behaviors. Its supplementation had no negative effects on eating rate and FI when mixed well with concentrates. Additionally, it did not impair either rumen fermentation or apparent total tract digestibility. Meanwhile, this phytogenic combination reduced CH_4 emissions from sheep in a typical dose-dependent manner up to 12.8% when added at 10 g/kg DM. Therefore, Mootral supplementation as a natural combination is a promising candidate to reduce CH_4 emissions from ruminants.

References

Ahmed, E., Fukuma, N., Hanada, M., Nishida, T., 2021. The efficacy of plant-based bioactives supplementation to different proportion of concentrate diets on methane production and rumen fermentation characteristics *in vitro*. Animals 11 (4), 1029. https://doi.org/10.3390/ani11041029.

Altmann, J., 1974. Observational study of behavior: sampling methods. Behaviour 49 (3), 227–267. https://doi.org/10.1163/156853974x00534.

AOAC International, 1995. Official methods of analysis of AOAC International: Association of Official Analytical Chemists. AOAC International, Arlington Va.

Bacqué-Cazenave, J., Bharatiya, R., Barrière, G., Delbecque, J.-P., Bouguiyoud, N., Di Giovanni, G., Cattaert, D., Deurwaerdère, P. de, 2020. Serotonin in animal cognition and behavior. Int. J. Mol. Sci. 21 (5). https://doi.org/10.3390/ijms21051649.

Beauchemin, K.A., Ungerfeld, E.M., Eckard, R.J., Wang, M., 2020. Review: Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. Animal 14 (S1), s2-s16. https://doi.org/10.1017/S1751731119003100.

Belanche, A., La Fuente, G. de, Newbold, C.J., 2014. Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiol. Ecol. 90 (3), 663–677. https://doi.org/10.1111/1574-6941.12423.

Brouwer, E., 1965. Report of sub-committee on constants and factors. in: Blaxter, K.E. (Ed.), Proceedings of the Third Symposium on Energy Metabolism, EAAP, 11. Academic Press, London, pp. 441–443.

Busquet, M., Calsamiglia, S., Ferret, A., Cardozo, P.W., Kamel, C., 2005. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. J. Dairy Sci. 88 (7), 2508–2516. https://doi.org/10.3168/jds.S0022-0302(05)72928-3.

Cabezas-Garcia, E.H., Krizsan, S.J., Shingfield, K.J., Huhtanen, P., 2017. Between-cow variation in digestion and rumen fermentation variables associated with methane production. J. Dairy Sci. 100 (6), 4409–4424. https://doi.org/10.3168/jds.2016-12206.

Cattani, M., Maccarana, L., Rossi, G., Tagliapietra, F., Schiavon, S., Bailoni, L., 2016. Dose-response and inclusion effects of pure natural extracts and synthetic compounds on *in vitro* methane production. Anim. Feed Sci. Technol. 218, 100–109. https://doi.org/10.1016/j.anifeedsci.2016.05.014.

Chagas, J.C., Ramin, M., Krizsan, S.J., 2019. *In vitro* evaluation of different dietary methane mitigation strategies. Animals 9 (12). https://doi.org/10.3390/ani9121120.

Chaves, A.V., Stanford, K., Dugan, M., Gibson, L.L., McAllister, T.A., van Herk, F., Benchaar, C., 2008. Effects of cinnamaldehyde, garlic and juniper berry essential oils on rumen fermentation, blood metabolites, growth performance, and carcass characteristics of growing lambs. Livest. Sci. 117 (2-3), 215–224. https://doi.org/10.1016/j.livsci.2007.12.013.

Colgan, P.W., 1978. Quantitative ethology. Wiley, New York, Chichester.

Da Silva, C.S., Souza, E.J.O. de, Pereira, G.F.C., Cavalcante, E.O., Lima, E.I.M. de, Torres, T.R., Da Silva, J.R.C., Da Silva, D.C., 2017. Plant extracts as phytogenic additives considering intake, digestibility, and feeding behavior of sheep. Trop. Anim. Health Prod. 49 (2), 353–359. https://doi.org/10.1007/s11250-016-1199-y.

Devant, M., Penner, G.B., Marti, S., Quintana, B., Fábregas, F., Bach, A., Arís, A., 2016. Behavior and inflammation of the rumen and cecum in Holstein bulls fed high-concentrate diets with different concentrate presentation forms with or without straw supplementation. J. Anim. Sci. 94 (9), 3902–3917. https://doi.org/10.2527/jas.2016-0594.

Durmic, Z., Blache, D., 2012. Bioactive plants and plant products: Effects on animal function, health and welfare. Anim. Feed Sci. Technol. 176 (1-4), 150–162. https://doi.org/10.1016/j.anifeedsci.2012.07.018.

Eger, M., Graz, M., Riede, S., Breves, G., 2018. Application of Mootral^{TM} reduces methane production by altering the archaea community in the rumen simulation technique. Front. Microbiol. 9, 2094. https://doi.org/10.3389/fmicb.2018.02094.

Haagensen, A.M.J., Sørensen, D.B., Sandøe, P., Matthews, L.R., Birck, M.M., Fels, J.J., Astrup, A., 2014. High fat, low carbohydrate diet limit fear and aggression in Göttingen minipigs. PLoS One 9 (4), e93821. https://doi.org/10.1371/journal.pone.0093821.

Hristov, A.N., Oh, J., Giallongo, F., Frederick, T.W., Harper, M.T., Weeks, H.L., Branco, A.F., Moate, P.J., Deighton, M.H., Williams, S.R.O., Kindermann, M., Duval, S., 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. U. S. A. 112 (34), 10663–10668. https://doi.org/10.1073/pnas.1504124112.

Jayanegara, A., Yogianto, Y., Wina, E., Sudarman, A., Kondo, M., Obitsu, T., Kreuzer, M., 2020. Combination effects of plant extracts rich in tannins and saponins as feed additives for

mitigating *in vitro* ruminal methane and ammonia formation. Animals 10 (9). https://doi.org/10.3390/ani10091531.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73 (8), 2483–2492. https://doi.org/10.2527/1995.7382483x.

Kaur, C., Kapoor, H.C., 2002. Anti-oxidant activity and total phenolic content of some Asian vegetables. Int. J. Food Sci. Technol. 37 (2), 153–161. https://doi.org/10.1046/j.1365-2621.2002.00552.x.

Kim, J.Y., Ghassemi Nejad, J., Park, J.Y., Lee, B.H., Hanada, M., Kim, B.W., Sung, K.I., 2018. *In vivo* evaluation of garlic (*Allium sativum*) supplementation to rice straw-based diet on mitigation of CH_4 and CO_2 emissions and blood profiles using crossbreed rams. J. Sci. Food Agric. 98 (14), 5197–5204. https://doi.org/10.1002/jsfa.9055.

Klevenhusen, F., Zeitz, J.O., Duval, S., Kreuzer, M., Soliva, C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Anim. Feed Sci. Technol. 166-167, 356–363. https://doi.org/10.1016/j.anifeedsci.2011.04.071.

Kraimi, N., Dawkins, M., Gebhardt-Henrich, S.G., Velge, P., Rychlik, I., Volf, J., Creach, P., Smith, A., Colles, F., Leterrier, C., 2019. Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review. Physiol. Behav. 210, 112658. https://doi.org/10.1016/j.physbeh.2019.112658.

Ley, J.P., Krammer, G., Kindel, G., Gatfield, I.-L., Müller, M., 2014. Use of hydroxyflavanones for masking bitter taste. Google Patents. https://patents.google.com/patent/US8685436B2/en.

Ma, T., Chen, D., Tu, Y., Zhang, N., Si, B., Deng, K., Diao, Q., 2016. Effect of supplementation of allicin on methanogenesis and ruminal microbial flora in Dorper crossbred ewes. J. Anim. Sci. Biotechnol. 7 (1), 1. https://doi.org/10.1186/s40104-015-0057-5.

Manchope, M.F., Casagrande, R., Verri, W.A., 2017. Naringenin: an analgesic and anti-inflammatory citrus flavanone. Oncotarget 8 (3), 3766–3767. https://doi.org/10.18632/oncotarget.14084.

Martinez-Fernandez, G., Denman, S.E., Yang, C., Cheung, J., Mitsumori, M., McSweeney, C.S., 2016. Methane inhibition alters the microbial community, hydrogen flow, and fermentation response in the rumen of cattle. Front. Microbiol. 7, 1122. https://doi.org/10.3389/fmicb.2016.01122.

Mason, G., Rushen, J., 2006. Stereotypic animal behaviour: Fundamentals and applications to welfare, 2nd ed. CABI, Wallingford.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S.E., McSweeney, C.S., 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108 (3), 482–491. https://doi.org/10.1017/S0007114511005794.

Moss, A.R., Jouany, J.-P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49 (3), 231–253. https://doi.org/10.1051/animres:2000119.

Oskoueian, E., Abdullah, N., Oskoueian, A., 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. BioMed Res. Int. 2013. https://doi.org/10.1155/2013/349129.

Paniagua, M., Crespo, J., Arís, A., Devant, M., 2019. *Citrus aurantium* flavonoid extract improves concentrate efficiency, animal behavior, and reduces rumen inflammation of Holstein bulls fed high-concentrate diets. Anim. Feed Sci. Technol. 258, 114304. https://doi.org/10.1016/j.anifeedsci.2019.114304.

Paniagua, M., Crespo, J., Bach, A., Devant, M., 2018. Effects of flavonoids extracted from *Citrus aurantium* on performance, eating and animal behavior, rumen health, and carcass quality in Holstein bulls fed high-concentrate diets. Anim. Feed Sci. Technol. 246, 114–126. https://doi.org/10.1016/j.anifeedsci.2018.08.010.

Panthee, A., Matsuno, A., Al-Mamun, M., Sano, H., 2017. Effect of feeding garlic leaves on rumen fermentation, methane emission, plasma glucose kinetics, and nitrogen utilization in sheep. J. Anim. Sci. Technol. 59 (1), 14. https://doi.org/10.1186/s40781-017-0139-3.

Patra, A., Park, T., Kim, M., Yu, Z., 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. J. Anim. Sci. Biotechnol. 8 (1), 13. https://doi.org/10.1186/s40104-017-0145-9.

Patra, A.K., 2010. Meta-analyses of effects of phytochemicals on digestibility and rumen fermentation characteristics associated with methanogenesis. J. Sci. Food Agric. 90 (15), 2700–2708. https://doi.org/10.1002/jsfa.4143.

Patra, A.K., Kamra, D.N., Bhar, R., Kumar, R., Agarwal, N., 2011. Effect of *Terminalia chebula* and *Allium sativum* on *in vivo* methane emission by sheep. J. Anim. Physiol. Anim. Nutr. 95 (2), 187–191. https://doi.org/10.1111/j.1439-0396.2010.01039.x.

Patra, A.K., Kamra. D, N., Agarwal, N., Chatterjee, P.N., 2007. Effect of *Terminalia chebuaa* and *Allium sativum* on rumen fermentation, enzyme activities and microbial profile in buffaloes. Indian J. Anim. Nutr. 24 (4), 251–255.

Pen, B., Takaura, K., Yamaguchi, S., Asa, R., Takahashi, J., 2007. Effects of *Yucca schidigera* and *Quillaja saponaria* with or without β 1–4 galacto-oligosaccharides on ruminal fermentation, methane production and nitrogen utilization in sheep. Anim. Feed Sci. Technol. 138 (1), 75–88. https://doi.org/10.1016/j.anifeedsci.2006.11.018.

Roque, B.M., van Lingen, H.J., Vrancken, H., Kebreab, E., 2019. Effect of Mootral—a garlic-and citrus-extract-based feed additive—on enteric methane emissions in feedlot cattle. Transl. Anim. Sci. 3 (4), 1383–1388. https://doi.org/10.1093/tas/txz133.

Strickland, V.J., Fisher, J.S., Potts, W.T., Hepworth, G.W., 2009. Lack of response to garlic fed at different dose rates for the control of *Haemonchus contortus* in Merino wether lambs. Anim. Prod. Sci. 49 (12), 1093. https://doi.org/10.1071/AN09057.

Takahashi, J., Chaudhry, A., Beneke, R., Young, B., 1999. An open-circuit hood system for gaseous exchange measurements in small ruminants. Small Ruminant Res. 32 (1), 31–36. https://doi.org/10.1016/S0921-4488(98)00163-1.

Ungerfeld, E.M., 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6, 37. https://doi.org/10.3389/fmicb.2015.00037.

Veneman, J.B., Muetzel, S., Hart, K.J., Faulkner, C.L., Moorby, J.M., Perdok, H.B., Newbold, C.J., 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS One 10 (10). https://doi.org/10.1371/journal.pone.0140282.

Vyas, D., McGinn, S.M., Duval, S.M., Kindermann, M.K., Beauchemin, K.A., 2018. Optimal dose of 3-nitrooxypropanol for decreasing enteric methane emissions from beef cattle fed high-forage and high-grain diets. Anim. Prod. Sci. 58 (6), 1049. https://doi.org/10.1071/AN15705.

Wanapat, M., Khejornsart, P., Pakdee, P., Wanapat, S., 2008. Effect of supplementation of garlic powder on rumen ecology and digestibility of nutrients in ruminants. J. Sci. Food Agric. 88 (13), 2231–2237. https://doi.org/10.1002/jsfa.3333.

Wang, C., Wang, S., Zhou, H., Glindemann, T., 2007. Effects of forage composition and growing season on methane emission from sheep in the Inner Mongolia steppe of China. Ecol. Res. 22 (1), 41–48. https://doi.org/10.1007/s11284-006-0191-9.

Wang, C.J., Wang, S.P., Zhou, H., 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Anim. Feed Sci. Technol. 148 (2-4), 157–166. https://doi.org/10.1016/j.anifeedsci.2008.03.008.

Yang, W.Z., Benchaar, C., Ametaj, B.N., Chaves, A.V., He, M.L., McAllister, T.A., 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. J. Dairy Sci. 90 (12), 5671–5681. https://doi.org/10.3168/jds.2007-0369.

Zhu, Z., Mao, S., Zhu, W., 2012. Effects of ruminal infusion of garlic oil on fermentation dynamics, fatty acid profile and abundance of bacteria involved in biohydrogenation in rumen of goats. Asian Australas. J. Anim. Sci. 25 (7), 962–970. https://doi.org/10.5713/ajas.2011.11442.

Chapter Three

The Efficacy of Mootral Supplementation to Different Proportion of Concentrate Diets on Methane Production and Rumen Fermentation Characteristics *in vitro*

Abstract

This *in vitro* study was conducted to investigate the impacts of Mootral, a plant-bioactives extract, on methane production, rumen fermentation, and digestibility in different feeding models. The dietary treatments were 1000 g grass/kg ration + 0 g concentrate/kg ration (100:0), 80:20, 60:40, 40:60, and 20:80. Mootral was supplemented at 200 g/kg of the feed. Each group consisted of 6 replicates. The experiment was performed as *in vitro* batch culture for 24 h at 39 °C. This procedure was repeated in 3 consecutive runs. The results of this experiment showed that supplementation with Mootral strongly reduced methane production in all kinds of feeding models ($P < 0.001$). Its efficacy in reducing methane/digestible dry matter was 44% in the 100:0 diet, and this reduction power increased up to a 69.2% with the inclusion of CON in the 20:80 diet. The Mootral application significantly increased gas and carbon dioxide production and the concentration of ammonia-nitrogen, but decreased the pH ($P < 0.001$). In contrast, it did not interfere with organic matter and fiber digestibility. Supplementation with Mootral was effective in altering rumen fermentation toward less acetate and more propionate and butyrate ($P < 0.001$). Additionally, it improved the production of total volatile fatty acids in all feeding models ($P < 0.001$). In conclusion, the Mootral combination showed effective methane reduction by improving rumen fermentation characteristics without exhibiting adverse effects on fiber digestibility. Thus, Mootral could be used with all kinds of feeding models to effectively mitigate methane emissions from ruminants.

1. Introduction

Mootral have showed the ability to reduce CH_4 production without impairing rumen fermentation characteristics in two *in vitro* studies (Eger et al. 2018; Chapter 1). We hypothesized that Mootral supplementation might have the same mode of action and the potentiality to reduce CH_4 regardless grass:concentrate ratio. However, there are still limitations to proving the efficacy of this new combination with different feeding models (grass:concentrate ratios). Therefore, in this chapter, this study was conducted to investigate the potential of Mootral to be used as an antimethanogenic feed supplement with different feeding styles of ruminants, considering its impact on rumen fermentation and nutrient digestibility.

2. Materials and Methods

2.1- Donor animals and rumen fluid collection

The animals used in this experiment were kept and cared for by Field Science Center, Obihiro University of Agriculture and Veterinary Medicine, Japan. The animal management and sampling procedures were approved by the Obihiro University of Agriculture and Veterinary Medicine, Animal Care and Use Committee (Approval number, 20-119).

At 3 h after the morning feeding, approximately 3 L of rumen fluid was collected from two ruminally fistulated, nonlactating Holstein cows (880 kg average body weight). The cows were maintained on a daily diet of Orchard grass (*Dactylis glomerata*) hay (OM, 980 g/kg; CP, 132 g/kg; NDF, 701 g/kg; ADF, 354 g/kg; ADL, 40 g/kg; on DM basis) with free access to clean drinking water and mineral blocks (KOEN® E250 TZ, Nippon Zenyaku Kogyo Co., Fukushima, Japan). The rumen fluid from each cow was collected from four different locations in the rumen. The collected rumen fluid was strained through four layers of surgical gauze into a thermos flask that was prewarmed to 39 °C and then immediately transferred to the laboratory within 15 minutes.

2.2- Experimental treatments and in vitro incubation technique

Prior to the *in vitro* incubation, ten experimental groups, with six replicates each, were prepared with approximately 500 mg (fresh matter) of ground substrate comprised of Kleingrass (*Panicum coloratum*) hay and commercial concentrate mixture at different ratios with and without Mootral inclusion. The Mootral mixture is composed of 90% garlic granules (Allicin) and 10% citrus extract powder (Naringin, Naringenin, Neohesperidin, Rhoifolin, and Neoeriocitrin). The garlic powder used for Mootral preparation was sourced from cultivated and carefully processed and

dried non-GMO garlic of Chinese origin. The dried garlic granules were standardized to contain 1% (w/w) Allicin potential (S-Prop-2-en-1-yl prop-2-ene-1-sulfinothioate). Allicin concentration was determined by HPLC as described in details by Eger et al. (2018). The citrus components for the Mootral mixture (Naringin, Naringenin, Neohesperidin, Rhoifolin, and Neoeriocitrin) were developed from commercially available citrus extracts (Khush Ingredients, Oxford, United Kingdom) mainly extracted from bitter oranges (*Citrus aurantium*). The total polyphenol content of the citrus extract was standardized to 45% (w/w) by the Folin-Ciocalteau method (Kaur and Kapoor 2002). Flavonoid concentrations were analyzed by HPLC using standards from (Sigma-Aldrich Ltd., Dorset, United Kingdom). Further information on the Mootral preparation was described in details by Eger et al. (2018). This mixture is known commercially as Mootral and was provided by a Swiss company (Mootral SA, Rolle, Switzerland).

The experimental diets were as follows: 1- 1000 g grass/kg ration + 0 g concentrate/kg ration (100:0), 2- 100:0 + 200 g Mootral /kg of substrate (20 MT), 3- 80:20, 4- 80:20 + 20 MT, 5- 60:40, 6- 60:40 + 20 MT, 7- 40:60, 8- 40:60 + 20 MT, 9- 20:80, and 10- 20:80 + 20 MT. Five hundred milligrams of each of the experimental substrates (GRS and CON) was added to preweighed ANKOM filter bags (F57, ANKOM Technology, Macedon, NY, USA), which were heat-sealed and placed in 120 mL glass bottles, whereas the Mootral mixture was added directly to the bottles (out of filter bag) one day before incubation. The dosage of Mootral used in the current study was based on the effective dose that altered the bacterial and archaeal communities in our previous *in vitro* study (Chapter 1). Therefore, that effective dosage has to be used in further *in vitro* trials for better understanding the mode of action of this new mixture. The chemical composition of the substrates and Mootral are described in Table 1.

Table 1: Chemical composition of ration and plant-bioactives extract (g/kg of dry matter) used in 24 h *in vitro* incubation.

(g/kg dry matter)	Kleingrass hay	Concentrate	Mootral
Dry matter (g/kg fresh matter)	844.9	843.0	871.5
Organic matter	904.7	934.2	955.5
Crude ash	95.3	65.8	44.5
Crude protein	134.8	223.1	210.7
Ether extract	38.4	38.0	17.1
Neutral detergent fiber	662.5	232.6	35.5
Acid detergent fiber	362.6	109.1	33.7
Acid detergent lignin	52.2	30.1	1.8

The procedure of *in vitro* batch culture was performed as described by Menke and Steingass (1988). In the laboratory, the collected rumen fluids from the two cows were mixed together in one beaker under a constant stream of CO_2. Forty milliliters of fresh buffer solution at a pH of 6.8 prepared according to McDougall (1948) with twenty mL of rumen fluid was added to each 120 mL bottle under continuous CO_2 flushing to maintain anaerobic conditions. Thereafter, the fermentation bottles were flushed with CO_2 before sealing with butyl rubber stoppers and aluminum caps (Maruemu Co., Ltd, Osaka, Japan). All bottles were incubated for 24 h at 39 °C. This batch culture procedure was repeated in three consecutive runs on three different days. In each run, two blanks without substrate (empty filter bag plus 60 mL of buffered rumen fluid) were included to be used for digestibility and gas production correction. In total, 180 bottles plus 6 blank bottles were examined in this study.

2.3- Sample collection

After 24 h of incubation, the total gas production was measured, and gas samples were collected from the headspace of the glass bottles into vacutainer tubes (BD Vacutainer®, Becton Drive, USA). The tubes were stored at room temperature until CH_4 and CO_2 determination. Thereafter, the bottles' caps were removed, and the pH of each tube was recorded using a pH meter (LAQUA F-72, HORIBA Scientific, Kyoto, Japan). Then, aliquots of the culture fluid were transferred into 1.5 mL Eppendorf tubes and centrifuged at 16,000×g and 4 °C for 5 minutes. The supernatant was collected and transferred into a new Eppendorf tube® (Eppendorf AG, Hamburg, Germany), which was stored at −20 °C until use for volatile fatty acid (VFA) and ammonia nitrogen (NH_3-N) analysis. The bags were removed from the bottles, washed under running tap water until the draining fluid became clear, and then dried at 60 °C for 48 h to determine the *in vitro* dry matter digestibility (IVDMD). After IVDMD determination, the bags were used for the determination of *in vitro* organic matter digestibility (IVOMD), *in vitro* neutral detergent fiber digestibility (IVNDFD), and *in vitro* acid detergent fiber digestibility (IVADFD). The residues in the fermentation bottles were discarded.

2.4- Chemical analysis

The chemical composition of the GRS, CON, Mootral and remaining substrate in the bags was determined following the standard procedure of AOAC (1995). DM content was measured by drying the samples in an air-forced oven at 135 °C for 2 h (930.15). OM and ash were measured by placing the samples into a muffle furnace at 500 °C for 3 h (942.05). Nitrogen (N) was measured according to the method of Kjeldahl (984.13) using an electrical heating digester (DK 20, VELP Scientifica, Usmate (MB), Italy) and an automatic distillation apparatus (UDK 129 VELP Scientifica, Usmate (MB), Italy), and then CP was estimated as N × 6.25. The NDF, ADF, and

ADL were measured and expressed as inclusive residual ash using an ANKOM200 Fiber Analyzer (Ankom Technology Methods 6, 5 and 8, respectively; ANKOM Technology Corp., Macedon, NY, USA). The NDF was measured using sodium sulfite with heat-stable α-amylase.

2.5- Gas composition analysis

The concentrations of CH_4 and CO_2 in the gas samples were determined by injection of 1 mL using a Hamilton gastight syringe (Hamilton Company, Reno, Nevada, USA) into a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan). The carrier gas was helium. The temperatures of the infuser port, column, and detector were 70 °C, 150 °C, and 150 °C, respectively. The identification of CH_4 and CO_2 was based on the retention time.

2.6- Volatile fatty acids and ammonia-nitrogen analysis

The concentration of VFA was determined using high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) after diluting the supernatant 3 times with distilled water. Briefly, the analytical specifications were as follows: column, Shim-pak SCR-102H (7 mm, i.d. 8.0 mm×300 mm, Shimadzu Corp., Kyoto, Japan); eluent flow rate and mobile phase for organic acid analysis (Shimadzu Corp., Kyoto, Japan) at 0.8 mL/min; column temperature, 40 °C; reaction reagent and flow rate, pH buffer for organic acid analysis (Shimadzu Corp., Kyoto, Japan) at 0.8 mL/min; conductivity detector (CDD-10AVP, Shimadzu Corp., Kyoto, Japan). Quantification of the VFA concentration was performed using an external standard quantitation method (Chapter 1).

The NH_3-N concentration was measured by diluting samples 50 times with 0.1 M phosphate buffer (pH 5.5) and then they were analyzed following the procedure of the modified Fujii-Okuda method (Kawasaki et al. 2015) using an NH_3 kit (FUJIFILM Wako Pure Chemical Corp, Osaka,

Japan). The plate was read by a microplate reader (SH-1000 Lab, Corona Electric Co., Ltd., Japan) at an optical density of 630 nm.

2.7- Statistical analysis

All variables were analyzed using PROC MIXED by SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The model included the treatment (diet) effect, Mootral effect, and their interaction as fixed effects, whereas the experimental runs were considered random effects. Least square means and SEM were calculated, and the differences of means were estimated by pairwise t-tests (PDIFF option of PROC MIXED). Significance was declared at $P < 0.05$, and a tendency toward significance was declared when the P value was between 0.05 and 0.10.

3. Results

3.1- Effect of Mootral supplementation on in vitro pH, gas production, and gas composition

Supplementation of Mootral to all feeding models reduced the pH ($P < 0.001$) when compared with its corresponding group without Mootral supplementation in the same feeding model (Table 2). Moreover, the inclusion of Mootral increased the absolute total gas production when correlated with DM and digestible DM in all feeding styles ($P < 0.001$, Table 2).

Adding Mootral to all feeding styles decreased the proportion of CH_4 but increased the proportion of CO_2 in the produced gas ($P < 0.001$, Table 3). Furthermore, the CH_4/CO_2 ratio in the produced gas (mL/mL) decreased in all feeding models due to Mootral's effect ($P < 0.001$, Table 3). Mootral inclusion was effective with all diets in reducing the production of CH_4/DM (mL/g) ($P < 0.001$); moreover, it reduced the production of CH_4/digestible DM (mL/g) by 44.2%, 48.2%, 59.7%, 63.7%, and 69.2% in 100:0, 80:20, 60:40, 40:60, and 20:80, respectively, ($P < 0.001$). In contrast, the production of CO_2/DM and CO_2/digestible DM (mL/g) increased ($P < 0.001$) due to the effect of Mootral in all feeding styles (Table 3).

3.2- Effect of Mootral supplementation on in vitro nutrient digestibility and ammonia-nitrogen concentration

Mootral supplementation did not affect the IVDMD in the different experimental diets except in the 80:20 and 60:40 diets, where adding Mootral to these styles reduced the IVDMD ($P < 0.01$, Table 4). However, IVOMD, IVNDFD, and IVADFD did not show any differences when Mootral was added as compared with their corresponding groups without Mootral supplementation ($P > 0.05$, Table 4). Mootral inclusion increased the NH_3-N concentration ($P < 0.01$) in 100:0 and 60:40

and it tended to increase in 40:60 (P = 0.088), but there was a non-significant numerical increase in 80:20 (P = 0.106) and 20:80 (P = 0.32) (Table 4).

3.3- Effect of Mootral supplementation on in vitro volatile fatty acids

Mootral supplementation did not show any effect on the acetate concentration in all feeding models; however, the interaction between Mootral and treatment showed a difference for Mootral supplemented groups to be increased in 100:0 ($P < 0.001$) and to be decreased in 20:80 ($P < 0.05$) compared with its corresponding treatment without Mootral inclusion (Table 5). In contrast, the acetate ratio decreased in all feeding models due to Mootral supplementation ($P < 0.01$), whereas the interaction between Mootral and treatment did not have a significant effect ($P > 0.05$, Table 5). The concentration and the ratio of propionate and butyrate increased ($P < 0.001$) by adding Mootral in all feeding models. Additionally, the concentration of total volatile fatty acids (TVFA) showed the same finding (Table 5). The acetate/propionate (A/P) ratio decreased ($P < 0.001$) with Mootral supplementation in all feeding styles (Table 5).

Table 2: Effect of Mootral (MT) supplementation on gas production and pH in different feeding styles after 24 h *in vitro* incubation (n = 18).

Parameter	Treatments[1]										SEM	*P*-value		
	100:0		80:20		60:40		40:60		20:80					
	0	20	0	20	0	20	0	20	0	20		Trt	MT	Trt×MT
Gas production (mL)	30.06	45.53***	40.18	51.19***	43.50	53.08***	44.17	57.14***	45.94	54.47***	0.74	< 0.001	< 0.001	0.07
Gas/DM[2] (mL/g)	71.17	107.67***	94.90	120.93***	102.89	125.44***	104.31	135.09***	108.68	128.87***	1.76	< 0.001	< 0.001	0.07
Gas/Digestible DM (mL/g)	191.13	281.87***	207.46	288.09***	209.23	247.66***	200.41	266.66***	197.98	241.21***	3.18	< 0.001	< 0.001	< 0.001
pH	6.58	6.50***	6.55	6.47***	6.53	6.44***	6.51	6.41***	6.50	6.40***	0.01	< 0.001	< 0.001	0.36

[1] grass:concentrate ratio; 0: 0 g MT/kg; 20: 200 g MT/kg of substrate. [2] DM: dry matter. Asterisks in 20 mean significant difference between 0 and 200 g MT/kg in the same feeding model, *** ($P < 0.001$). Trt: treatment; MT: Mootral; Trt×MT: interaction between treatment and Mootral. SEM: standard error of the mean.

Table 3: Effect of Mootral (MT) supplementation on CH_4 and CO_2 production in different feeding styles after 24 h *in vitro* incubation (n = 18).

Parameter	Treatments[1]										SEM	*P*-value		
	100:0		80:20		60:40		40:60		20:80					
	0	20	0	20	0	20	0	20	0	20		Trt	MT	Trt×MT
CH_4 (%)	5.19	1.99***	5.59	2.07***	5..87	1.84***	5.83	1.59***	5.89	1.51***	0.16	0.006	< 0.001	< 0.001
CO_2 (%)	94.81	98.01***	94.41	97.93***	94.13	98.16***	94.17	98.41***	94.11	98.49***	0.16	0.006	< 0.001	0.21
CH_4/CO_2 ratio (mL/mL)	0.055	0.020***	0.059	0.021***	0.062	0.019***	0.062	0.016***	0.063	0.015***	0.002	0.004	< 0.001	< 0.001
CH_4/DM[2] (mL/g)	3.68	2.09***	5.36	2.57***	6.11	2.30***	6.09	2.16***	6.48	1.91***	0.16	< 0.001	< 0.001	< 0.001
CO_2/DM (mL/g)	67.45	105.58***	89.50	118.36***	96.78	123.14***	98.22	132.93***	102.20	126.40***	1.78	< 0.001	< 0.001	0.12
CH_4/digestible DM (mL/g)	9.88	5.51***	11.62	6.02***	12.35	4.98***	11.64	4.23***	11.66	3.59***	0.29	< 0.001	< 0.001	< 0.001
CO_2/digestible DM (mL/g)	181.26	276.36***	195.85	282.07***	196.89	269.68***	188.77	262.43***	186.33	237.60***	3.35	< 0.001	< 0.001	< 0.001

[1] grass:concentrate ratio; 0: 0 g MT/kg; 20: 200 g MT/kg of substrate. [2] DM: dry matter. Asterisks in 20 mean significant difference between 0 and 200 g MT/kg in the same feeding model, *** ($P < 0.001$). Trt: treatment; MT: Mootral; Trt×MT: interaction between treatment and Mootral. SEM: standard error of the mean.

Table 4: Effect of Mootral (MT) supplementation on digestibility and ammonia-nitrogen in different feeding styles after 24 h *in vitro* incubation (n = 18).

Parameter	Treatments[1]										SEM	*P*-value		
	100:0		80:20		60:40		40:60		20:80					
	0	20	0	20	0	20	0	20	0	20		Trt	MT	Trt×MT
IVDMD[2]	0.38	0.38	0.45	0.42**	0.49	0.46**	0.52	0.51	0.55	0.53	0.56	< 0.001	0.001	0.10
IVOMD[3]	0.43	0.41	0.51	0.49	0.57	0.52	0.59	0.59	0.64	0.61	0.02	< 0.001	0.03	0.82
IVNDFD[4]	0.36	0.36	0.40	0.38	0.38	0.32	0.36	0.34	0.32	0.35	0.01	0.61	0.56	0.76
IVADFD[5]	0.21	0.24	0.25	0.21	0.21	0.23	0.20	0.23	0.22	0.23	0.01	0.97	0.62	0.64
NH_3-N[6] (mg/dL)	4.46	6.43***	6.30	7.09	6.14	7.51**	6.01	6.84	7.35	7.83	0.18	< 0.001	< 0.001	0.17

[1] grass:concentrate ratio; 0: 0 g MT/kg; 20: 200 g MT/kg of substrate. [2] IVDMD: In vitro dry matter digestibility. [3] IVOMD: In vitro organic matter digestibility. [4] IVNDFD: In vitro neutral detergent fiber digestibility. [5] IVADFD: In vitro acid detergent fiber digestibility. [6] NH_3-N: ammonia-nitrogen. Asterisks in 20 mean significant difference between 0 and 200 g MT/kg in the same feeding model, ** ($P < 0.01$), *** ($P < 0.001$). Trt: treatment; MT: Mootral; Trt×MT: interaction between treatment and Mootral. SEM: standard error of the mean.

Table 5: Effect of Mootral (MT) supplementation on volatile fatty acids in different feeding styles after 24 h *in vitro* incubation (n = 18).

Parameter	Treatments[1]										SEM	*P*-value		
	100:0		80:20		60:40		40:60		20:80					
	0	20	0	20	0	20	0	20	0	20		Trt	MT	Trt×MT
Acetate (mmol/L)	62.54	66.02**	64.81	66.12	64.81	65.66	65.61	65.91	66.80	64.15*	0.54	0.45	0.21	0.01
Propionate (mmol/L)	15.24	20.19***	18.57	22.95***	19.38	24.34***	20.61	26.82***	22.21	26.78***	0.29	< 0.001	< 0.001	0.20
Butyrate (mmol/L)	7.14	10.86***	8.53	11.38***	9.01	12.36***	9.17	12.71***	9.84	12.89***	0.16	< 0.001	< 0.001	0.21
TVFA[2] (mmol/L)	84.91	97.07***	91.92	100.45***	93.20	102.36***	95.40	105.44***	98.85	103.81*	0.78	< 0.001	< 0.001	0.07
Acetate (mol/100 mol)	73.64	67.93***	70.53	65.67***	69.47	64.00***	68.72	62.39***	67.54	61.59***	0.30	< 0.001	< 0.001	0.07
Propionate (mol/100 mol)	17.95	20.80***	20.20	22.92***	20.86	23.86***	21.66	25.49***	22.46	25.92***	0.20	< 0.001	< 0.001	0.04
Butyrate (mol/100 mol)	8.41	11.27***	9.27	11.41***	9.67	12.14***	9.62	12.12***	10.00	12.50***	0.13	< 0.001	< 0.001	0.50
A/P ratio[3]	4.11	3.29***	3.49	2.88***	3.34	2.69***	3.19	2.46***	3.02	2.40***	0.04	< 0.001	< 0.001	0.03

[1] grass:concentrate ratio; 0: 0 g MT/kg; 20: 200 g MT/kg of substrate. [2] TVFA: total volatile fatty acids. [3] A/P: Acetate/Propionate. Asterisks in 20 mean significant difference between 0 and 200 g MT/kg in the same feeding model, * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). Trt: treatment; MT: Mootral; Trt×MT: interaction between treatment and Mootral. SEM: standard error of the mean.

4. Discussion

The CH_4 emissions from ruminants are not only a serious environmental issue but also a significant source of energy loss to the animals. Different kinds of anti-methanogenic compounds have already been studied to investigate their potential to reduce CH_4 production (Patra et al. 2017); however, there are limitations to their use due to their negative impacts on rumen fermentation characteristics (Jafari et al. 2019; Lee et al. 2020), and they exhibited inconsistent efficiency with different feeding styles (Vázquez-Carrillo et al. 2020; Suybeng et al. 2020; Jayanegara et al. 2020; Lee et al. 2020). Therefore, sustainable and immediate CH_4 mitigation strategies for the livestock industry are in high demand. Combining different plant extracts to achieve effective and sustainable CH_4 reduction is relatively a new and promising approach (Jayanegara et al. 2020). Mootral, a novel plant-based combination of garlic and citrus extracts, showed promising results when used as a feed supplement for CH_4 mitigation from ruminants (Eger et al. 2018; Roque et al. 2019). Therefore, this study was performed to evaluate the efficacy of Mootral with different kinds of feeding styles in ruminants. Although the current study provides interesting information about the effect of Mootral on rumen fermentation, digestibility and CH_4 production, the microbial analysis which is very important for proper interpretation the current findings has not been done. Microbial characteristic is very important to obtain the whole picture of fermentation, what was underlined by Pers-Kamczyc et al. (Pers-Kamczyc et al. 2011), however our study to be considered as a pilot, carried out following the positive result of the tested Mootral effect on *in vitro* rumen fermentation. Forthcoming research considering the microbial analysis is strongly needed to be done for better understanding the potential of this new mixture. Importantly, it must be taken into account when interpreting the findings of the current study that some of the

differences in the results might arise from the differences in the nutrient composition due to Mootral addition rather than the plant-bioactives present in Mootral.

Similar to the findings of the current study, Mootral increased gas production when used as a feed supplement with rumen fluid collected from sheep, which may reflect a stimulating effect of Mootral on rumen microbes (Chapter 1). This finding has been reported previously from a 48 h *in vitro* gas production study conducted by Hansen and Nielsen (Hansen and Nielsen 2018). Furthermore, Mootral increased the concentration of ruminal NH_3-N, which might be due to the role of Mootral in enhancing the proteolysis process. This nitrogen source can be captured and used by rumen microorganisms to build their own protein (Marcos et al. 2020), which in turn would be used as a protein source for the animal (Wang et al. 2018b). A similar effect has been reported when this Mootral was used as a feed supplement with a 70 forage:30 concentrate diet in the RUSITEC system (Brede et al. 2019). The same finding has also been observed with garlic oil with a 50 forage:50 concentrate diet for 24 h incubation by Busquet et al. (2005b).

Mootral supplementation did not interfere with fiber degradability in all feeding models, which was similar to the findings of García-González et al. (2008), who reported that inclusion of garlic bulbs in the substrate in an *in vitro* trial did not affect IVNDFD, and Zhong et al. (2019), who declared that adding garlic powder to the basal diet did not change the NDF and ADF digestibility through an *in vivo* trial using lambs. Rumen microbiome analysis in upcoming studies would provide a better understanding of Mootral's effect on nutrient digestibility and proteolytic bacteria.

The synergism between the organosulfur compounds and flavonoids in the Mootral mixture was effective in decreasing CH_4 production in all feeding models. The reduction in CH_4 may be due to the direct inhibitory effect of Mootral on methanogenic archaea. Eger et al. (2018) reported a lower abundance of the family *Methanobacteriaceae,* which is the major CH_4 producer in the

rumen, in Mootral supplemented treatment. This was attributed to the toxicity of organosulfur compounds of garlic, such as diallyl sulfide and allicin, to inhibit certain sulfhydryl-containing enzymes essential for the metabolic activities of methanogenic archaea (Patra and Yu 2015). Moreover, it has been reported that flavonoids have the ability to reduce CH_4 production as they have antimicrobial activities through interfering with cellular integrity of some bacteria as well as protozoa (Hassan et al. 2020). It has been established that ruminal ciliated protozoa could enhance methanogenesis, as they are major H_2 producers in the rumen and are in symbiotic relationships with methanogens (Levy and Jami 2018). Although the impact of Mootral on protozoa has not yet been investigated, allicin and flavonoids have shown toxic effects on protozoa (Oskoueian et al. 2013; Miron et al. 2000). Any effect of Mootral on protozoa has to be confirmed in additional studies.

It is well established that CH_4 formation has been positively associated with more acetate production and negatively associated with increased propionate production (Vargas et al. 2020). The allicin and flavonoids in Mootral were able to shift rumen fermentation toward less acetate and more propionate and butyrate. This increase in propionate may be due to the role of Mootral in increasing the abundance of the *Prevotellaceae* and *Veillonellaceae* families, which was confirmed in Chapter 1. *Prevotellaceae* is one of the dominant families in rumen fluid, and it is well known to produce propionate by utilizing H_2 produced during the fermentation of carbohydrates (Denman et al. 2015). This pathway is the main pathway for H_2 consumption and it represents a competitive and alternative pathway to methanogenesis (Ungerfeld 2015; Wang et al. 2018a). Moreover, the family *Veillonellaceae* showed high relative abundance due to the effect of flavonoids extracted from citrus (Oskoueian et al. 2013), and it was associated with propionate production (Chen et al. 2019). Supplementation of steers with garlic powder reduced the A/P ratio

(Wanapat et al. 2008). Similarly, the current study showed the same finding. An increase in butyrate was also associated with a reduction in CH_4 production when the basal diet of ewes was supplemented with garlic extract (Ma et al. 2016).

Reports about the effects of garlic and flavonoid components on TVFA are inconsistent. Some studies reported that they had no effect on TVFA (Busquet et al. 2005a; Seradj et al. 2014; García-González et al. 2008; Klevenhusen et al. 2011), but others reported an adverse effect (Dey et al. 2021; Busquet et al. 2005b; Oskoueian et al. 2013) using an *in vitro* batch culture system. In contrast, in the current study, the Mootral formulation increased the production of TVFA what suggest improved feed efficiency. This phenomenon has also been observed previously in studies using *in vitro* batch culture (Chapter 1) and the RUSITEC system (Eger et al. 2018). That improvement in TVFA production might be occurred as the produced H_2 from fermentation was utilized by microorganisms to produce more propionate and butyrate in Mootral supplemented groups while in control groups, part of H_2 was utilized by methanogens to produce CH_4 (energy loss). Therefore, Mootral supplemented groups were more effective to redirect H_2 toward producing beneficial byproducts (energy source). Another theory would be attributed to the role of Mootral in stimulating the metabolic activity of some rumen microbes to utilize Mootral particles as a feed to produce more TVFA, which may be proven by increasing the production of total gas and CO_2. The latter theory could be supported by what was recently reported that flavonoids could be used as a source of carbon for metabolism in the rumen (Hassan et al. 2020). This finding has to be confirmed and discovered in upcoming researches.

5. Conclusion

In the present *in vitro* study, we investigated the efficiency of the Mootral mixture on CH_4 production, rumen fermentation, feed efficiency, and digestibility in different feeding styles. According to the design of this *in vitro* study, Mootral at level of 20%/substrate was potential to effectively reduce CH_4 production with all feeding styles. Mootral showed a high reducing power up to 69% when the amount of CON comprised up to 800 g/kg of the ration. Moreover, 20% Mootral supplementation improved the production of TVFA and shifted the fermentation profile toward less acetate and more propionate and butyrate. Additionally, Mootral did not impair fiber digestibility. Therefore, Mootral could be used as a feed supplement with all feeding styles to efficiently reduce CH_4 production by ruminants. The hypothesis and design of this trial should be taken into consideration when interpreting the experimental results. The Mootral dosage used in the current *in vitro* trial would not be feasible for practical feeding. Further long-term *in vivo* trials with optimum dosage have to be done to confirm the current findings

References

AOAC, 1995. Official methods of analysis, 16th edn. Arlington, VA: Association of Official Analytical Chemists.

Brede, J., Eger, M., Breves, G., 2019. Dose-dependent effects of a garlic-citrus powder on methane production and fermentation parameters of rumen microbial metabolism. International Symposium on Ruminant Physiology, September 3rd – 6th. Available online : https://m00tral.s3.amazonaws.com/Publications/Poster_ISRP_TiHo.pdf (accessed on 05 February 2021).

Busquet, M., Calsamiglia, S., Ferret, A., Cardozo, P.W., Kamel, C., 2005a. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. J Dairy Sci 88, 2508–2516. https://doi.org/10.3168/jds.S0022-0302(05)72928-3.

Busquet, M., Calsamiglia, S., Ferret, A., Carro, M.D., Kamel, C., 2005b. Effect of garlic oil and four of its compounds on rumen microbial fermentation. J Dairy Sci 88, 4393–4404. https://doi.org/10.3168/jds.S0022-0302(05)73126-X.

Chen, L., Shen, Y., Wang, C., Ding, L., Zhao, F., Wang, M., Fu, J., Wang, H., 2019. *Megasphaera elsdenii* lactate degradation pattern shifts in rumen acidosis models. Front Microbiol 10, 162. https://doi.org/10.3389/fmicb.2019.00162.

Denman, S.E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., McSweeney, C.S., 2015. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.01087.

Dey, A., Paul, S.S., Lailer, P.C., Dahiya, S.S., 2021. Reducing enteric methane production from buffalo (Bubalus bubalis) by garlic oil supplementation in *in vitro* rumen fermentation system. SN Appl Sci 3. https://doi.org/10.1007/s42452-021-04264-6.

Eger, M., Graz, M., Riede, S., Breves, G., 2018. Application of Mootral^{TM} reduces methane production by altering the archaea community in the rumen simulation technique. Front Microbiol 9, 2094. https://doi.org/10.3389/fmicb.2018.02094.

García-González, R., López, S., Fernández, M., Bodas, R., González, J.S., 2008. Screening the activity of plants and spices for decreasing ruminal methane production *in vitro*. Anim Feed Sci Technol 147, 36–52. https://doi.org/10.1016/j.anifeedsci.2007.09.008.

Hansen, H., Nielsen, M., 2018. Impact of Mootral on rumen digestion. University of Copenhagen. Available online: https://ivh.ku.dk/nyheder/2018/impact-of-mootral-on-rumen-digestion/ (accessed on accessed 05 February 2021).

Hassan, F.-U., Arshad, M.A., Li, M., Rehman, M.S.-U., Loor, J.J., Huang, J., 2020. Potential of Mulberry leaf biomass and its flavonoids to improve production and health in ruminants: Mechanistic insights and prospects. Animals 10. https://doi.org/10.3390/ani10112076.

Jafari, S., Ebrahimi, M., Goh, Y.M., Rajion, M.A., Jahromi, M.F., Al-Jumaili, W.S., 2019. Manipulation of rumen fermentation and methane gas production by plant secondary metabolites (saponin, tannin and essential oil) – A review of ten-year studies. Ann Anim Sci 19, 3–29. https://doi.org/10.2478/aoas-2018-0037.

Jayanegara, A., Yogianto, Y., Wina, E., Sudarman, A., Kondo, M., Obitsu, T., Kreuzer, M., 2020. Combination effects of plant extracts rich in tannins and saponins as feed additives for mitigating *in vitro* ruminal methane and ammonia formation. Animals 10. https://doi.org/10.3390/ani10091531.

Kaur, C., Kapoor, H.C., 2002. Anti-oxidant activity and total phenolic content of some Asian vegetables. Int J Food Sci Tech 37, 153–161. https://doi.org/10.1046/j.1365-2621.2002.00552.x.

Kawasaki, K., Min, X., Li, X., Hasegawa, E., Sakaguchi, E., 2015. Transfer of blood urea nitrogen to cecal microbial nitrogen is increased by fructo-oligosaccharide feeding in guinea pigs. Anim Sci J 86, 77–82. https://doi.org/10.1111/asj.12238.

Klevenhusen, F., Zeitz, J.O., Duval, S., Kreuzer, M., Soliva, C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Anim Feed Sci Technol 166-167, 356–363. https://doi.org/10.1016/j.anifeedsci.2011.04.071.

Lee, S.J., Lee, Y.J., Eom, J.S., Kim, H.S., Choi, Y.Y., Jo, S.U., Kang, S.N., Park, H.Y., Kim, D.H., Lee, S.S., 2020. Effects of the appropriate addition of antioxidants from *Pinus densiflora* and *Mentha canadensis* extracts on methane emission and rumen fermentation. Animals 10. https://doi.org/10.3390/ani10101888.

Levy, B., Jami, E., 2018. Exploring the prokaryotic community associated with the rumen ciliate protozoa population. Front Microbiol 9, 2526. https://doi.org/10.3389/fmicb.2018.02526.

Ma, T., Chen, D., Tu, Y., Zhang, N., Si, B., Deng, K., Diao, Q., 2016. Effect of supplementation of allicin on methanogenesis and ruminal microbial flora in Dorper crossbred ewes. J Animal Sci Biotechnol 7. https://doi.org/10.1186/s40104-015-0057-5.

Marcos, C.N., Carro, M.D., Fernández-Yepes, J.E., Arbesu, L., Molina-Alcaide, E., 2020. Utilization of Avocado and Mango fruit wastes in multi-nutrient blocks for goats feeding: *in vitro* evaluation. Animals 10. https://doi.org/10.3390/ani10122279.

McDOUGALL, E.I., 1948. The composition and output of sheep's saliva. Biochem J 43, 99–109.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analyses and gas production using rumen fluid. Anim Res Develop 28, 7–55.

Miron, T., Rabinkov, A., Mirelman, D., Wilchek, M., Weiner, L., 2000. The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity. Biochim Biophys Acta-Biomembr 1463, 20–30. https://doi.org/10.1016/S0005-2736(99)00174-1.

Oskoueian, E., Abdullah, N., Oskoueian, A., 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. Biomed Res Int 2013, 1–8. https://doi.org/10.1155/2013/349129.

Patra, A., Park, T., Kim, M., Yu, Z., 2017. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. J Animal Sci Biotechnol 8. https://doi.org/10.1186/s40104-017-0145-9.

Patra, A.K., Yu, Z., 2015. Effects of adaptation of *in vitro* rumen culture to garlic oil, nitrate, and saponin and their combinations on methanogenesis, fermentation, and abundances and diversity of microbial populations. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.01434.

Pers-Kamczyc, E., Zmora, P., Cieślak, A., Szumacher-Strabel, M., 2011. Development of nucleic acid based techniques and possibilities of their application to rumen microbial ecology research. J. Anim. Feed Sci. 20, 315–337. https://doi.org/10.22358/jafs/66189/2016.

Roque, B.M., van Lingen, H.J., Vrancken, H., Kebreab, E., 2019. Effect of Mootral—a garlic- and citrus-extract-based feed additive—on enteric methane emissions in feedlot cattle. Transl Anim Sci 3, 1383–1388. https://doi.org/10.1093/tas/txz133.

Seradj, A., Abecia, L., Crespo, J., Villalba, D., Fondevila, M., Balcells, J., 2014. The effect of Bioflavex® and its pure flavonoid components on *in vitro* fermentation parameters and methane production in rumen fluid from steers given high concentrate diets. Anim Feed Sci Technol , v. 197, 85–91. https://doi.org/10.1016/j.anifeedsci.2014.08.013.

Suybeng, B., Charmley, E., Gardiner, C.P., Malau-Aduli, B.S., Malau-Aduli, A.E.O., 2020. Supplementing Northern Australian beef cattle with desmanthus tropical legume reduces *in-vivo* methane emissions. Animals 10. https://doi.org/10.3390/ani10112097.

Ungerfeld, E.M., 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.00037.

Vargas, J.E., Andrés, S., López-Ferreras, L., Snelling, T.J., Yáñez-Ruíz, D.R., García-Estrada, C., López, S., 2020. Dietary supplemental plant oils reduce methanogenesis from anaerobic microbial fermentation in the rumen. Sci Rep 10, 1613. https://doi.org/10.1038/s41598-020-58401-z.

Vázquez-Carrillo, M.F., Montelongo-Pérez, H.D., González-Ronquillo, M., Castillo-Gallegos, E., Castelán-Ortega, O.A., 2020. Effects of three herbs on methane emissions from beef cattle. Animals 10. https://doi.org/10.3390/ani10091671.

Wanapat, M., Khejornsart, P., Pakdee, P., Wanapat, S., 2008. Effect of supplementation of garlic powder on rumen ecology and digestibility of nutrients in ruminants. J Sci Food Agric 88, 2231–2237. https://doi.org/10.1002/jsfa.3333.

Wang, K., Nan, X., Chu, K., Tong, J., Yang, L., Zheng, S., Zhao, G., Jiang, L., Xiong, B., 2018a. Shifts of hydrogen metabolism from methanogenesis to propionate production in response to replacement of forage fiber with non-forage fiber sources in diets *in vitro*. Front Microbiol 9, 2764. https://doi.org/10.3389/fmicb.2018.02764.

Wang, R., Wang, M., Ungerfeld, E.M., Zhang, X.M., Long, D.L., Mao, H.X., Deng, J.P., Bannink, A., Tan, Z.L., 2018b. Nitrate improves ammonia incorporation into rumen microbial protein in

lactating dairy cows fed a low-protein diet. J Dairy Sci 101, 9789–9799. https://doi.org/10.3168/jds.2018-14904.

Zhong, R., Xiang, H., Cheng, L., Zhao, C., Wang, F., Zhao, X., Fang, Y., 2019. Effects of feeding garlic powder on growth performance, rumen fermentation, and the health status of lambs infected by gastrointestinal nematodes. Animals 9, 102. https://doi.org/10.3390/ani9030102.

General Summary

The animal production industry is a significant source of GHGs. Ruminants represent the source of approximately 80% of the total GHG emissions from that sector. The most important GHG is CH_4. The production of CH_4 is formed mainly by enteric fermentation in the rumen. Emitted CH_4 represents up to a 15% loss of feed energy. Numerous CH_4 mitigation technologies have been investigated, with promising findings from dietary interventions. However, most of these strategies have only been investigated *in vitro* without being confirmed *in vivo* in addition to the adverse effects of some approaches on animal performance, rumen fermentation and behavior. Therefore, the main aim of this thesis was to provide a comprehensive evaluation of one of the novel and promising strategies (Mootral, a garlic and citrus extract) to mitigate CH_4 missions from ruminants through *in vitro* and *in vivo* applications.

In Chapter 1, the first study was performed to understand Mootral's mode of action and to investigate its impacts on gas production, fermentation profile, and rumen microbiome. The experiment was a 24-h small-scale *in vitro* trial using rumen fluid collected from sheep. Mootral was supplemented in two dosages, 10% and 20% of the substrate (50% grass: 50% concentrate). Mootral impacts showed a dose-dependent effect on the investigated parameters. Supplementation with Mootral led to increased production of total gas and total volatile fatty acids. Mootral supplementation reduced the proportion of CH_4 in the produced gas by 22% and 54% for 10 and 20% Mootral, respectively. The fermentation profile was shifted toward more propionate and less acetate due to the addition of Mootral. The bacterial community showed an increase in the relative abundance of the *Prevotellaceae* family and *Veillonellaceae* family, while there was a decrease in the relative abundance of some hydrogen-producing bacteria by Mootral supplementation. The alteration in the archaeal community due to Mootral was obvious, where the *Methanobacteriaceae*

family decreased and the *Methanomassiliicoccaceae* family increased in a dose-dependent manner. Thus, this preliminary study showed the ability of this novel combination to alter the rumen fermentation profile and reduce microbial groups associated with CH_4 production, indicating the potential of this promising natural mixture to mitigate CH_4 emissions from ruminants.

The aforementioned *in vitro* findings were further evaluated through an *in vivo* trial conducted in sheep, as described in Chapter 2, to confirm the potential of this new combination. The aim of this study was to determine the optimal effective dosage of Mootral for reducing CH_4 production while considering its impact on behavior, rumen fermentation characteristics, digestibility, and growth performance. The experiment was carried out using the same feeding style applied *in vitro*, while Mootral was evaluated at three dosages: 1, 5, and 10 g/kg DM, mixed thoroughly with concentrates and supplemented once daily in the morning feeding. The experimental design was a 4 × 4 Latin square design in which sheep were kept in individual metabolic cages. Mootral supplementation at all dosages had no adverse impact on feed intake, health status, growth performance, rumen fermentation, or nutrient digestibility. Mootral showed a dose-dependent manner in reducing CH_4 production yield per digestible DM intake up to 12.8% with the highest dosage. The results of this study also indicated that Mootral supplementation improved animal welfare by reducing the incidence of abnormal behaviors. Therefore, it can be concluded that the natural combination of garlic and citrus has the potential to reduce CH_4 emissions without any negative effects on animal health or behavior.

The experiment described in Chapter 3 was performed to investigate Mootral's potential to reduce CH_4 production across different kinds of feeding styles (forage:concentrate ratios) in a different ruminant model: cows. The experiment was conducted as a batch culture for 24 h with

rumen fluid collected from Holstein cows. Five experimental diets (forage:concentrate) were used: 100:0, 80:20, 60:40, 40:60, and 20:80. Mootral was supplemented at 200 g/kg of the substrate, which was selected based on the effective dosage from the first *in vitro* trial (Chapter 1). Each treatment had 6 replicates, and the experiment was repeated in three consecutive runs. The results of this trial confirmed the ability of Mootral to effectively reduce CH_4 production for all tested feeding styles. The reduction potential per digestible DM was 44% in the high forage diet, and this reduction power increased with the inclusion of concentrate in the diet to reach up to 69% in the high concentrate diet. Mootral supplementation improved rumen fermentation by increasing the production of total volatile fatty acids and shifting the fermentation profile toward more propionate and less acetate. Additionally, Mootral had no adverse effects on nutrient digestibility. Thus, Mootral combination could work effectively as a CH_4 inhibitor candidate in all feeding styles for ruminants.

要約

畜産業は、温室効果ガスの重要な排出源である。反芻動物は、畜産業から排出される温室効果ガス総量の約80%を占めている。その内で最も重要な温室効果ガスはメタン(CH_4)である。CH_4の生成は、主にルーメン内での微生物発酵によって行われる。排出されたCH_4は、飼料エネルギーの最大15%の損失となる。数多くのCH_4生成緩和技術が研究されており、飼養管理に関する研究で有望な結果が得られている。しかし、これらの戦略のほとんどはin vitroでしか研究されておらず、in vivoでは確認されていない。また、いくつかのアプローチは動物の生育、ルーメン発酵、行動に悪影響を及ぼす。したがって、本論文の主な目的は、反芻動物からのCH_4生成を軽減するための新規かつ有望な素材の1つ（Mootral®、ニンニクと柑橘類の抽出物、Mootral社、スイス）について、in vitroおよびin vivo実験を通じて総合的な評価を行うことである。

第1章では、Mootralの作用機序を理解し、ルーメンでのガス産生、発酵特性、微生物叢への影響を調査するための研究を行った。実験は、ヒツジから採取したルーメン液を用いた24時間のin vitro試験である。基質は牧草50%：濃厚飼料50%の混合物とし、Mootral を10%と20%の2水準で添加した。Mootralの影響は、調査した項目に対して用量依存的な効果を示した。Mootralの添加により、総ガスと総揮発性脂肪酸の生産量が増加した。Mootralの添加により、生産されたガス中のCH_4の割合が、Mootral 10%と20%でそれぞれ22%と54%減少した。発酵特性としては、Mootralの添加により、プロピオン酸が多く、酢酸が少ない方向にシフトした。細菌群は、Prevotellaceae familyとVeillonellaceae familyの相対量が増加したが、Mootralの添加により、いくつかの水素産生菌の相対量が減少した。Mootralによる古細菌群集の変化は明らかであり、Methanobacteriaceae familyが減少し、Methanomassiliicoccaceae familyが用量依存的に増加した。このように、本予備的研究では、Mootral添加がルーメン発酵特性を変化させ、CH_4生成に関連する微生物群を減少させる能力があることを示し、反芻動物からのCH_4排出を緩和するこの有望な天然混合物である可能性を示した。

第2章では、この添加物の可能性を確認するために、前述のin vitroでの知見をさらにヒツジで実施したin vivo試験で評価した。本試験の目的は、行動、ルーメン発酵特性、消化率、成長成績への影響を考慮しながら、CH_4生成量を低減するためのMootralの最適有効投与量を決定することである。実験は、Mootralは1, 5, 10 g/kg DMの3水準の投与量とし、濃厚飼料と十分に混合し、1日1回、朝の給餌時に添加した。実験方法は4×4ラテン方格法で、ヒツジは個別の代謝ケージで飼育された。すべての投与量でMootralを補給しても、飼料摂取量、健康状態、成長成績、ルーメン発酵、栄養成分消化率に悪影響はなかった。Mootralは用量依存的に、可消化乾物摂取量あたりのCH_4産生量を、最高用量(10 g/kg DM)で12.8%減少させた。また、本研究の結果は、Mootralの添加が異常行動の発生率を減少させることで動物の福祉を向上させることを示した。したがって、ニンニクと柑橘類の天然物質の組み合わせは、動物の健康や行動に悪影響を及ぼすことなく、CH_4排出量を削減できる可能性があると結論づけることができる。

第3章では、異なる反芻家畜であるウシにおいて、異なる種類の給餌スタイル（粗飼料：濃厚飼料の比率）でのMootralのCH_4生成量削減の可能性を調査するために行われた。実験は、ホルスタイン種のウシから採取したルーメン液を用いて、24時間のバッチ培養で行った。5種類の実験飼料（100:0、80:20、60:40、40:60、20:80、粗飼料：濃厚飼料）を使用した。Mootralは、最初のin vitro試験（第1章）で得られた効果的な投与量に基づいて選択された200g/kgの基質を補充した。培養実験は3回反復した。本試験の結果、すべての給餌スタイルにおいて、MootralがCH_4生成量を効果的に削減できることが確認された。可消化DMあたりの削減量は、高粗飼料区で44%であり、この削減量は濃厚飼料の比率が高くなるに従って増加し、高濃厚飼料区では最大69%に達した。Mootralの添加は、総揮発性脂肪酸の生産量を増加させ、発酵特性をプロピオン酸が多く、酢酸が少ない方向にシフトさせた。さらに、Mootralは栄養分の消化率に悪影響を及ぼさなかった。

以上の結果から、本研究は天然物質であるニンニクと柑橘類からできているMootralは、反芻動物のあらゆる給餌スタイルにおいて効果のある、他に悪影響を及ぼさない安全・安心なCH_4阻害剤の候補としての可能性があることを明らかにした。

General Conclusion

Although many dietary strategies have demonstrated strong antimethanogenic efficacy *in vitro*, they fail to show CH_4 reduction *in vivo*. The novel technology evaluated in this thesis, Mootral, a combination of garlic and citrus, has indicated its potential to mitigate CH_4 production *in vitro* as well as *in vivo* despite the different reduction powers between *in vitro* and *in vivo* trials. Mootral supplementation at 20% substrate in an *in vitro* trial achieved CH_4 reduction up to 69%, while in the *in vivo* trial, it was up to 12.8% with 1% DM. Therefore, more research is required to further evaluate the effect of higher Mootral dosages on reduction potential while considering its impacts on animal health, fermentation, digestibility, and behavior. The advantage of this combination is that it is natural and safe. No adverse effects have been observed on animal health, growth performance, fermentation or digestibility. An additional advantage has been observed: supplementation with Mootral led to a reduction in the incidence of abnormal behaviors. Further research must be conducted to confirm this finding and understand the mechanism by which this mixture modulates animal behavior. Furthermore, Mootral supplementation showed the ability to work effectively with different feeding styles as well as different ruminant species. Although the *in vitro* study conducted with rumen fluid collected from cows showed effective reduction with Mootral supplementation, these findings must also be confirmed *in vivo*.

This natural combination provides an innovative and promising strategy to reduce CH_4 emissions from ruminants. The mixture of garlic and citrus can play a pivotal role in ensuring the sustainability of the livestock production industry in meeting rapidly increasing consumer needs in the near future.

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