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Original Article

Effect of Concentrate: Roughage Ratio and the Addition of Kefir on the Production Characteristics of Ruminant *in vitro*

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Abstract

The stability of the gut ecosystem, especially the rumen, is an important area of research that has an impact on the use of feed additives and is associated with a number of diseases. The current study aimed to survey the effect of concentrate: roughage (C: R) ratio and the addition of kefir on the production characteristics of *in vitro* ruminant fermentation. In a 5x6 factorial order, six ratios of C: R (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100) and five doses of kefir (0, 0.8, 1.6, 2.4, and 3.2 ml) were used, respectively. Gas production during incubation was estimated at 0- 96 h. During inoculation, the rumen fluid was obtained at 0, 4, 8, 12, and 24 h of incubation. Cumulative gas production, GPDM, GPOM, and GPNDF at 24 h was highest at the C: R ratio of 100:0 (67.82 mL, 75.92 mL/ 200 mg, 1979.15 mL/200 mg, and 11.11 mL/ 200 mg, respectively). The kefir addition improved the kinetics and gas production significantly. The highest *in vitro* dry matter and organic matter digestibility (IVDMD and IVOMD) were obtained at the C: R ratio of 100:0 (9.26% and 182.2% higher than those in C: R ratio of 0:100, respectively). The increase of concentrate diet ratio improved the overall volatile fatty acids (TVFA). No interaction effect on the gas production was detected between the C: R ratio and kefir. The microorganism populations were influenced neither by the level of concentrate nor by the level of kefir. Consequently, the high concentrate-to-roughage ratio and the addition of 1.6 mL kefir to the overall dietary substrate could promote rumen fermentation and feed digestibility without affecting microbe counts.

Keywords: Concentrate: Roughage, Gas production, *In vitro* ruminal fermentation, Kefir

1. Introduction

Feed additives are extremely important in livestock ration due to the increase in nutrient utilization, change in rumen fermentation, and optimization of efficiency in livestock production processes. Due to the prohibition of synthetic hormones and antibiotics in livestock feed additives in many countries, the use of probiotics, feed enzymes, herbs, and other “natural" supplements is becoming increasingly popular and considerable. In addition to increasing the efficacy, these natural food additives minimize the transmission risk of human infections, reduce antibiotic use and risk of developing antibiotic resistance, and limit the removal of contaminants. Recently, many additives have been examined to replace or reduce the use of antibiotics, such as probiotics (1). Probiotic bacteria are live bacterial feed additives that increase the microbial balance of the host animal. It has been shown that probiotics have multiple functions, including the prevention of young animals from enteropathy diseases, enhancement of feed quality, animal growth, and immunity system (2-4).

Kefir is saline, thick, lightly carbonated, and fermented milk mixture that is often cultured with bacteria and yeasts. Kefir is made from the kefir grains through the inoculation of cow, sheep, or goat milk (5).

It includes proteins, polysaccharides, ethyl alcohol, lactic acid, salt, minerals, and vitamins (5). Kefir grains are comprised of lactic acid bacteria, acetic acid bacteria (e.g., species *Lactobacillus sp., Lactobacillus acidophilus, Leuconostoc sp., Acetobactersp*. and *Streptococcus sp.*), yeasts (e.g., *Saccharomycessp*., *Torula sp.*),and other microorganisms.

The health effects of kefir on mice, rats, fowl, and goat kids have been investigated widely (6, 7). However, there has been very little research regarding the usage of kefir in calves.

Manipulation of the rumen microbial environment and the creation of optimal conditions have always been important for animal nutritionists. Yeast in ruminants helped to keep the rumen pH stable and allowed low pH-sensitive cellulolytic bacteria to grow. Rumen yeast assisted in the protection of required anaerobes from feed ingested in the rumen as well as feed consumption. In sheep, total VFA production, acetate to propionate ratio, and dry matter digestibility increased *in vitro* (8). Accordingly, the use of feed additives "probiotics and enzymes" have been considered due to the lack of long-term consequences. The current study aimed to investigate the efficiency of kefir in vitro fermentation and kinetics of various concentrate and roughage ratios combinations as a probiotic source using cattle rumen liquor.

2. Materials and Methods

Experimental diets were designed to evaluate *in vitro* digestion of six concentrate to forage ratios (100: 0, 80:20, 60:40, 40:60, 20:80 and 0: 100, respectively). Kefir was used in diet in the amount of 0, 0.8, 1.6, 2.4, and 3.2 ml. The concentrate feed consisted of corn (61.00%), soybean (27.00%), wheat bran (8.50%), and minerals mixture (3.5%, including calcareous [0.79%], sodium bicarbonate (0.99%), di-calcium phosphates (0.59%), trace premix (0.40%), and salt (0.79%). Table 1 presentsthe chemical composition data for the feed ingredients and the tested ratios.

Table 1. Percentages of concentrate and roughage feed chemical components

Item	Alfalfa hay	Concentrate feed mixture
Dry matter	88.95	89.09
Organic matter	87.87	93.37
Crude protein	20.85	15.73
Ether Extract	2.84	4.74
Crude fiber	46.06	18.43
Ash	12.13	6.63
Non-fiber carbohydrate	18.12	54.47

2.1. Gas Production

The quantity of the *in vitro* gas production was estimated according to Menke, Raab (9). Fluids from rumen were gathered from two bulls slaughtered at the slaughterhouse of the Basrah Governorate. The rumen fluid was poured into a sealed container that was pre-warmed and filtered using sterile gauze. Rumen fluid was washed and fixed well mixed with CO2 stream. For each experiment, 200 mg of samples from each diet were collected into 125 ml glass bottles. A buffer solution was prepared as described by Tilley and Terry (10). During sample inoculation, CO_2 was constantly pumped at 39°C. The syringes were immediately filled with buffered rumen fluid (30 ml) and placed in a shaking water bath set at 39°C. Cumulative sample volume measurements from the three replicates were read manually at 0, 4, 6, 8, 12, 24, 48, 72, and 96 h of incubation. Fermentation syringes without samples (blanks) were included to control group gas generated directly from rumen fluid. The data were fitted to the exponential model used by Ørskov and McDonald (11) and the equation (Y= A+B(1-exp-ct)) following the subtraction of gas output from blanks, where 'Y' is the cumulative gas produced over time in ml; 't' is time in hours; 'A' and 'B' parameters are used to explain the fermentation potential ('A+B' represents the maximum fermentation potential), and 'c' is the constant gas output rate used to explain fermentation speed. Gas production was calculated as followed after 24 h:

GPDM= total gas production (ml)/ substrate DM (g)
GPOM= total gas production (ml)/ substrate OM (g)
GPNDF= total gas production (ml)/ substrate NDF (g)
GPADF= total gas production (ml)/ substrate ADF (g)

Metabolizable energy was estimated using the method adopted by Menke, Raab (9): ME in MJ= 2.20 + 0.136 GP + 0.057 CP. The short chain fatty acid (SCFA) output (mM) was calculated as SCFA= 0.0239 GP-0.0601,using the equation described by Getachew, Makkar (12).According to Menke, Raab (9), *invitro* digestibility of organic matter (ivOMD, g/kg OM) was calculated as ivOMD= 14.88 + 0.889 GP+ 4.5 CP (%) + 0.0651 ash (%), where GP is a net GP in ml after 24 h of incubation from 200 mg of dry sample.

2.2. *in vitro* Degraded Dry Matter (*iv*TDDM)

Using methods described by Anele, Südekum (13),*in vitro* degraded dry matter (ivTDDM) was determined and was calculated as *iv*TDDM= feed (DM) incubated − residue (DM) recovered in the crucibles/feed (DM) incubated (14). Partitioning factor (PF, a measure of fermentation efficiency) was calculated as: PF= *iv*TDDM (mg) /GP_{24} (mL). Following the method of Blümmel, Makkar (15), the *in vitro* microbial mass production can be calculated as Microbial mass (mg)= *iv*TDDM (mg) - (GP_{24} (mL)×2.2),where: 2.2: stoichiometric Ally factor, according to the amounts (mg) of C, H, and O required for the production of 1 mM of SCFA and associated 1 mL gas.

2.3. Statistical Analysis

Data on *in vitro* degradability and fermentation parameters have been statistically analyzed using SPSS software (version 20) (16) through a two-factor general linear model (six levels of concentrate: roughage and five kefir levels). The separation of means was achieved using the Bonferroni test within the same statistical program. Regression analysis of various parameters was also carried out. A p-value less than 0.05 (P<0.05) was considered statistically significant.

3. Results and Discussion

The ratio of concentrate and roughage (C: R) and the addition of kefir on the gas output parameters, including soluble gas production (a), insoluble gas (b), and constant gas production rate (P<0.05) were evaluated in this study. Insoluble gas production (c), and the potential degree of gas production (a plus b) are tabulated in tables 2 and 3. The total gas production produced in 96 h was presented in figures 1, 2, and 3. In the C: R ratio, accumulated gas output was highest at 96 h, at 100:0, and 80:20 (Figure 1). There was no interaction effect between the ratios of C: R and kefir (P>0.05) on gas kinetics (Table 4).

Table 2. Effect of concentrate and roughage(C: R) ratio on the gas kinetics

Concentrate%	Gas Kinetics ±standard deviation			
	A	B	C	A+B
100	44.71^{a}±0.51	98.67^{ab}±1.52	0.065^{d}±0.008	143.38^{ab}±1.07
80	41.57^{bc}±0.83	102.67^{a}±3.64	0.091^{a}±0.010	144.24^{a}±2.90
60	41.86^{b}±1.13	98.01^{b}±3.98	0.088^{a}±0.010	139.88^{bc}±2.85
40	40.62^{c}±1.13	98.99^{ab}±4.13	0.074^{bc}±0.008	139.61^{c}±3.02
20	36.59^{d}±0.66	100.77^{ab}±4.07	0.072^{cd}±0.007	137.37^{c}±4.50
RLSD (P<0.05)	1.40	5.30	0.012	3.70

Means in the same column with different superscriptsaresignificantly different (P<0.05)

Table 3. Effect of kefir supplements on the gas kinetics

Kefir (ml)	Gas Kinetics (±standard deviation)			
	A	B	C	A+B
0	40.28^{b}±3.36	103.07^{a}±3.97	0.072^{c}±0.007	143.35^{a}±2.42
0.8	40.63^{b}±3.173	101.78^{a}±2.76	0.074^{c}±0.009	142.41^{a}±2.39
1.6	41.01^{ab}±1.13	100.46^{ab}±2.00	0.075^{bc}±0.014	141.47^{ab}±2.83
2.4	41.66^{a}±3.15	97.68^{bc}±2.08	0.085^{ab}±0.013	139.34^{b}±3.75
3.2	41.77^{a}±2.75	96.14^{c}±3.19	0.089^{a}±0.015	137.91^{b}±4.50
RLSD (P<0.05)	1.03	4.10	0.011	3.07

Means in the same column with different super scripts are significantly different (P<0.05)

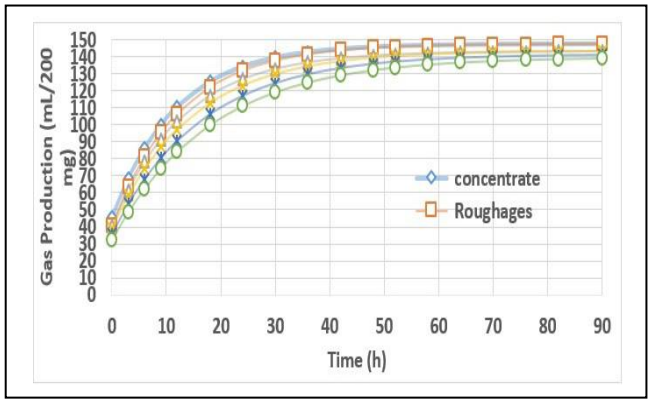

Figure 1. Gas production (mL/ 200 mg) for different ratios of concentrates: roughages
(Concentrate=100% concentrate, 80CR=80% concentrate, 60CR=60% concentrate, 40CR=40% concentrate, 20CR=20% concentrate, and Roughage=0% concentrate: 100%alfalfa hay).

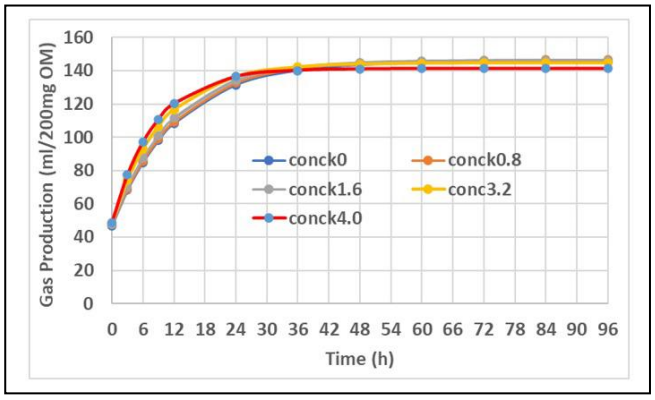

Figure 2. Gas production (mL/ 200 mg) for different levels of kefir and 100% concentrates (conck0=100 %concentrat+0 kefir, conck 0.8= 100% concentrate+ 0.8 ml kefir, = 100%concentrate+ 1.6 ml kefir, = 100% concentrate+ 3.2ml kefir, and conck 4.0= 100% concentrate+ 2.4 ml kefir)

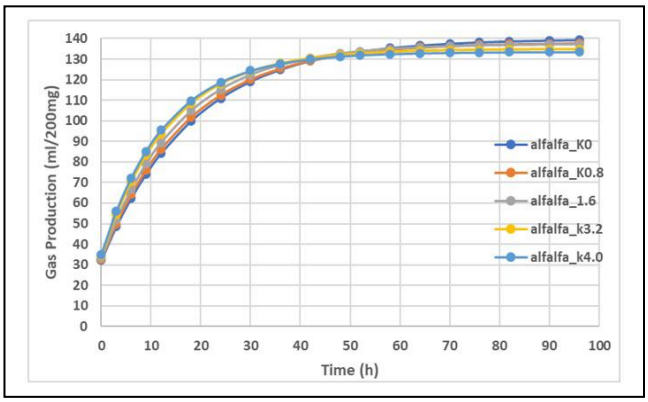

Figure 3. Gas production (mL/200 mg) for different levels of kefir and 100% Alfalfa hay (alfalfak0=100 %alfalfa+0 kefir, alfalfak0.8= 100% alfalfa+ 0.8 ml kefir, alfalfak1.6 = 100% alfalfa + 1.6 ml kefir, alfalfak3.2= 100% alfalfa + 3.2 ml kefir and alfalfk4.0= 100%alfalfa+2.4 ml kefir)

Table 4. Effect of C: R ratio and kefir supplementation on the ecology of the rumen and *in vitro* digestibility

Level of kefir	Equation coefficients		
	A	B	C
Concentration: Alfalfa (100:0)			
0	45.20	96.59	0.075
0.8	45.00	97.89	0.065
1.6	44.98	98.85	0.054
2.4	44.38	99.49	0.065
3.2	43.99	100.55	0.067
Concentration: Alfalfa (80:20)			
0	40.52	106.90	0.080
0.8	41.04	105.11	0.084
1.6	41.56	103.38	0.089
2.4	42.60	99.86	0.099
3.2	42.12	98.10	0.104
Concentration: Alfalfa (60:40)			
0	40.56	102.62	0.076
0.8	41.10	100.70	0.081
1.6	41.65	98.78	0.086
2.4	42.73	94.94	0.095
3.2	43.28	93.02	0.100
Concentration: Alfalfa (40:60)			
0	39.32	103.78	0.068
0.8	39.86	101.70	0.073
1.6	40.41	99.62	0.078
2.4	41.49	96.46	0.087
3.2	42.04	93.38	0.092
Concentration: Alfalfa (20:80)			
0	35.83	105.46	0.061
0.8	36.15	103.50	0.066
1.6	36.47	101.64	0.071
2.4	37.12	97.62	0.080
3.2	37.43	95.66	0.085

The presence of a high concentrate ration improved the rate and duration of fermentation (17). Similarly, Kang, Wanapat (18) observed that total gas production improved with the increase in the percentage of concentrate in the diet. The addition of kefir increased gas dynamic and aggregation which may be attributed to the fact that kefir might activate the rumen microbes and increase the incubated substratum digestibility, leading to progress in the kinetics of gas output.

Tang, Tayo (19) reported that accumulated gas production was improved by supplementation of a yeast culture. These findings agreed with those obtained by Wang, He (20), indicating that probiotic supplementation improved overall gas output following the incubation of various types of diets. In addition to improving the overall production of gas, the incorporation of yeast can also lead to qualitative improvements in the gases (21).

Table 5 demonstrated the influence of the C: R ratio and the incorporation of kefir on *in vitro* digestibility rumen pH, and NH3-N. Total gas production at 24 h of incubation decreased significantly (P<0.05) with the rise of roughage percentage in the ratio, from 67.82 to 52.97 ml for 100% concentrate to 0% alfalfa hay, respectively. Both IVDMD and IVOMD improved with the increase of concentrate proportions (P<0.05). Consistently, the results of the studies conducted by Phesatcha, Phesatcha (22) and Kang, Wanapat (18) revealed that digestibility can be optimized by the elevated percentage of concentrated feeds in the ration. This may be attributed to the resultant induction of microorganisms' growth, which led to increased digestibility. The inclusion of kefir dosage improved the analysis of IVDMD and IVOMD as well. The maximal IVDMD and IVOMD were obtained at 24 h of incubation in the R: C ratio of 80:20 withthe addition of Kefir, which was higher than those in the group of control by 4.76% and 3.15%.Wang, He (20), observed that the increase in IVDMD and *in vitro* NDF (IVNDFD) occurred due to the inclusion of Kefir. Tang, Tayo (19) showed that the addition of yeast increased digestibility *in vitro* with low-quality roughage. The inclusion of *Saccharomyces cerevisiae* (*S. cerevisiae*) improved the NDF and digestibility of both extracts in bovine animals at 2.5 g/ d (23).Cagle, Fonseca (24) reported that the addition of dry yeast at 10 g/d improved the digestibility of DM and NDF in finishing beef cattle (24). The fact that the digestibility of nutrients has been increased with the addition of kefir may be attributed to the enhancement of the rumen microorganisms. In addition, *S.cerevisiae* was proposed to be able to scavenge the available oxygen on the tops of freshly consumed feed to sustain metabolism activity which in turn reduced the ability of rumen redox (25-27).

Table 5. Effect of gas kinetics and accumulated gas output of the concentrate on roughage (C: R) ratio with kefir supplementation

Item	Concentrate feed mixture%					Alfalfa hay
	100	80	60	40	20	
Gas Pro 24h (mL)	67.82[a]	65.325[ab]	63.591[b]	59.71[c]	55.84[d]	52.97[e]
GPDM (mL/200 mg)	75.92[a]	73.95[b]	73.80[b]	72.32[b]	68.93[c]	65.54[d]
GPOM (mL/200 mg)	1979.15[a]	1926.88[b]	1877.42[c]	1839.80[c]	1768.65[d]	1697.87[e]
GPNDF (mL/200 mg)	11.11[a]	10.93[a]	10.54[b]	10.34b[c]	9.94[c]	9.54[c]
GPADF (mL/200 mg)	10.60	8.82	10.30	10.21	9.94	9.66
IVOMD%	66.60[a]	68.10[a]	52.29[b]	38.396[c]	29.51[d]	23.60[d]
ivTDDM	224.832	242.21	238.84	249.28	276.46	302.80
PF (mL/mg)	0.747	0.73	0.73	0.72	0.69	0.66
ME (MJ/Kg DM)	1.10	1.11	1.15	1.21	1.23	1.25
Microbial mass (g/kg DM)	11.51	11.18	10.95	10.43	9.91	9.52
SCFA (µM)	598.44[a]	594.35[a]	591.84[a]	588.56[a]	566.45[b]	543.05[c]

The beneficial effect of kefir and its mode of action can be summed up in the changes occurring in the rumen fermentation rates and forms. Certain probiotics are successful in increasing and stabilizing the pH by stimulating and inducing the protozoa to rapidly consume the starch and thereby with the bacteria that digest the starch and generate lactate (17). The reduction of rumen acidity increased the growth and activity of fibrolytic bacteria (28). Prado, Blandón (29) demonstrated that kefir was made through the fermentation of milk with kefir starting grain that contain a symbiotic consortium of microbes impacted by grain origin and growth conditions. However, there are variations in the total number of microorganisms in grains (29), including lactic acid bacteria (LAB) (*Lactobacillus spp*.), yeast (*Saccharomyces spp., Kluyveromyces spp., Kazachstania spp.,* and *Lachancea spp*.), and acetic acid bacteria (*Acetobacter spp*.) (30). Proteins, lipids, and lactose, as well as ethanol and lactic acid produced kefir. In the diet, kefir could be a rich source of calcium, essential amino acids, and vitamins. Some microbial strains of kefir community have been used as probiotics or antibacterial substance producers in the past (31). Although there has been few studies on the use of milk kefir in livestock or companion animals (32), it has been suggested that kefir can be a protein-rich livestock feed (33) or probiotics in both ruminant and non-ruminant herbivores. Several studies have described the anti-inflammatory, anti-allergic (34), and probiotic effects of kefir on the digestive systems (5). Moreover, kefir has been shown to interact with the gut microbiota and reduce pathogenic diseases, such as *Clostridium difficile* (35).

The calculation of the relationship between the produced gases, the *in vitro* organic matter digestibility coefficient, and the percentage of the concentrated diet showed a positive linear relationship with an accuracy of 0.9862 for gas production and 0.9140 for the digestibility coefficient of organic matter (Figure 4). The increase in the percentage of concentrated feed by one unit increased the percentage of gas by an average of 0.153 ml/200 mg and the digestibility coefficient of organic matter by 0.0978%.Moreover, ivTDDM correlated positively with less accuracy than the previous parameters (0.8730). The mean increase in ivTDDM was 0.0008 mg after increasing the concentrated feed by one unit (Figure 5). On the other hand, PF had a linear and negative correlation with concentrated feed with high accuracy (0.9648) and the mean of reduction in PF was 0.0016 ml/mg after the increase of concentrate by one unit.

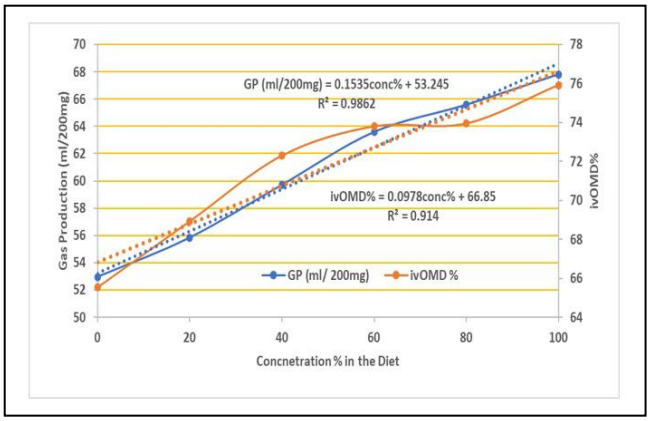

Figure 4. Gas production (GP) and *in vitro* organic matter digestibility (IVOMD %) associated with percentages of dietary concentration.

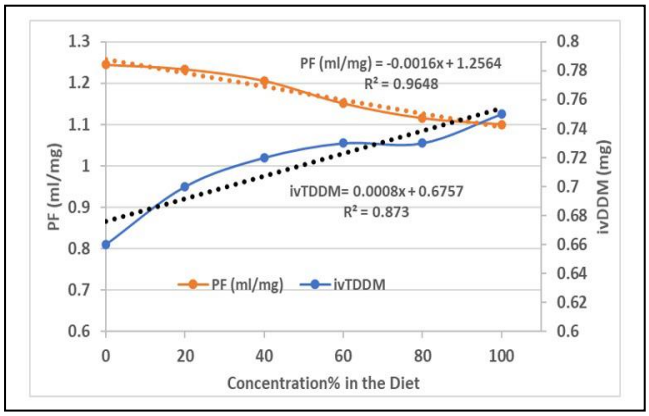

Figure 5. Association between partitioning factor (PF) and *in vitro* total dry matter digestibility (ivTDDM) with concentration percentages in the diet.

Animal efficiency is the most direct indicator for measuring the quality of nutrition. Performance data, on the other hand, is hard to determine and describe the rumen's potential interactions. Only a few studies exanimated cause-effect interactions of roughage combination in terms of energy or protein substrate involvement, rumen-degrading chemicals, and potentially associated effects that contribute to favorable or poor animal performance results. The need to evaluate VFAs in *in vivo* digestibility tests was crucial to determine the concentration, difference, and uptake of fermented VFAs. However, VFA levels were only determined at the end of the 48-hdigestion cycle in the phase of *in vitro* gas digestibility, providing useful information when concentrations were comparable in a variety of feed forms. As the most abundant source of energy which accounts for at least half of all digestible energy, VFAs were formed by fermentation of the rumen substrate and consumed subsequently (36).

In conclusion, the high ratio of concentrate to roughage and the addition of 1.6 ml of kefir to the overall dietary substrate could improve the fermentation of the rumen and boost the digestibility of the feed with no changes in the counts of microorganisms.

Authors' Contribution

Study concept and design: H. A. J. A. and M. S. A.
Acquisition of data: H. A. J. A. and M. S. A.
Analysis and interpretation of data: H. A. J. A. and M. S. A.
Drafting of the manuscript: H. A. J. A. and M. S. A.
Critical revision of the manuscript for important intellectual content: H. A. J. A. and M. S. A.
Statistical analysis: H. A. J. A. and M. S. A.
Administrative, technical, and material support: H. A. J. A. and M. S. A.

Ethics

This study was ethically approved by the Institutional Animal Care and Use Committee at the University of Basrah, Basrah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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