



## Effect of adding probiotic on experimental in vitro digestion and rumen fluid characteristics of cows

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### Abstract

The aim of this study was to demonstrate effect use of probiotic that contains many of bacteria such as *Clostridium butyricum*, *Bacillus subtilis*, *Bacillus licheniformis* estimate the digestion coefficient of dry and organic matter. The concentration of ammonia nitrogen, use of 3 treatments, to measurement of percentage of dry matter, organic matter, the amount of metabolized energy, the percentage of protein, and the total numbers of anaerobic bacteria in the rumen fluid. The results showed that there were no significant differences between all treatments in the character the percentage of organic matter digestibility (OM%) and the percentage of dry matter and organic matter (DM), and no differed significantly from each other in the organic matter digestibility coefficient (IVOMD). When the result for (IVDMD) recorded high differed between treatment so each treat of probiotic (T2 and T3) superiority on treat control (T1). also the results of the statistical analysis to the (NH<sub>3</sub>N) showed a highly significant superiority of the two probiotic treatments over the control treatment, as the averages reached 0.48, 0.69, and 0.81 for both the control treatment and the probiotic at concentrations of 0.02 and 0.04, respectively. The results of the statistical analysis of the protein percentage showed a highly significant value of  $p < 0.01$ . The probiotic treatment with a concentration of 0.04 and 0.02 was significantly different from the control treatment the control treatment and the probiotic for both concentrations reached 2.97, 4.30 and 5.07%, respectively. The statistical analysis indicated that there was no significant effect of using a probiotic in estimating the amount of metabolized energy for each treatment, The results of the statistical analysis for the number of microorganisms showed that the two coefficients were superior. The probiotic treatment was significantly superior to the control treatment, as the numbers of bacteria reached 72.33, 196.66, and 228.33 Bacterial colony units/ml of rumen fluid for both the control and probiotic treatments at concentrations of 0.02 and 0.04, respectively.

**Keywords:** probiotic, *Bacillus subtilis*, *Bacillus licheniformis*, digestion experiment, number of anaerobic bacteria.

## Introduction

In last year used of probiotic substitute of antibiotics purpose of increasing animal growth, improve production and its products by improving to health of the rumen and increased content of microbial, probiotic includes all microbial such as bacteria, fungi, and yeasts [1,2,3]. Many microorganisms are used in direct feeding of ruminants or feeding on probiotics, but the most widely used are *Propionibacterium* and *Lactobacillus bacteria*, as they have been used in many studies of dairy cows and have proven their efficiency in increasing milk production and increasing the percentage of its components represented by protein, fat, and lactose [4].

Probiotics are microorganisms that include different types of bacteria, algae, or yeasts that do not harm the health of the host, but rather help stabilize the microorganisms in the host, which has a positive effect on the animal [5]. Probiotic increased to creating amicrobial balance in the digestive system that has been to used in feeding ruminants and increased efficiency and ability functions of the intestinal wall and maintain environmental balance of microbial in the digestive system [6].

The study of the rumen environment aims to know the productivity of the rumen and improve the health of the host The percentage of dry matter in the rumen is 10-18% the pH is 6.9-5.5 with a temperature of 6.4 and the temperature is 38-41°C. The bacteria are about  $10^{11}$ - $10^{10}$  colonies/ml, which includes 200 species, the Autophagic bacteria are  $10^7$ - $10^9$  cfu/ml, and the protozoa are  $10^6$ - $10^4$  colonies/ml [7]. most of important criteria set for manufacturing a probiotic is its content of viable bacteria and their number. This is reflected in the rate of growth and production and the positive effect on the digestive system. The strain of bacteria is chosen depending on the type and age of the host animals and it is manufactured in the form of powder, tablets, granules, or packaged in capsules [8]. probiotic make to improve the nutrit value of poor-quality feed, which is integrated in increasing what is suitable for these feed materials, and the digestion of solid ready-made matter is accompanied by fermentation processes within the rumen.[9] also conducted a digestion experiment using direct feeding of two types of probiotic compared with the control treatment in its effect on fiber fermentation and the amount of organic matter, the first probiotic contained two types of bacteria, *Bacillus subtilis* and *Bacillus licheniform*, at a concentration of  $5.9 \times 10^7$  cfu.

The second probiotic contained two types of bacteria, *Ligilactobacillus* animals and *Propionibacterium freudenreichii* at a concentration of  $1.5 \times 10^7$  cfu. The results showed that both treatments were significantly superior to the control treatment in the percentage of organic matter, reaching 81.4, 80.7, and 78.3% for each of the two probiotic treatments and the control treatment, respectively.

The study of microbiology in the rumen is one of the most important modern sciences, as bacteria constitute 95% of these organisms out of the total microbial community in the rumen [10] conducted a study on the use of *Bacillus subtilis* bacteria to estimate ammonia nitrogen, pH, lactic acid, volatile fatty acids, and methane gas. They used them at two concentrations: 10 billion colonies and 60 billion colonies. The results showed the role of bacteria in reducing methane productionIt has a role in digesting feed materials compared to the control treatment, as the treatment with a

concentration of 60 billion colonies was significantly superior to both treatments in producing ammonia nitrogen and did not differ significantly from each other in the rest of the mentioned characteristics. Through the above, the study aimed to demonstrate the importance of probiotics in improving rumen functions and increasing its content of microbial, this study considered the basis for the purpose of conducting a field experiment on holstein cow's milk producing and pregnant cows by dosing them with the probiotic after placing it in gelatin capsules in season summer.

### Materials and Methods

This study included used of probiotic that contains of anaerobic bacteria in experiment laboratory digestion to demonstrate of effect on the characteristics of rumen fluid cow.

Used in the experience of digestion probiotic imported company from Chinese (HebeiWeierli Animal Pharmaceutical Group Co.,LTD )

The Bacterial in probiotic :-

*Clostridium butyricum*

*Bacillus subtilis*

*Bacillus licheniformis*

*Lactic acid bacteria*

*Lactobacillus inactivated cells*

Number of microorganisms in the probiotic:-

*Clostridium butyricum*  $1.3 \times \text{CFU } 10^7 / \text{g}$

*Bacillus subtilis*  $1.2 \times \text{CFU } 10^{10} / \text{g}$

### Sampling

The quantity of the probiotic used in the laboratory digestion experiment was estimated based on the rumen capacity, that capacity amounted to 11 liters. Sampling including T1 Control without addition of probiotic, T2(0.02) mg probiotic and T3(0.04) mg probiotic mixed with rumen fluid and artificial saliva . The quantity of the probiotic that would be used for the cows was estimated at a laboratory concentration of 5 grams, at a concentration of 0.02 mg, according to the rumen capacity of the cow that was slaughtered, and the quantity of the probiotic that would be used for the cows, at a laboratory concentration of 10 grams, at a concentration of 0.04 mg during the estimation of the weight of the rumen contents of the feed materials consumed by the cow after slaughter, as the capacity of the rumen contents reached (11) liters, and thus the amount used of the probiotic was estimated at two different levels, as the amount of the probiotic used estimated The concentration of 5 gm used for cows in the field experiment corresponds to 0.02 in the digestion experiment, and the concentration of 10 gm used for cows in the field experiment corresponds to 0.04 in the digestion experiment. These two levels are compared with the control treatment.

### Traits studied in the digestion experiment

(Dry matter digestibility coefficient, organic matter digestibility coefficient, dry matter percentage, organic matter percentage, protein percentage, estimate of the

number of microorganisms, estimate of ammonia nitrogen, amount of metabolized energy).

The digestion experiment included two stages: microbial digestion for 48 hours and enzymatic digestion for another 48 hours. 40 ml of artificial saliva and 10 ml of filtered rumen fluid were prepared for each tube, and 3 test tubes of 50 ml of artificial saliva were made for each tube and used as a plank. The treatments are T1 without adding the probiotic, T2 adding the probiotic at a concentration of 0.02 g, and T3 adding the probiotic at a concentration of 0.04 to the rumen fluid.

### **Preparation of artificial saliva**

Artificial saliva consists of two solutions. The first solution is called McDougalls Budder Solution and consists of: -

1. 49 g of sodium bicarbonate  $\text{NaHCO}_3$
2. 18.6 g of disodium phosphate  $\text{Na}_2\text{HPO}_4$

Dissolve in 800 ml of distilled water

The second solution, called Chloride Solution, consists of:

1. 28.5 g of potassium chloride  $\text{KCl}$
2. 23.5 g of sodium chloride  $\text{NaCl}$
3. 6 g of hydrated magnesium sulfate  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
4. 2 g calcium chloride  $\text{CaCl}_2$

These materials are dissolved in 1 liter of distilled water, and 100 ml of (the second solution) is taken from it and added to 800 ml of (the first solution), then the volume is completed to one liter. Then 40 ml of it is taken and 10 ml of rumen fluid is added to it, in 3 repetitions for each treatment.

All of them were placed in digestion tubes, consisting of 10 digestion tubes, including 3 tubes for the control treatment, which is rumen fluid and artificial saliva, 3 digestion tubes for the probiotic treatment with a concentration of 0.02 grams, rumen fluid and artificial saliva, and 3 digestion tubes for the probiotic treatment with a concentration of 0.04 grams mixed with rumen fluid and artificial saliva. There are 3 digestion tubes for treating the booster with a concentration of 0.04 g mixed with rumen fluid and artificial saliva, and 3 digestion tubes containing artificial saliva only, which represent the plank. The proportions of the probiotic were estimated based on the volume of the rumen components of the cow, which amounted to 11 litres. These tubes were placed in a water bath at a temperature of 39 °C and  $\text{CO}_2$  gas was added to the tubes twice a day. The tubes were covered with a tight lid and placed in a dark room and shaken every 6 hours for up to 48 hours according to the method of [11]. After the end of the microbial digestion stage, samples were taken from all treatments for the purpose of bacterial counting, and the samples were kept at 4 °C until counting. As for the rest of the treatments: Two drops of hydrochloric acid were placed on each treatment for the purpose of stopping the microbial digestion process. The samples were placed in the centrifuge at 3000 rpm for 20 minutes. The sediment was taken and the filtrate was discarded. Then 50 ml of the 2% pepsin enzyme dissolved in hydrochloric acid was added and the tubes were incubated for another 48 hours to represent the enzymatic digestion process. The tubes are then centrifuged, the sediment is taken, and the filtrate, which represents the products of microbial and enzymatic



digestion, is discarded. Samples are taken for the purpose of measuring ammonia nitrogen, and the remainder is used to calculate the dry matter digestibility coefficient and the organic matter digestibility coefficient.

### Measurement of NH<sub>3</sub>N concentration

After the completion of the microbial and enzymatic digestion stages, samples were taken and placed in plastic tubes and frozen, then left to thaw. Then they were placed in a centrifuge at a speed of 3000 rpm for 20 minutes until a pure yellow fluid free of impurities was obtained. 2 ml were withdrawn of the samples, 3 replicates for each treatment, and the samples were placed in 250 ml Kjeldahl digestion tubes. The samples were digested with 98% concentrated sulfuric acid in the Micro Kjeldahl device. 2 ml of the sample was added to the 250 ml digestion tube, with the addition of 0.5 g of copper sulphate and 0.5 g of potassium chloride, to which 20 ml were added of sulfuric acid for each replicate at a temperature of 250°C. Digestion continues until the color of the samples changes to a clear color. After that, the samples are transferred to the distillation stage for each replicate separately using a steam distillation method using sodium hydroxide to collect the liberated ammonia. The resulting ammonia is received with 5 ml of ammonia reception solution consisting of 2 ml of boric acid with a concentration of 2% with 0.5 g of zinc and methyl orange dye (1 g of the dye is mixed in 100 ml of distilled water and used for all experimental samples). After the end of the distillation stage, the solution is done with hydrochloric acid with a concentration of 35-38%. The acid is prepared in a quantity of 5 ml in 1000 ml of distilled water. All analysis steps are performed, but without adding rumen fluid to measure the plank and calculate the number of consumed volumes of acid, the ammonia concentration is known according to the following equation. The ammonia nitrogen concentration was calculated as a percentage of the total nitrogen and according to the method [12].

$$\text{NH}_3\text{-Nmg}/100\text{ML} = \frac{(\text{ml acid titration volume} - \text{ml Blank}) \times \text{concentration acid titration}(0.014) \times 14.008 \times 100}{\text{ml sample volume}}$$

The protein percentage was calculated through the following equation:-

$$\text{protein percentage} = \text{N} \times 6.25$$

### Estimation of IVDMD

Estimation of in vitro digestibility coefficient of dry matter (IVDMD).

After laboratory digestion is carried out in two successive stages, the remaining (undigested) is dried at a temperature of 105°C for 24 hours using an electric oven according to the method (11) using the equation:

$$\text{DM digestibility coefficient}\% = \frac{\text{DM in the sample} - (\text{remaining undigested DM} - \text{Blank})}{\text{Dry matter in the sample}} \times 100$$

(Dry matter in the sample) represents taking a sample of the dry matter ingested from the rumen of the animal after slaughter, and the probiotic was mixed at concentrations of 0.02, 0.04 at a rate of 3 replicates for each concentration with the treatment representing the control and without adding the probiotic at a rate of 3 replicates as



well. Measurement of the laboratory digestibility factor of organic matter IVOMD. The laboratory digestibility factor of organic matter is calculated by burning the dried samples in the previous stage at a temperature of 600°C for 2-3 hours to estimate the percentage of Ash. The organic matter is calculated through the following equation: –

$$\text{OM digestibility coefficient \%} = \frac{\text{OM in the sample} - (\text{remaining undigested OM} - \text{Blank}) \times 100}{\text{Organic matter in the sample}}$$

### Dry matter ratio%

The dry matter was estimated by drying the samples at 105°C for 24 hours, then the percentage of dry matter was calculated according to the following equation:

$$\text{DM}(\%) = (\text{weight of remaining sample after drying} \div \text{weight of sample}) \times 100$$

### Percentage of organic matter

The percentage of organic matter was estimated by burning the samples in an incinerator at a temperature of 550°C for 4 hours and then calculated according to the following equation:

$$\text{Organic matter}(\%) = 100 - \text{ASh} \%$$

Estimating the amount of metabolized energy:

It was calculated using the equation:

$$\text{Metabolic energy (MJ/kg dry matter)} = 0.15 \times \text{Laboratory DM digestibility factor} \%$$

[13]

### Calculating the total numbers of anaerobic bacteria

Nutrient agar was prepared for counting total bacteria by dissolving 28 gm of culture medium powder according to the manufacturer's conditions at a ratio of (28 gm per 1000 ml) of distilled water in a glass beaker. The culture medium solution was sterilized for half an hour at a temperature of 121°C. The work surface was cleaned using a suitable disinfectant, and the dishes were poured with (15-20 ml) of the culture medium. Prepared in each dish, the method of counting colonies in the cultured dishes was adopted, as the number of colonies growing in the cultured media represents the total number of anaerobic bacteria whose numbers were obtained, and this count depends on the approved dilution rate, as a series of dilutions were used in the dilution. In it, 1 ml of the original sample is added to 9 ml of distilled water and shaken well. Then 1 ml of the new solution is taken and added to 9 ml of distilled water again. Then the process is repeated until we obtain four different solutions whose dilution ratio ranges from 1/10 to 1/10,000 the method of dilution described by [14]. Then 1 ml of the diluted solutions was transferred to the Petri dishes containing the sterile culture medium Nutrient agar (manufactured by the India company Himedia India and its components are: peptone, beef extract, sodium chloride and agar) for each of the dishes mentioned. The dishes were gently moved clockwise and counter-clockwise to mix the diluted sample with the culture medium. Then the plates were

incubated anaerobically upside down with CO<sub>2</sub> gas pumped for 24-48 hours at a temperature of 37°C.

According to the method [15], after which the colonies were counted according to the following equation :Number of bacterial cells/ml = number of colonies in the dish x reciprocal of dilution/sample volume ml.

### **Detection of microorganisms (bacteria) that have been added to the probiotic.**

The presence of microorganisms in the probiotic used in the digestion experiment was detected in two ways:

**First:** included the use of a hydrogen peroxide test (catalase test).

The reagent was brought from the Green chemicals Processing Office in Baquba and is of Turkish origin. The test is approved On the ability of bacteria to produce the enzyme catalase, which decomposes hydrogen peroxide H<sub>2</sub>O<sub>2</sub> into water and oxygen. When hydrogen peroxide is added to the bacterial colony, oxygen bubbles will appear, which indicates that the bacterial colony contains the enzyme catalase, which indicates that the result is positive, and not The appearance of bubbles indicates that the bacteria do not produce the catalase enzyme, that is, they are negative for catalase. The test was applied by placing a drop of the catalase reagent on the slide and taking a sample of the probiotic in which the presence of bacteria was to be detected after diluting the sample, as it was observed that air bubbles formed.

**Second:** The use of a gram stain is used to determine whether the bacteria are positive or negative for the gram stain . Several Gram stains were used, including the following steps:-

#### **Sample preparation.**

A sample of the bacteria that had been grown in the culture medium for 36 hours was taken and the sample was placed on a sterile glass slide.

#### **Install the scan.**

The negative side was pressed against the flame in order to pin the bacteria to the side.

#### **Pigmentation with crystal purple dye.**

It was applied without going to crystal violet only to remove all the bacteria in the eye with the purple stain, and then the side was washed with an alcohol solution to remove the color from the gram negative bacteria.

**After that, staining was done with safranin dye, which gives the red color to the Gram-negative bacteria, while the positive bacteria retain the purple color**

The slide is then examined under an electron microscope using an oil lens with 100x magnification.

#### **Statistical analysis**

The data analysis is based on a completely randomized design (CRD), according to the following mathematical model, and using the SAS Statistical Analysis System [16] program to analyze the data, and the significant differences between the means are compared with the multinomial test [17]:

$$Y_{ij} = \mu + t_i + e_{ij}$$

$\mu$  = the general average of the studied characteristic.

$Y_{ij}$  = the effect of viewing j for transaction i.

$t_i$  = effect of treatment  $i$ .

$e_{ij}$  = experimental error that is normally and randomly distributed with a mean equal to zero and a variance of  $\delta^2$ .

## Results and Discussion

### Percentage OM, DM and IVDMD and IVOMD

The results of the statistical analysis in Table (1) showed that there was no significant effect of the probiotic on the digestibility coefficient of the OM and DM%. The number of averages to OM% was 20.33, 23.80, 18.24% to T0, T1 and T2 so to DM% 73.25, 83.17, 80.36% respectively, the results for IVOMD showed that there were no significant differences between the treatments and the number of averages was 66.75, 78.51, 79.36% to T1, T2 and T3 respectively. The result of IVDMD observe high significant to treatment of probiotic compar with control the average was 70.39, 76.81, and 79.62% for both the control treatment and the probiotic at a concentration of 0.02 and 0.04, respectively.

**Table (1):** The effect of using the probiotic in the digestion experiment on both the digestibility coefficient of dry and organic matter and the percentage of dry and organic matter compared with the control treatment.

Treatment	OM%	DM%	%IVOMD	%IVDMD
T1(0)	20.33±2.60	73.25±2.26	66.75±5.55	70.39 ±1.44 b
T2(0.02)	23.80±4.95	83.17±1.92	78.51±6.45	76.81 ±1.41 a
T3(0.04)	18.24±1.58	80.36±4.93	79.36±3.97	79.62 ±1.17 a
General average	20.79±5.82	78.92±5.76	74.87±9.39	74.91±3.80
Significant analysis of variance	NS	NS	NS	*

T1(control), T2(0.02 probiotic), T3(0.04 probiotic).

OM (Organic Matter), DM (Dry Matter), IVOMD (In Vitro Organic Matter Digestibility), IVDMD (In Vitro Dry Matter Digestibility).

NS indicates that there are no significant differences between the coefficients within one column.

\* Indicates that there are significant differences between the coefficients at a sig level ( $p < 0.05$ ).

### NH<sub>3</sub>-N (mg/100 ml) and percentage of protein.

The results of the statistical analysis in Table (2) showed that the probiotic treatment with a concentration of 0.04 was superior to the control treatment, and the concentration of 0.02 did not differ significantly from the control treatment and the probiotic treatment with a concentration of 0.04, as the ammonia nitrogen concentration reached 0.48, 0.69, and 0.81 mg/100 ml for each of the control treatments and the probiotic at a concentration of 0.02 and 0.04, respectively. These results agreed with [26] who conducted a laboratory digestion experiment using the probiotic at a concentration of  $1 \times 10^9$  cfu. The results to NH<sub>3</sub>N also agreed with [10], who showed the moral superiority of the two probiotic treatments over Treatment to control the con-

centration of ammonia nitrogen by using the probiotic containing *Bacillus subtilis* bacteria. The results agreed with [27], who indicated that the two probiotic treatments were significantly superior to the control treatment, as the probiotic was used at two concentrations of 0.5 and  $1 \times 10^{11}$  g. The results did not agree with [28] who indicated that there was no significant effect of the probiotic on the concentration of ammonia nitrogen, as the probiotic was used at a concentration of  $1 \times 10^{10}$  cfu. The results also did not agree with what was reported by [22], who indicated that the control treatment was significantly superior to the probiotic treatment in ammonia nitrogen concentration. The results agreed with [29] who indicated that the probiotic treatments were superior to the control treatment in ammonia nitrogen concentration, reaching 13.35, 22.08, 20.51, and 22.40 for both the control treatment and the probiotic at a concentration of 10, 20, and 30 ml, respectively. The results agreed with [21] who indicated that the probiotic treatment was significantly superior to the probiotic treatment Control treatment in ammonia nitrogen concentration, as the concentrations for both the control treatment and the probiotic treatment reached 25.88 and 29.17 mmol/L, respectively. The results did not agree with [25] who indicated that there were no significant differences between the control and probiotic treatments in the concentration of ammonia nitrogen, as it reached 14.42 and 12.96 mg/dL.

The results agreed with [30], who pointed out The probiotic treatment was superior to the control treatment in ammonia nitrogen concentration when the probiotic was used in the laboratory fermentation process using rumen fluid for 72 hours, as the probiotic contained *Lactobacillus sakei* bacteria at a concentration of  $1 \times 10^9$  cfu and the ammonia nitrogen concentration reached 7.28 and 6.50 mg/dL for the probiotic treatment and control, respectively. The results did not agree with [31].

who indicated that there were significant differences between all treatments in the concentration of ammonia nitrogen during the fermentation process for the first 8 hours and during the 24 hours, as the averages reached 1.69, 1.65, 1.77 mlmol/L net for the full 24 hours 12.70, 12.83, 13.16 mmol/L. [32] conducted a study on the use of the booster in a laboratory experiment containing *Clostridium butyricum* bacteria at concentrations of 0.5, 1 and  $2 \times 10^6$  cfu, which then outperformed  $2 \times 10^6$  cfu. Moving forward, probiotic was a concentration of 0.5, as Belgium developed 14.0, 14.5, 15.7, 15.5 mg/100 ml. The results of the statistical analysis of the protein percentage showed that the probiotic treatment with a concentration of 0.04 was significantly superior to the control treatment, and the T2 treatment did not differ significantly from the control treatment and the T3 treatment, as the protein percentage reached 2.97, 4.30, and 5.07% for each of the T1, T2, and T3 treatments, respectively, as in Table (2). The results did not agree with [33] who indicated that the control treatment was superior to the 2 g probiotic treatment, which contains a group of bacteria, the most important of which are *Bacillus licheniform*, *Bacillus subtilis*, and *Clostridium butyricum*, when used with sheep's rumen fluid, as the averages reached 30.50 and 26.63 mg/100 ml for both the control and the probiotic. so the table(2)show the results of protein ratio high significant superiority T1 and T2 on T0 , the averages was 2.97 , 4.30 and 5.07 for each to T0 , T2 and T3 respectively, this result agree with [34] how explain the treats to probiotic of protein ratio high significant superi-

ority on control the average was 6.11 and 6.22% when used of probiotic contain of bacteria *Clostridium butyricum* that concentration  $2.5 \times 10^8$  cfu . [27-35] show that bacteria *Clostridium butyricum* that produce of NH<sub>3</sub>N in rumen and the concentration of NH<sub>3</sub>N is indicators that reflect the function of rumen and stability of its internal balance.

**Table (2):** The effect of using the probiotic in the digestion experiment on the concentration of ammonia nitrogen the protein percentage, the moisture percentage, and the amount of metabolized energy. (Means±standard error).

Treatment	Protein percentage %	NH <sub>3</sub> -N mg/100 ml
T1(0)	2.97±0.47 b	0.48±0.07 b
T2(0.02)	4.30±0.37 a	0.69±0.06 a
T3(0.04)	5.07±0.08 a	0.81±0.01 a
General average	4.10±0.61	0.65±0.09
Significant analysis of variance	**	**

T1(control), T2(0.02 probiotic), T3(0.04 probiotic).

\*\* Indicates that there are significant differences between the coefficients at a sig level ( $p < 0.01$ ).

### The energy metabolized and the numbers of anaerobic microbial.

Table (3) shows the amount of metabolized energy and the total numbers of bacteria in the rumen fluid. The results of the statistical analysis indicated that the metabolized energy to treatments was no significant between treatments, data of this trials recording 11.94 , 11.52 and 10.96 for T1 , T2 and T3. This indicates that is no effect of bacteria contained in the probiotic on the amount of metabolic energy, as all the coefficients did not differ from each other significantly, So not agree with [10] how mentioned bacteria *Bacillus subtilis* improve concentration of energy production in treatment of probiotic than control in vitro experimental digestion, when used of probiotic that concentration  $1 \times 10^9$  and  $6 \times 10^9$  . Also, this result not agree with [37] how mentioned that when used of probiotic in vitro experiment to estimate the amount of energy produced from fiber fermentation the reason is due to bacteria *Pseudo butyrvibrio* compare with control. The results of the statistical analysis for the number of microorganisms showed that the two coefficients were superior. The probiotic treatment was significantly superior to the control treatment, as the numbers of bacteria reached 72.33, 196.66, and 228.33 Bacterial colony units/ml of rumen fluid for both the control and probiotic treatments at concentrations of 0.02 and 0.04, respectively. These results are consistent with the results of [28] indicating that the probiotic treatment was significantly superior to the control in the number of bacteria. The results did not agree with [29], who indicated that there were no significant differences between all treatments, which included the control treatment and the probiotic with 3 different concentrations in the total numbers of bacteria. The reason for the increase number of microbial to treatment of probiotic than treatment of control due to bacteria *Clostridium butyricum* that work to increase microbial to diversity in the digestive system [34]. The presence of bacteria in rumen is necessary for the sta-

bility of fermentations as there are bacteria decompose cellulose, starches and proteins these types of bacteria convert feed that ingested to energy [38].

**Table (3):** The effect of adding the probiotic to rumen fluid in the digestion experiment on the amount of metabolized energy (MJ/kg dry matter) and the number of total anaerobic bacteria  $\times 10^4$  cfu/ml.

Treatment	number of total anaerobic bacteria $\times 10^4$ cfu/ml.	amount of metabolized energy (mikagol/kg dry matter)
T1(0)	72.33 $\pm$ 3.71      b	11.94 $\pm$ 0.17
T2(0.02)	196.66 $\pm$ 14.52      a	11.52 $\pm$ 0.21
T3(0.04)	228.33 $\pm$ 37.67      a	10.96 $\pm$ 0.96
<b>General average</b>	165.77 $\pm$ 40.55	11.47 $\pm$ 1.00
<b>Significant analysis of variance</b>	**	NS

T1(control), T2(0.02 probiotic), T3(0.04 probiotic).

NS indicates that there are no significant differences between the coefficients within one column.

\*\* Indicates that there are significant differences between the coefficients at a sig level ( $p < 0.01$ ).

### Percentage OM, DM and IVDMD and IVOMD

This study aimed to evaluate the extended effects of supplementing of probiotic on characteristics of rumen fluid cow, studies previous show importance of using bacteria improve all characteristics of digestion, the current study there were no observed improvements in OM, DM and IVDMD. [24] indicate no significant differences between treatments in percentage of OM and DM when used of probiotic that contain of bacteria *Lactic acid bacteria* in vitro digestion experiment, so [22] who indicated that there were no significant differences between all treatments, when used of probiotic at a concentration of  $1 \times 10^6$  cfu in vitro experimentally. [9] who explain to important of bacteria *Bacillus subtilis* and *Bacillus licheniformis* to increasing of percentage OM were significantly superior to the control treatment. However [23], indicated that was no significant effect of the probiotic on the DM percentage, as all treatments when the concentration of bacteria was  $3.2 \times 10^9$  cfu when using the laboratory digestion experiment, as the probiotic contained two types of bacteria *Bacillus subtilis* and *Bacillus licheniformis*. [25] observed the percentage of OM was significantly superior to probiotic on control treatment, while no observed any different between treatments of percentage DM. [21] found that there were no significant differences between all treatments in the character of the IVOMD, when conducting the laboratory digestion experiment, as the averages of the IVOMD reached 55.07 and 54.17% for both the control treatment and the probiotic with a concentration of  $1.7 \times 10^9$  cfu, which contains *Lactobacillus mucosae* bacteria. The results showed that there were highly significant differences between the treatments to IVDMD, When the treatment of probiotic gives significant results than treatment of control as the two probiotic treatments outperformed on the control treatment. [18] reported improved of IVDMD when used the probiotic treatment at a concentration of  $3.2 \times 10^9$  cfu when contains of bacteria *Bacillus subtilis* and *Bacillus licheniformis* in direct-fed microbial on in vitro and [19]

who indicated that the probiotic treatment at different concentrations was superior on control treatment in percentage of DM and OM, when used of probiotic contains on the bacteria *Lactobacillus plantarum* at four concentrations: 0.3, 0.6, 0.9 and 1.2, compared with the control treatment in experimental in vitro digestion used of fluid rumen goat. Additionally [20] indicated that the probiotic treatment at a concentration of  $1 \times 10^{10}$  was superior to the control treatment, reaching 58.28 and 54.02 for both the probiotic and the control treatment, respectively.

### **NH<sub>3</sub>-N (mg/100 ml) and percentage of protein**

It was found [34] high superiority to probiotic than control in concentration of NH<sub>3</sub>N in a laboratory digestion experiment that the contained bacteria at a concentration of  $2.5 \times 10^8$  cfu *Clostridium butyricum* the reason for the increase in NH<sub>3</sub>N concentration is the presence of bacteria *Clostridium* when [35] was stated that *Clostridium* bacteria are gram-positive bacteria that produce amino acids and nitrogen. The amount of NH<sub>3</sub>N is an indicator of the efficiency of protein utilization through nitrogen metabolism and is also an indicator of the presence of a large amount of digested proteins in the rumen [36].

The results of the study indicated that use of probiotic in a laboratory digestion experiment led to an increase of IVDMD, percentage of protein, concentration of NH<sub>3</sub>N, and increase of microbial community therefore it will be accompanied by an increase in the mass of microbial protein which further improve the digestion process and utilization of nutrients. Can be increased of energy metabolized by increase amount of concentration probiotic in digestion experimental as previous studies show of improvement in the amount of energy produced.

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