Production of Lysozyme from Duck Egg and Testing its Usefulness in Reduction of Methane Emission in Ruminants

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By

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Abstract

The objectives of this study were to prepare lysozyme from duck egg white and test its usefulness in the reduction of methane emission in ruminants. Triplicates of treatment were added with the additive of lysozyme from duck egg white along with 0.5 g of commercial animal feed and 50 ml of buffered rumen fluid. Moreover, controls were added with 0.5 g of commercial animal feed, 50 ml of buffered rumen fluid but without lysozyme from duck egg white. Triplicates of treatment and control were set for 24 h and 48 h of incubation. Total gas (TG) production at different stages was measured for all treatment and control samples. Total gas produced was also used to measure the amount of CH_4 and CO_2 released during the incubation period. The optimum incubation period for the experiment (i.e. 24 h and 48 h) was determined after conducting *in vitro* fertilization for 6 h, 24 h and 48 h of incubation that followed standard procedures and data was recorded. The data suggested that there was no production of methane after 6 h of incubation, thus 6 h sampling was dropped for the actual *in vitro* experiment. After 24 h of incubation, the mean value of CH_4 production out of total gas production was higher (86.07%) in control as compared to treatment (76.80%). Similarly, after 48 h of incubation, the mean value of CH_4 production out of total gas production was also higher (55.20%) in control as compared to treatment (50.30%). At 95% confidence interval, there was no significant difference in CH_4 reduction in control as compared to treatment after 24 h and 48 h of incubation. Consequently, the study suggests that lysozyme from duck egg white supplementation may improve in vitro fermentation and reduce CH_4 emissions from ruminants.

Key words: Duck egg white lysozyme, *in vitro* fermentation, methane, ruminants, global warming

1. INTRODUCTION

This is a consensus that greenhouse gases (GHGs) such as methane, carbon dioxide, nitrous oxide, sulphur hexafluoride, per-fluorocarbons, and hydro-fluorocarbons have led changes in the earth climate and caused global warming. The environmental impacts of methane and other greenhouses gases are not new to us. Moreover, anthropogenic activities such as intensive rearing of ruminant livestock, agricultural practices, and burning of fossils fuels have contributed largely to the rise of atmospheric greenhouse gases. The increase in methane concentration is raising concerns over global warming and climate change (Biswas et al., 2014). There is a need to reduce methane and other greenhouse gases to mitigate the risks of global climate change.

1.1. Methane production by ruminants

Agriculture, forestry and other land use constitute about 24 percent of 2010 global greenhouse gas emissions. According to Intergovernmental Panel on Climate Change (IPCC), 2012 report, livestock and mostly ruminants produce about 35 percent of total anthropogenic methane. Radiative forcing of methane (CH_4) is higher than of carbon dioxide (CO_2) and its production by ruminant livestock is the major source of greenhouse gas emissions. Methane is important characteristics of farming productivity, as it is associated with the conversion of feed to the product. Therefore, measurement of enteric methane is a vital research subject (Hill et al., 2016).

Methane emission from ruminant livestock is estimated around 100 million ton each year. The production of methane is second highest after the rice agriculture that represents the biggest anthropogenic source of methane. The production of methane from ruminant livestock not only increases greenhouse gas in the atmosphere but also adds to global warming. Additionally, it is also problematic for farmers because methane produced is released into the atmosphere instead of converted into milk or meat (Reay).

Methane is produced from ruminant livestock's gut because of fermentation by methanogenic microorganisms such as *Archaea*. Different ruminants produce different amount of methane such as a dairy cow can produce up to 200 litres of methane but a sheep can produce about 30 litres of methane in each day. The composition of animal feed is an important factor in controlling the amount of methane production. With large consumption demand for dairy products and meat, intensive rearing methods have been developed to provide these products to the customers at a lower price. These lead to very high densities of ruminant livestock and strong methane point sources (Reay). In addition to these, anaerobic decomposition of animal waste from ruminant livestock also produces a large amount of methane due to high organic carbon content. Moreover, waste along with industrial and domestic water waste add up to between 14 and 25 million tons of methane emission per year, globally (Reay).

1.2. Egg white lysozymes

Egg white protein is among the traditional raw material that provides essential nutrients such as protection against bacteria for embryo development etc. Lysozyme is an antimicrobial enzyme, which can lyse bacterial cell walls by hydrolyzing the polysaccharide component (Salton, 1957; Biswas et al., 2016). It was found that egg white is the richest source of lysozyme. A gram-positive coccus called *Micrococcus lysodeikticus* was isolated by Fleming, which was particularly susceptible to the actions of lysozyme (Salton, 1957). Meyer et al. discovered that "lysozyme digestion of bacterial substrates" was added by the production of reducing groups that have the ability to lyse certain bacteria. As lysozyme action is confirmed by the freeing of reducing groups, it suggests that egg white, papaya extract, saliva have lysozyme (Salton, 1957).

1.3. Properties of egg white lysozymes

Lysozyme has been the topic for numerous reviews till date and a great deal of information on its properties and structure are available. The enzyme showed usefulness in bacterial studies and more accurate picture of its enzymatic properties are emerging. Most of the investigations on the action of lysozyme are done on microbial activity, which mostly used egg white lysozyme (Salton, 1957).

Meyer et al showed that purified egg white lysozyme is a basic protein. High degree of purification of lysozyme was obtained by crystalline preparations by Abraham and Robinson (Salton, 1957). Heterogeneities of crystalline preparations have found by immunological and chromatographic methods of analysis. Lysozyme showed a progressive change in the proportions of the chromatographic components when prepared in carbonate form and stored in the dry state (Salton, 1957).

1.4. Objectives

The objectives of the study were to produce lysozyme from duck egg white and testing the effect of produced lysozyme on the reduction of methane (CH_4) emission in ruminants.

2. REVIEW OF LITERATURE

2.1. Global climate change – reality check and future predictions

The fifth IPCC assessment report has put a strong emphasis on global warming and its unprecedented impacts of ocean acidification, diminishing snow and ice covers. Anthropogenic activities are the main driving forces behind high rate of emission of global warming gases, which started since the dawn of pre-industrial era and most likely the main culprit for recorded global warming. Change in climate has both short term and long term impacts on the natural system which has begun to be observed now. For example, change in melting pattern of ice-covers has altered the hydrological system, crop yields have been affected by high level of GHG and migration patterns of numerous fresh water and marine water species has shown significant alterations in recent decades. IPCC has predicted an

increase in extreme climatic events in the wake of changing global climate such as heat waves, cold waves, droughts etc. (IPCC, 2014).

Changes in the climate system will be drastic if the present emission rate of greenhouse gases continues. According to fifth IPCC report, there will be an increase in global mean surface temperature under all RCP scenarios. These climatic changes will increase the number of vulnerable people and communities. The disadvantaged groups will face severe risks and challenges because of lack of infrastructure and coping mechanism. Changes will be irreversible and more drastic as the global warming increases. To reduce and manage climate change risks, carbon emission has to be reduced over next few decades. Risk management plan can be achieved using analytical approaches like economic assessment, risks & benefits, etc. (IPCC, 2014). Mitigation plans are not full proof as it also poses some risks, if not implanted properly, but to say the least it will limit the negative impacts of climate change. Similarly, adaptation measures work but with limited effectiveness if climate change impact is already high. There are numerous mitigation pathways to reduce carbon emission which separately poses different economic, social, technological and institutional challenges (IPCC, 2014).

Adaptation and mitigation will address climate change issue given that necessary policies and cooperation at all levels is available. Effective governance, innovation, investment in eco- friendly technologies, sustainable livelihood and lifestyle changes are pre-requisites for adaptation and mitigation measures to be effective. Different adaptation and mitigation plans pose different sets of challenges, which differ across sectors and regions. To make adaptation and mitigation responses effective, multi-level cooperation, international, regional, national, sub-national, and necessary policies are needed to be at the place (IPCC, 2014). The greenhouse effect of methane is about 20 to 50 times of carbon dioxide (Kim et al., 2013).

2.2. Lysozyme and *in vitro* fermentation

As the rising problem will add to global warming, researchers are conducting studies to identify to mitigate methane emissions through manipulating ruminant feed. Protein, plant cell wall, and starch are consumed by ruminants are mostly hydrolyzed by microorganisms such as bacteria, protozoa, and fungi to produce amino acids and sugars. Primary or secondary microorganisms further break down amino acids and sugars leading to the production of volatile fatty acids, carbon, hydrogen and ammonia (Kim et al., 2013). The end product of fermentation does not have hydrogen because bacteria such as methanogens utilize hydrogen to produce methane.

The study done by Mamaud et al. on the effect of fumarate reducing bacteria on *in vitro* rumen fermentation, methane mitigation, and microbial diversity suggests that production, utilization and the activity of methanogens are important characteristics that should be considered in controlling methane production. One of the interventions used by Mamaud et al. was hydrogenotrophic microorganisms such as fumarate reducing bacteria to reduce methane production. Fumarate is an intermediate compound used in the citric acid cycle that produces energy in the form of adenosine triphosphate (ATP).

Fumarate is reduced to succinate by fumarate reductase in the rumen. H_2 compete with methanogens in its utilization, as it serves as an electron donor for the reduction of fumarate to succinate. This study used *Mitsuokella jalaludinii,* as an addition to treatment on *in vitro* rumen fermentation and the amount of methane produced was compared with the control. It found that *M. jalaludinii* was significantly able to reduce methane at 48 h and 72 h of incubation. The results proposed that addition of fumarate bacteria i.e. *M jalaludinii* was successful in reducing methane production, increases succinate and changes the microbial diversity of the rumen (Mamaud et al., 2014). A study conducted by Hattori et al. found that a diversity of fumarate reducing bacteria are present in the rumen. These bacteria are analyzed

by using different "culture dependent and independent methodologies." Additionally, about 39 strains were isolated and analyzed using different media (Hattori et al., 2008). Soriano et al., in his study suggests that lactobacillus facilitates the growth of microorganisms in rumen, which are adapted to lactic acid present in the rumen that in turns reduces ruminal acidity. This acidity prevents the occurrence of ruminal acidosis in cows (Soriano et al., 2014). Mamuad et al. also suggests that some bacterium such as *L. mucosae* is capable of producing CO_2 and H_2 when grown in MRS medium. It is because the gas products from the anaerobic fermentation of glucose in the MRS medium. These gases are main substrates for methanogens that produce CH4 and could let to higher CH4 production in groups that have *L. mucosae* an addition (Mamuad et al., 2014).

Another study conducted by Biswas et al. studied the use of lysozyme as a feed additive on *in vitro* rumen fermentation and its effect on methane emission. The study used *in vitro* rumen fermentation technique and utilized commercial concentrate and rice straw. Antimicrobials are used in animal production sector at low doses to promote growth for the bacterial effect. Although, the side effects of the undiscerning use of antibiotics on animals and environment leave the animal husbandry in a vulnerable condition (Biswas et al., 2016). Because of bacteriolytic and enzymatic characteristics of the lysozyme, the study used lysozyme from Celltech co. to identify the alternatives to antibiotics for sustainable animal production.

The study used different lysozyme concentration i.e. 2000 U, 4000 U, and 8000 U along with 1 g of dry matter (DM) and buffered rumen fluid. Inclusion level of lysozymes was measured based on previous research. Based on inclusion level, 13.33 µl, 26.66 µl, and 53.33 µl lysozyme were used with 1 g of dry matter for different treatments. The study found that the addition of lysozyme had an effect on reduction of CH_4 after 24 h of incubation. After 24 h of incubation, pH was highest in 8000 U lysozyme followed by the 4000 U, 2000 U, and

no lysozyme. After 24 h of incubation, the CH_4 concentration was lowest in the 8000 U lysozyme and highest in sample without lysozyme. Additionally, the lysozyme increased the acetate, propionate and decreased CH_4 concentration (Biswas et al., 2016).

3. MATERIALS AND METHODS

The research was conducted to evaluate the effectiveness of lysozyme purified from duck egg white in reducing methane production *in vitro* fermentation under laboratory conditions. All chemicals and laboratory apparatus used in this study were collected from AUW laboratory or purchased from Jamuna Scientific, Dhaka, Bangladesh.

Materials and reagents

- Beakers, gradated cylinders, duck egg, centrifugation machine, sodium phosphate dibasic, sodium phosphate monobasic, cheesecloth and paraflim for lysozyme preparation
- Commercial feed, mortar and sieve for powdering commercial feed
- K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, trypticase peptone, yeast extract, and cysteine·HCl, rumen fluid, CO_2 cylinder and hot bath to prepare buffered rumen fluid.
- Glass serum bottles, feed, lysozyme, buffered rumen, shaking incubator, parafilm to measure methane measurement

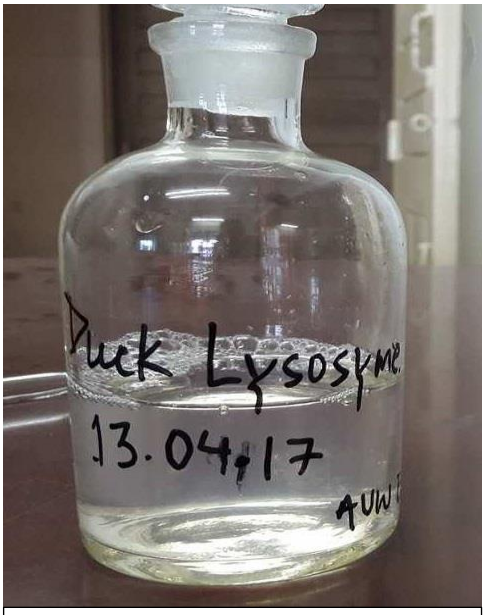

Figure 1: Duck lysozyme

3.1. Purification of lysozyme from duck egg white

Peking duck (*Anas platyrhynchos*) eggs, freshly laid, were bought from Khulshi Mart, Chittagong, Bangladesh. Duck eggs were carefully cracked, egg whites were separated into a beaker and yolk was discarded. Four times folded cheesecloth was fixed over

a breaker and egg white was filtered to have sediment free solution. Then, 3 ml of filtered egg white was transferred into a conical tube and 12 ml of 0.1 M Phosphate (0.5 M Sodium phosphate dibasic and 0.5 M Sodium phosphate monobasic at pH 7.2) buffer was added. The solution of egg white and phosphate buffer was mixed gently avoiding any bubbles. The conical

tubes were put into a balanced centrifuge at 15000 rpm for 5 min. Tubes were removed from centrifuge and supernatant were collected into a clean and labeled tube as shown in figure 1 (Worcester Polytechnic Institute, 2010).

3.1.1. Preparation of 0.1 phosphate buffer of pH 7.2

A stock of sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4) were prepared by adding 35.5 g of sodium phosphate dibasic and 30 g of anhydrous sodium phosphate monobasic with 500 ml of H_2O respectively in different beakers. 80 ml of sodium phosphate dibasic stock (0.5 M) and 30 ml of sodium phosphate monobasic stock (0.5 M) were added with 320 ml and 120 ml of H_2O in different beakers to make 0.1 M solutions. Then, 0.1 M sodium phosphate dibasic was added with as much as 0.1 M sodium phosphate monobasic solution until the solution reaches a of pH 7.2 as shown in figure 2 (Cold Spring Harbor Protocol, 2010).

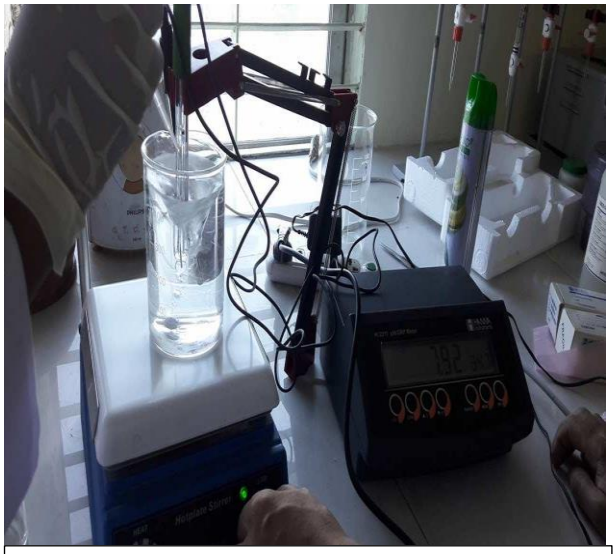

Figure 2: Using pH meter to get pH of 7.2 while making phosphate buffer

Figure 3: Commercial feed was powdered to <1mm size using mortar

3.2. Proximate composition of feed

The proximate composition of the commercial feed used in the experiment suggested that it contained 75-77% total digestible nutrients (TDN), 14-15% crude protein (CP), 1.1% calcium (minimum), 0.8% phosphorous (minimum), and 90% dry matter (DM). Feed powder of less than 1mm (<1mm) was prepared using mortar as shown in figure 3.

3.3. Preparation of buffer for rumen fluid

The buffer for rumen fluid used 0.45 g K_2HPO_4, 0.45 g KH_2PO_4, 0.9 g $(NH_4)_2SO_4$, 0.12 g $CaCl_2 \cdot 2H_2O$, 0.19 g $MgSO_4 \cdot 7H_2O$, 1.0 g trypticase peptone, 1.0 g yeast ex- tract, and 0.6 g cysteine·HCl per litre (Mamuad et al., 2014) by maintaining a pH of 6.9. All these chemicals were added in measured distilled water that was put on a hotplate to avoid granules as shown in figure 4. 2 litres of the buffer prepared for the experiment that was dispensed with 100% CO_2 atmosphere manually by a small CO_2 cylinder. Then the buffer was autoclaved at 121^oC for 15 min and cooled for *in vitro* fermentation experiment.

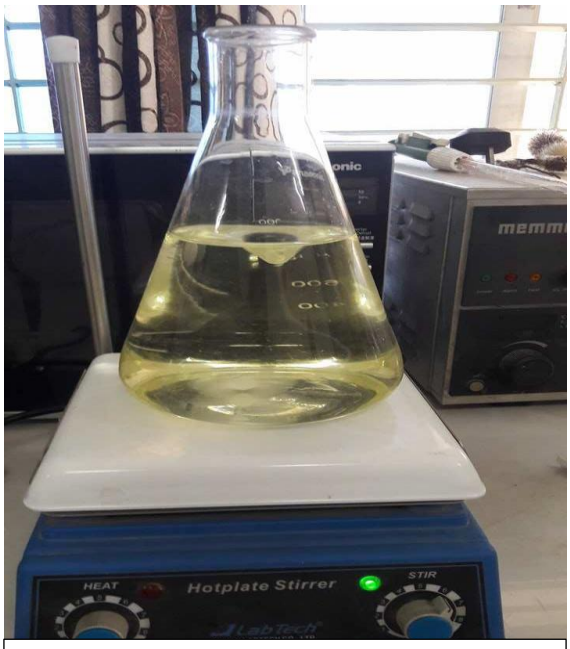

Figure 4: Preparation of buffer for rumen using hot plate

3.4. Collection of rumen fluid

Rumen fluid was collected from Holstein cow, which was fed with rice straw and commercial feed concentrates twice a day. The cow breed, type of feed and frequency of feed

Figure 5: Collection of rumen fluid after filtering with cheesecloth

each day was confirmed with the owner of the slaughterhouse. Rumen fluid was collected from the stomach of a freshly slaughtered cow. Rumen fluid was collected from ingesta of the cow stomach and cheesecloth was used to filter the fluid as shown in figure 5. The filtered rumen fluid was sealed into an airtight flask at 39°C to and immediately transported to the laboratory for the *in vitro* fermentation (Mamuad et al., 2014). In the laboratory, the rumen fluid was dispensed with 100% CO_2 using small a CO_2 cylinder. The rumen fluid was shaken vigorously by hands until the experiment started.

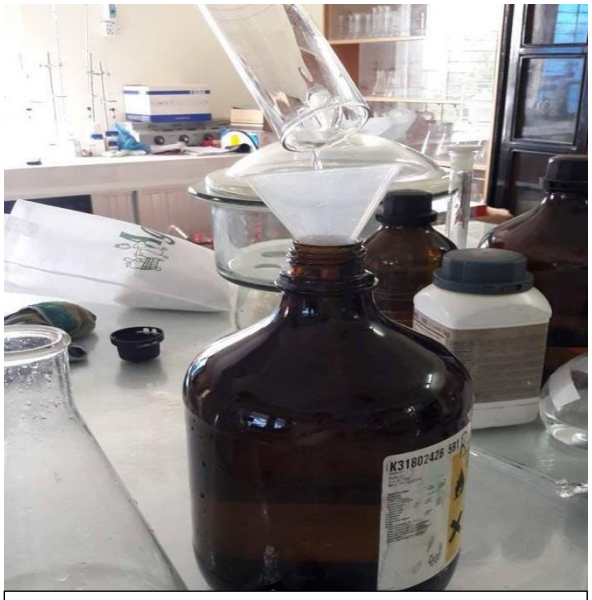

Figure 6: Rumen fluid is transferred into bottle that was added with buffer and CO2 atmosphere

Figure 7: Using a small CO_2 cylinder to transfer 100% $CO_2$to make the condition anaerobic

3.5. Preparation of buffered rumen fluid

The rumen fluid was mixed with buffer in 3:1 ratio and transferred into a closed bottle under an O_2 free condition as shown in figure 6. The bottle containing buffered rumen fluid was dispensed under 100% CO_2 atmosphere after every 3 min to make it O_2 free (Mamuad et al., 2014; Mruthunjaya et al., 2010).

3.6. Creating anaerobic condition

Firstly, an anaerobic condition was created by manually dispensing CO_2 from a small cylinder after every 3 min during the preparation of buffered rumen fluid to the transfer of buffered rumen in the serum bottle (as shown in figure 7) for optimum incubation period as well as actual experiment period of 24 h and 48 h. After the buffered rumen fluid was transferred into the serum bottles, rubber cap was put and bottle cap was sealed tightly with numerous layers of parafilm. Even during the experiment, serum bottles were checked to ensure any leakage of oxygen inside the bottle or methane outside the bottle and parafilm was put according to the need.

3.7. Determination of optimum incubation period

In vitro fertilization was conducted for 6 h, 24 h and 48 h incubation period to determine the suitable incubation period for the experiment. All standard procedures were followed for the pilot test and results were recorded. The result suggested that there was no production of methane (CH_4) gas in 6 h incubation period sample, as the time is less to start the process of fermentation and to produce methane gas. Although, methane (CH_4) gas were produced in both 24 h and 48 h samplings and standard process were used to measure the amount of CH_4**.** Based on these results, this study used only 24 h and 48 h samples for the actual *in vitro* fermentation experiment.

Figure 8: Control and treatment set for incubation for 24 h & 48 h

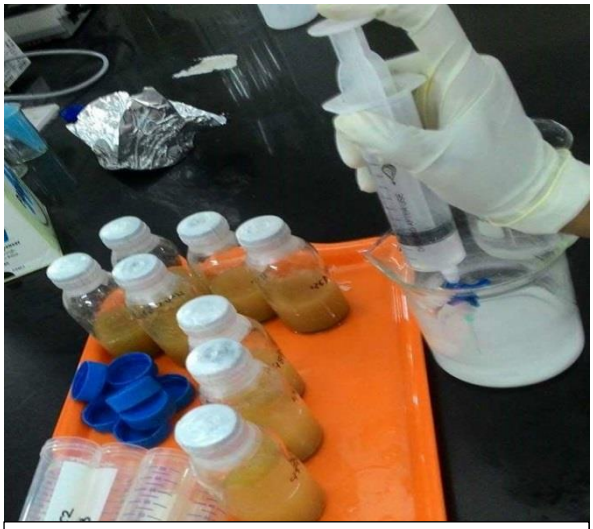

Figure 9: Using calibrated syringe to measure total gas and CH_4

3.8. *In vitro* gas production

Triplicates of treatment contained 50 µl of lysozyme from duck egg white mixed with 0.5 g air-dried commercial feed sample and 50 ml of buffered rumen fluid in autoclaved serum bottles. Control triplicates contained 0.5 g air-dried commercial feed sample with 50 ml of buffered rumen fluid except the lysozyme (Mamuad et al., 2014). *In vitro* gas production was determined by incubating treatment and control triplicates samples at 37^{o}C temperature in shaking incubator for 24 h and 48 h. Total gas was recorded using calibrated syringes at 24 h and 48 h as shown in figure 8. Falcon tubes were used to store the incubated samples in a refrigerator for further analysis of pH and nutrient composition (Mruthunjaya et al, 2010).

4. RESULTS

4.1. Total gas and CH_4 produced after 24 h of incubation

To study the amount of total gas and methane produced during the *in vitro* fermentation for 24 h of incubation. Results of production of total gas and methane produced after 24 h of incubation period are summarized in Table 1. In Table 1, C1, C2, C3 stand for three control samples without added lysozyme purified from duck egg white and T1, T2, T3 represent three treatment samples added with duck egg lysozyme.

Table 1: Total gas and CH_4 produced after 24 h of incubation

Sample	Total gas in ml	CH_4 in ml	CH_4 %	Average CH_4 %
C1	16	14	87.50	86.07
C2	20	17	85.00	
C3	21	18	85.70	
T1	18.5	13	70.30	76.80
T2	14.5	12	82.80	
T3	22	17	77.30	

4.2. Total gas and CH_4 produced after 48 h incubation

To study the amount of total gas and methane produced during the *in vitro* fermentation for 48 h of incubation. Results of production of total gas and methane produced after 48 h of incubation are summarized in Table 2.

Table 2: Total gas and CH_4 produced after 48 h of incubation

Sample	Total gas in ml	CH_4 in ml	CH_4 %	Average CH_4 %
C1	25	14	56.00	55.20
C2	20	11	55.00	
C3	22	12	54.50	
T1	18	10	55.60	50.30
T2	19	9	47.40	
T3	23	11	47.80	

4.3. Group statistics for control and treatment samples for 24 h and 48 h of incubation

Group statistics was calculated to study the amount of total gas and methane produced during in samples of control and treatment after the *in vitro* fermentation by using IBM SPSS Statistics, 2014. Results are summarized in Table 3.

Table 3: Group statistics for control and treatment samples for 24 h and 48 h of incubation

	Type	N	Mean	Std. Deviation	Std. Error Mean
Total_gas_24	control	3	19.0000	2.64575	1.52753
	experiment	3	18.3333	3.75278	2.16667
Total_gas_48	control	3	22.33	2.517	1.453
	experiment	3	20.00	2.646	1.528
Methane_24	control	3	16.33	2.082	1.202
	experiment	3	14.00	2.646	1.528
Methane_48	control	3	12.33	1.528	.882
	experiment	3	10.00	1.000	.577

4.4.Comparison of total gas and CH_4 produced after 24 h and 48 h of incubation

To compare the amount of total gas and methane produced during the *in vitro* fermentation after 24 h and 48 h of incubation. Results are summarized in figure 10.

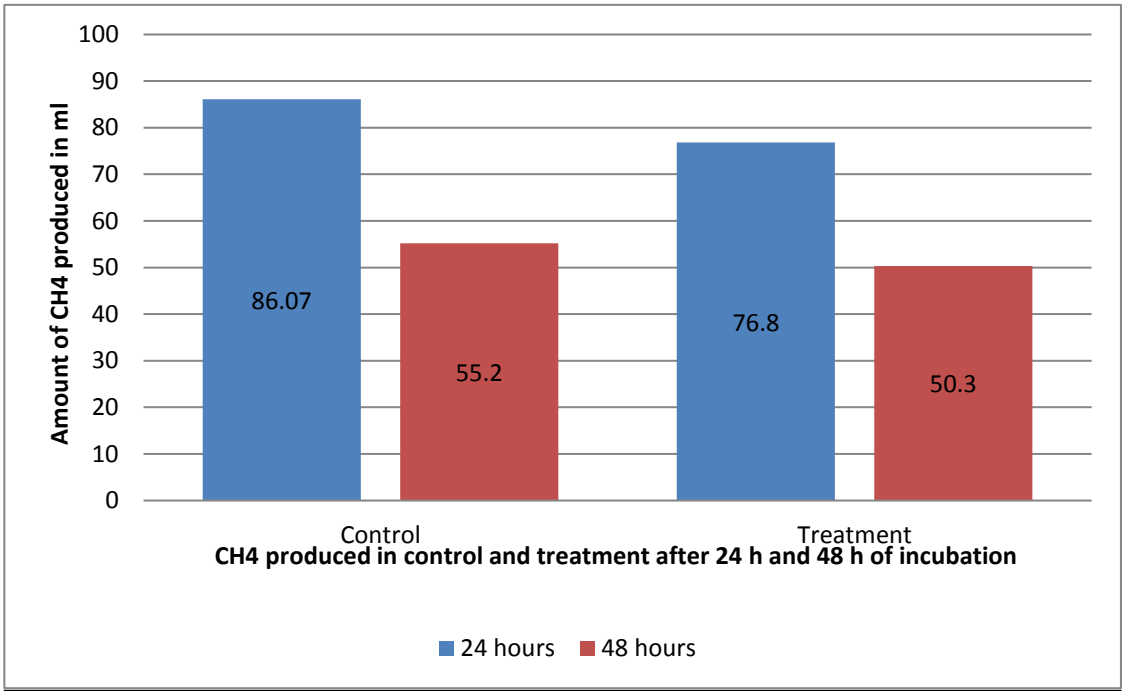

Figure 10: Comparison of total gas and CH_4 produced in control and treatment after 24 h and 48 h of incubation

4.5. Independent t-test to compare mean of control and treatment

Independent t-test was done to compare the mean of total gas and methane produced in control and treatment during the *in vitro* fermentation for 24 h and 48 h. Independent t-test was calculated in IBM SPSS Statistics, 2014 version and results are summarized in Table 4. The test used 95% confidence interval to find the significant difference.

Table 4: Independent sample test to compare mean of control and treatment samples for 24 h and 48 h of incubation

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Total_gas_24	Equal variances assumed	.175	.697	.251	4	.814	.66667	2.65100	-6.69368	8.02701
	Equal variances not assumed			.251	3.594	.815	.66667	2.65100	-7.03319	8.36653
Total_gas_48	Equal variances assumed	.057	.823	1.107	4	.330	2.333	2.108	-3.520	8.187
	Equal variances not assumed			1.107	3.990	.331	2.333	2.108	-3.526	8.192
Methane_24	Equal variances assumed	.348	.587	1.200	4	.296	2.333	1.944	-3.063	7.730
	Equal variances not assumed			1.200	3.790	.300	2.333	1.944	-3.183	7.850
Methane_48	Equal variances assumed	.727	.442	2.214	4	.091	2.333	1.054	-.593	5.260
	Equal variances not assumed			2.214	3.448	.102	2.333	1.054	-.788	5.454

5. DISCUSSION

Lysozyme is an antimicrobial enzyme, which can digest bacterial cell walls by hydrolyzing it. However, most of the gram-negative bacteria are not vulnerable to lysozyme because of the presence of thick protective outer layer. Additionally, lysozymes have

digestive abilities and can be added as a digestive enzyme to some animals to increase/improve their digestion (Biswas et al., 2016). Methane emissions are produced from enteric fermentation that takes place in the digestive systems of ruminants and to some extent of non-ruminants as well (Tubiello et al., 2014).

The mean of total gas produced in control after 24 h and 48 h incubation are 19 ml and 22.33 ml respectively. Similarly, the mean of total gas produced in treatment after 24 h and 48 h incubation are 18.33 ml and 20 ml respectively. There is an increasing trend in production of total gas with the incubation period. The increase in total gas amount found in this study aligns with the findings of Robinson et al., 1989 and Mamuad et al., 2014, which explained that total gas increase with incubation time because of microbial activities. Higher total gas production may also due to high content of breakable high crude protein coming from feed, which bacteria from rumen can easily break. After 24 h of incubation, the mean production of CH_4 was 76.80 % for treatment and 86.07% for the control. Table 1 suggests that there is 9.25% reduction in production of methane gas and this reduction is due to the added lysozyme from duck egg white.

The mean of CH_4 produced in control after 48 h of incubation is 55.20% as compared to 50.30% in treatment. There is a reduction of 4.90% of CH_4 in treatment as compared to control as shown in table 2. The decreasing trend in methane production suggests lysozyme maybe effective in reducing CH_4. The group statistics done in IBM SPSS Statistics, 2014 shows the means, standard deviation and standard error mean for total gas and methane production after 24 h and 48 h of incubation as shown in Table 3. Group statistics suggests that the standard deviation for control was 2.082 as compared to 2.646 for treatment for methane production after 24 h of incubation. In addition, the standard deviation for control was 1.528 as compared to 1.00 for treatment for methane production after 48 h of incubation. Similar methods were used in earlier done studies but none of these explained the standard

deviation for methane production. Therefore, due to lack of information about the standard deviation, the result of this study is not comparable to other studies.

The independent sample test was conducted to check significance of methane reduction for 24 h and 48 h of incubation as shown in Table 4. The t-test suggests that there were no significant difference between mean of total gas and methane production in control and treatment for 24 h and 48 h of incubation. The study used 95% confidence interval to compare the means and result was insignificant. Although, at 90% of confidence interval, there is a significant difference in mean of methane produced in control and treatment for 48 h of incubation. Although, study conducted by Mamuad et al., found significant decrease in CH_4 concentration after 12 h and 24 h of incubation. Another study conducted by Biswas et al., also found significant reduction in methane concentration. That study also found that with the increase in amount of added lysozyme, the concentration of CH_4 decreased, suggesting a negative correlation between CH_4 and added lysozyme. Though the study, the insignificant reduction in methane maybe due to use of lysozyme purified from duck egg white, as standard lysozyme was used in Biswas et al.'s study and microorganisms were used in Mamuad et al.'s study as the additive.

The figure 10 compare the total gas and CH_4 produced in control and treatment after 24 h and 48 h of incubation. The mean of CH_4 produced was 86.07 ml in control as compared to 76.8 ml in treatment after 24 h of incubation. Moreover, we can see drop in CH_4 production after 48 h of incubation in both control and treatment. Other studies also suggested that microorganisms use CH4 and CO2 or break down into simpler form after the fermentation is completed (Mamuad et al., 2014). It maybe because fermentation is mostly completed within 24 h and then produced CH_4 started to break into simpler forms or used by microorganisms from the rumen.

Numerous factors affect the fermentation of rumen. Concentration of microorganisms in the rumen fluid, along with composition of feed such as rice straw or commercial feed, pH of the ingesta, and concentration of microbial metabolite are some of the main indicators of intestinal health and total gas production (Mamuad et al., 2014). The simultaneous study of these factors is very important to suggest that the addition of lysozyme from duck egg white was effective in reducing CH_4 and have potential to reduce CH_4 and global warming in general. As this research only studied total gas and CH_4 production due to limited time and resources as an undergraduate thesis, the result of this study cannot be compared with already done studies. The data suggests that lysozyme purified from duck egg white was effective but not significant in reducing the amount of methane. Therefore, due to lack of study of other variables, the study suggests further research on lysozyme purified form duck egg white to firmly able to conclude if duck lysozyme was useful in reducing methane or not. Similarly, lysozymes additives in ruminant livestock feed can be helpful in reduction of methane production and this reduces greenhouse gases in the atmosphere and slows down global warming.

5.1. Implication of the research

Some of the implications of my study are this study will help researchers who are interested in working with lysozyme and rumen fluid. Recommendation for an effective methodology for rumen fluid and reduction of methane study are to identify the exact apparatus to do *in vitro* fermentation under laboratory conditions. Secondly, this study will act as a base for future in-depth studies on different lysozymes and methane reduction techniques to be used in animal farms.

5.2. Recommendation for further studies

Future studies on rumen fermentation should focus on different parameters of *in vivo* environment and try to incorporate these in *in vitro* experiments. Identification of strength of

lysozyme produced from duck egg white would have helped in better designing the experiments. Additionally, 12 h, 18 h, 30 h, 36 h, and 42 h of incubation period would have provided accurate results and it would be easier to identify the trends. Moreover, proper anaerobic experimental setup and apparatus would have added to the accuracy in the experiments.

6. CONCLUSION

The conclusion of my study is that lysozyme purified from duck egg white also showed enzymatic and bacteriolytic characteristics. Secondly, treatment with duck egg white lysozyme showed a reduction in methane production after both 24 h and 48 h of the incubation. Although, there was no statistically significant difference in CH_4 reduction in control and treatment. Therefore, the study suggests that lysozyme from duck egg white supplementation may improve *in vitro* fermentation and reduce CH_4 emissions from ruminants. Additionally, further studies are recommended to

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