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Improvement of Rumen Fermentation Efficiency Using Different Energy Sources: In Vitro Comparison between Buffalo and Cow

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Abstract: During haymaking and ensilage, a significant loss of sugars occurs. The addition of a total mixed ratio (TMR) with a liquid feed might provide promptly utilisable energy and recover the nutrients lost during the conservation. Interesting results were already obtained by including liquid feed in a TMR in a dairy cow. However, the possibility to also utilize them in Italian Mediterranean buffalo is not yet supported by data. This study aimed to evaluate the in vitro fermentation characteristics and kinetics of different types of liquid feed, utilising bovine and buffalo rumen liquor as *inocula*. TMR supplemented with 0.025 g of four different liquid feeds was incubated with the TMR as control with buffalo and bovine rumen fluid using in vitro *gas* production technique. Considering bovine *inoculum,* all the experimental diets showed lower organic matter degradability and higher volatile fatty acid production than control TMR, while with buffalo rumen liquor, significant differences were observed between experimental and control diets in terms of gas production and fermentation kinetics. The tested liquid feeds can have different fermentation patterns depending on their ingredients and compositions. Supplementing liquid feeds to a standard diet seems to provide a source of energy that improves fermentation. No negative effects were observed on the in vitro fermentation at the dosage utilised.

Keywords: sugar; molasses; volatile fatty acids; gas production; by-products; additive

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1. Introduction

The growing global population has led to increasing demand for products of animal origin, such as meat, milk, and their derivatives [1]. Consequently, animal production systems underwent a transformation towards an indoor farming system with a large herd concentration [2]. As a result, livestock production has intensified even more, with the need for breeders to make genetic, structural, and nutritional choices to improve animals' performance. In this regard, cattle breeding has been affected by this progressive change, both for milk and meat production [3]. These variations led to a change in nutritional choices and feeding strategies. Nowadays, ruminant breeders are mainly oriented toward the use of a total mixed ratio (TMR) that guarantees a more consistent and uniform supply of nutrients in the rumen throughout the day, avoiding sudden decreases in rumen pH and satisfying properly energy requirements [4]. For several years, the presence of both forage and concentrate in the TMR has contributed to improving milk yield and quality, together with dry matter intake requirements in dairy cows [5]. However, forage is mainly administered to animals conserved as hay or silage, with the aim to have forage available

all year round. Notwithstanding the fact that either haymaking or ensiling favour nutrients' preservation in the green forage, during both conservation processes, a significant loss of sugars occurs [6,7]. In addition, Ùden [8] evidenced that the ensiling process alters the normal presence of organic acids and leads to a decrease in the efficiency of nitrogen utilisation in the rumen. In order to improve rumen efficiency and nutrient utilisation, an increasing interest in simple sugars has been recorded in the last years [9]. This strategy may be particularly useful if simple sugars are administered as liquid feed; the supplementation of TMR with a liquid feed could both obviate the lack of characteristics naturally present in fresh forage and lost in dried and ensiled ones [10]. The purpose of using liquid substances is to provide promptly utilisable energy, improve the diet palatability, and provide a higher content of organic acids, such as citric acid, phosphoric acid, and lactic acid [11]. Furthermore, the liquid feed does not increase the diet cost due to the low price of the raw materials and production technology [9]. The effectiveness of these products was recently tested on dairy cows, obtaining interesting results. In particular, several studies [9,12] have shown that easily fermentable sugars in dairy cow diets promote dry matter intake, increase diet palatability, and improve animal performance in terms of quantitative and qualitative milk production, by enhancing rumen microbial activity. However, these results still need to be verified in Italian Mediterranean buffalo, which, compared to a cow, present several differences in feeding behaviour and ability to utilise dietary components (i.e., protein, fibre, etc.) due to its tropical origin [13,14]. Therefore, the first aim of this investigation was to evaluate the in vitro fermentation characteristics and kinetics of different types of liquid feed included in diets for dairy ruminants. For this purpose, an in vitro cumulative gas production trial was carried out, utilising, as *inocula,* the rumen liquor collected from two donor species (bovine and buffalo) and the differences between the species were also investigated. The hypothesis is that the nutrients included in the tested liquid feed modulate rumen microbial activity and underline the differences between the two species.

2. Materials and Methods

2.1. Experimental Design

Four liquid feeds (LF_1, LF_2, LF_3, LF_4) produced by Sugar Plus® (ED&F Man Liquid Products Italia srl Bologna, Italy) and characterized by different nutrient contents (Table 1) were selected for the in vitro fermentation study [15]. Considering the dosages recommended by the producer for dairy cows and buffaloes (2.0–2.5 kg as feed/head/day for cattle and 1.0–2.0 kg as feed/head/day for buffaloes) and taking into account the average dry matter intake in dairy cow and buffalo, each liquid feed was added to a total mixed ratio (basal TMR) commonly used for dairy production with a concentration of 0.025% (EXP 1–4: experimental diet added with LF 1–4, respectively). The chemical composition values of the liquid feeds (LF 1–4) and the basal TMR (control diet, CTR) are reported in Table 2.

Table 1. Ingredients of four liquid feeds (Sugar Plus®).

Liquid Feed_1	Liquid Feed_2	Liquid Feed_3	Liquid Feed_4
Cane molasses	Cane molasses	Cane molasses	Cane molasses
-	-	Soluble condensed molasses	-
	Beet molasses	Beet molasses	Beet molasses
	-	-	Glycerol
-	Glucose syrup	Glucose syrup	Glucose syrup
-	Citrus molasses	-	-
-	Isomaltulose molasses	Isomaltulose molasses	Isomaltulose molasses

-	Barley malt	Barley malt	Barley malt
-	Sucrose	Sucrose	Sucrose
-	Sodium chloride	Sodium chloride	Sodium chloride
-	acetic and propionic acids	acetic and propionic acids	acetic and propionic acids

Table 2. Chemical composition (% as feed) of the tested liquid feeds (Sugar Plus®) and control TMR (CTR).

	Liquid Feed_1	Liquid Feed_2	Liquid Feed_3	Liquid Feed_4	Control Diet
Moisture	30.0	32.0	33.0	30.0	55.0
CP	8.00	7.50	12.5	5.00	13.7
EE	0.10	0.10	0.10	0.10	4.42
CF	0.10	0.10	0.10	0.10	19.9
NDF	-	-	-	-	44.2
Ash	8.50	7.00	8.00	5.50	9.00
NSC	40.0	41.0	32.0	32.0	28.7
NEl (Mcal/kg)	1.30	1.35	1.28	1.40	14.38

TMR: brewer grain, corn silage, corn mash feed, alfalfa hay-silage, complementary commercial feed, oat hay, Vitamin + Mineral supplement. CP: crude protein; EE: ether extract; CF: crude fibre; NDF: neutral detergent fiber; Ash: inorganic matter; NSC: no structural carbohydrates; NEl: net energy for lactation.

2.2. In Vitro Fermentation

Five substrates named EXP diets 1–4 (TMR supplemented with Liquid Feeds 1–4) and control diet (CTR: TMR without liquid feed supplementation) were incubated (1.0044 ± 0.0025 g) in a serum flask with buffered buffalo and/or bovine rumen fluid (10 mL). This entailed three replications per substrate, respectively, at 39°C under anaerobic conditions. The rumen liquors were collected at slaughterhouse according to EU legislation (EU Council, 2004). All procedures involving animals were approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II (Prot. 2019/0013729 of 08/02/2019). Rumen fluids were collected at the slaughterhouse from six healthy animals, placed inside pre-heated thermos, and transported to the laboratory of Feed Evaluation of the Department of Veterinary Medicine and Animal Production (University of Napoli, Federico II) within 2 h. The rumen fluid was pooled, mixed, and strained through four layers of cheese cloths and diluted in a buffered medium (75 mL). Subsequently, the reducing agent (4 mL) was added to the flasks [16]. The gas produced was recorded 21 times through 120 h of incubation (from 2 to 24 h of intervals) employing a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, USA). The cumulative volume of gas produced at 120 h of incubation was related to incubated organic matter (OMCV, ml/g). At the end of the incubation period, the fermentation liquor was analysed for pH using a pH meter (ThermoOrion 720 A+, Fort Collins, CO, USA). The organic matter degradability (OMD, %) was determined by weight difference of the incubated organic matter and the undegraded filtered throughout crucibles (porosity #2) and burned in muffle at 550°C [17].

2.3. End-Products Measurement

The fermentation liquor after 120 h of incubation was sampled and cooled at 4°C in order to determine the volatile fatty acid (VFA) content. For this purpose, the samples were centrifuged at 12,000 g for 10 min at 4°C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Melle-Neuenkirchen, Germany) and the supernatant (1 mL) was mixed with 1 mL of 0.06 mol oxalic acid. Different VFA were assessed by gas chromatography (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy) prepared with a fused silica

capillary column (30 m, 0.25 mm ID, 0.25 μm film thickness), using an external standard solution composed of pure acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acids. The percentage of branched-chain fatty acids (BCFA) was calculated as (iso-butyric acid + iso-valeric acid/VFA).

2.4. Data Processing

To estimate the fermentation kinetics, the gas production data were fitted to the sigmoidal model for each bottle [18]:

$$G = A/(1 + (B/t)^C) \quad (1)$$

where G is the total gas produced (ml per g of incubated OM) at time t (h), A is the asymptotic gas production (ml/g), B is the time at which half of A is reached (h), and C is the curve switch. Maximum fermentation rate (R_{max}, ml/h) and the time at which it occurs (T_{max}, h) were determined using model parameters [19]:

$$R_{max} = \frac{(A * C^B) * B \times T_{max}^{(B-1)}}{((1 + C^B) * (T_{max} - B))^2} \quad (2)$$

$$T_{max} = C * \left(\frac{B - 1}{B + 1}\right)^{1/B} \quad (3)$$

2.5. Statistical Analysis

Statistical analyses were performed on in vitro fermentation parameters (OMD, OMCV), kinetics (T_{max}, R_{max}), and end-products (VFAs, BCFA) by two-way ANOVA (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, USA, 1989–2019) to evaluate the effects of the substrate (EXP 1–4) and *inoculum* (bovine vs. buffalo) as fixed factors. The significance level was verified using HSD Tukey's test at $p < 0.01$ and $p < 0.05$. Post hoc Dunnett test was performed to observe the differences between control and experimental diets. The statistical comparison Shapiro–Wilk test for normally distributed data was performed.

3. Results

3.1. In Vitro Fermentation

The results registered by the Dunnett test with bovine *inoculum* are depicted in Table 3. All the experimental diets added with liquid feeds revealed significantly lower OMD parameters compared to the control counterpart (CTR). EXP 1 and EXP 4 showed significantly (p<0.01) higher and lower gas production (OMCV) values, respectively, compared to CTR. The time, which occurs for the highest fermentation rate (T_{max}), was significantly lower in EXP 1 and 3 (p<0.01 and p <0.05, respectively) compared to the control diet, while EXP 2 showed an opposite trend (p<0.01). Considering R_{max}, diets added with liquid feed 1 and 4 showed, respectively, significantly (p< 0.01) lower and higher values compared to the control diet.

Table 3. In vitro fermentation parameters: control vs. experimental diets with bovine *inoculum.*

Diet	OMD (%)	OMCV (mL/g)	T_{max} (h)	R_{max} (mL/h)
CTR	71.0	252	4.50	7.92
EXP 1	67.2	265	1.70	9.47
EXP 2	66.2	255	5.22	8.56
EXP 3	67.7	256	4.04	8.37
EXP 4	65.4	226	4.82	6.44
		CTR vs.		
EXP 1	**	***	***	***
EXP 2	**	NS	**	NS

EXP 3	*	NS	*	NS
EXP 4	***	***	NS	***
MSE	1.14	2.59	0.03	0.10

CTR: control diet without liquid feeds; EXP 1: experimental diet 1 containing liquid feed 1; EXP 2: experimental diet 2 containing liquid feed 2; EXP 3: experimental diet 3 containing liquid feed 3; EXP 4: experimental diet 4 containing liquid feed 4. OMD: Organic Matter Disappeared, OMCV: Organic Matter Cumulative Volume, T_{max}: maximum time which occurs R_{max}; R_{max}: maximum fermentation rate. *, **, ***, NS: $p<0.05$, $p<0.01$, $p<0.001$, not significant, respectively. MSE mean square error.

The results registered by the Dunnett test with buffalo *inoculum* are reported in Table 4. All the experimental diets with liquid feed showed higher ($p<0.01$) OMCV and a faster and more consistent fermentation process in terms of $T_{max,}$ which was lower ($p<0.01$) and R_{max}, which was higher ($p<0.01$) compared to the control diet (CTR).

Table 4. In vitro fermentation parameters: control vs. experimental diets with buffalo *inoculum.*

Diet	OMD (%)	OMCV (mL/g)	T_{max} (h)	R_{max} (mL/h)
CTR	70.6	209	10.8	4.81
EXP 1	70.8	235	7.93	6.12
EXP 2	69.9	253	4.89	7.32
EXP 3	70.8	255	5.72	7.40
EXP 4	70.9	249	4.13	7.27
		CTR vs.		
EXP 1	NS	***	**	***
EXP 2	NS	***	***	***
EXP 3	NS	***	***	***
EXP 4	NS	***	***	***
MSE	0.97	8.37	0.25	0.08

CTR: control diet without liquid feeds; EXP 1: experimental diet 1 containing liquid feed 1; EXP 2: experimental diet 2 containing liquid feed 2; EXP 3: experimental diet 3 containing liquid feed 3; EXP 4: experimental diet 4 containing liquid feed 4. OMD: Organic Matter Disappeared, OMCV: Organic Matter Cumulative Volume, T_{max}: maximum time which occurs R_{max}; R_{max}: maximum fermentation rate. **: 0.01; ***: $p<0.001$, NS: not significant; MSE mean square error.

The comparison between control and experimental diets in fermentation profile (gas production and fermentation rate) is reported separately for the two *inocula* in Figures 1–2 and Figures 3–4, for buffalo and cattle, respectively. Both profiles (gas production and fermentation rate) are quite superimposable, comparing the tested substrates with bovine *inoculum*. Only a few differences appear in the first hours of incubation for EXP 1 and EXP 4, which showed the fastest and slowest fermentation process, respectively. On the other hand, for buffalo, the diets supplemented with the liquid feeds showed a different fermentation process (with more gas, faster, and with a higher rate) concerning the control diet, starting from the first hour of incubation. This phenomenon was more evident for EXP 1 and EXP 2 compared to the control.

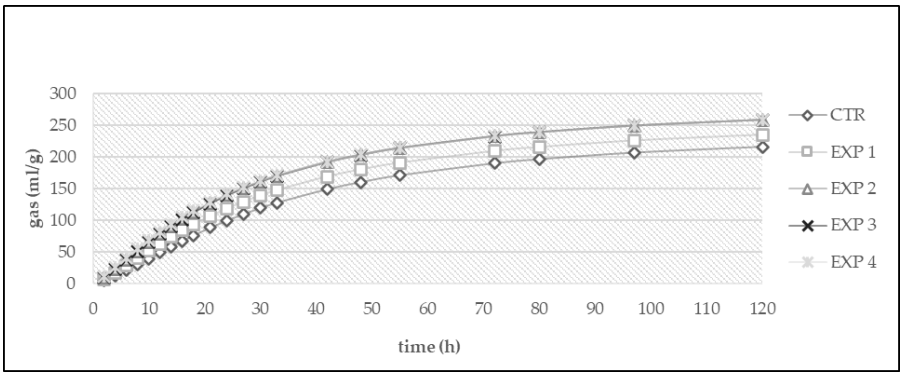

Figure 1. In vitro gas production over time in control and experimental diets with buffalo *inoculum*.

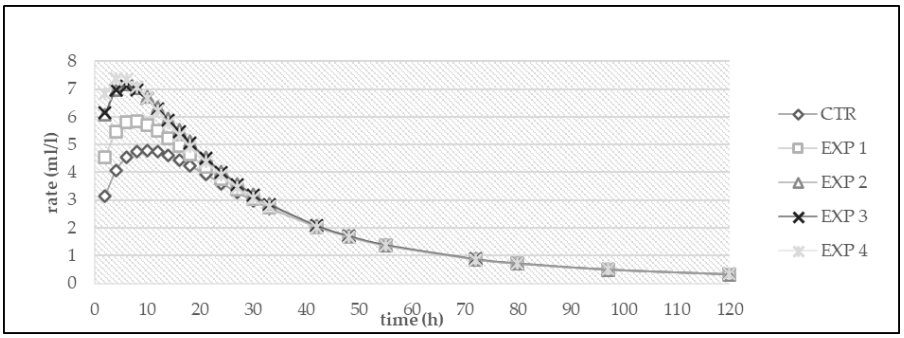

Figure 2. In vitro fermentation rate over time in control and experimental diets with buffalo *inoculum*.

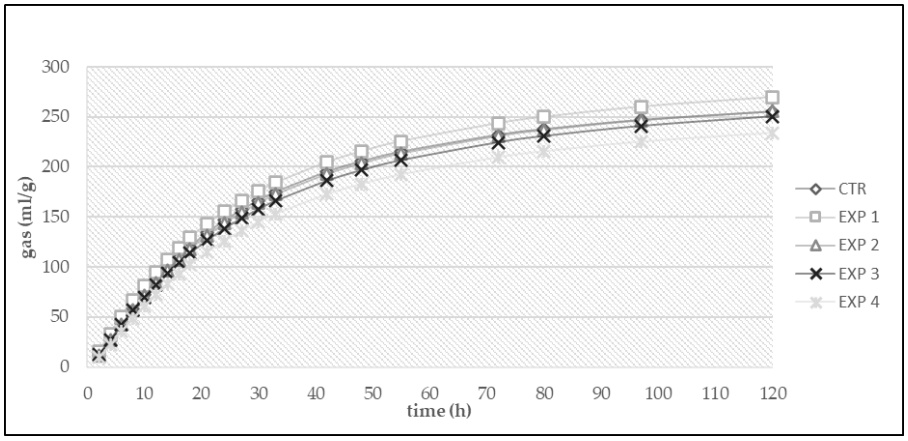

Figure 3. In vitro gas production over time in control and experimental diets with bovine *inoculum*.

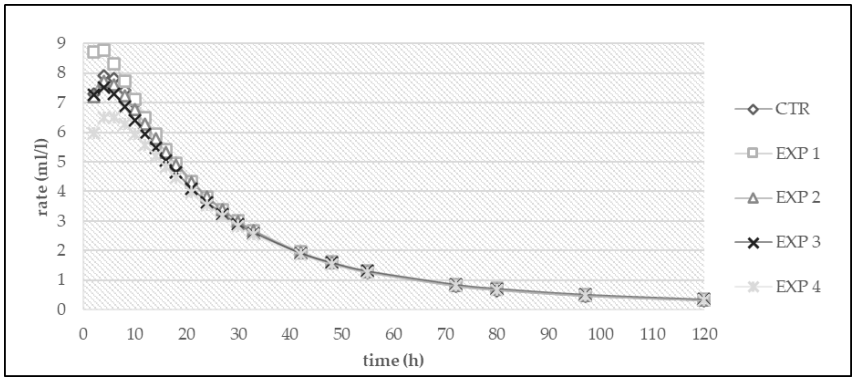

Figure 4. In vitro fermentation rate over time in control and experimental diets with bovine *inoculum*.

In Table 5, the least square means for the in vitro fermentation results at 120 h of incubation, considering the five substrates incubated with both *inocula*, are reported. Either *inoculum* or substrate, and their interaction, significantly (p<0.01) affect all the fermentation characteristics and kinetics parameters. Regarding the comparison between inocula, buffalo showed higher values of OMD and T_{max} and a lower gas production and fermentation rate compared to bovine.

Table 5. Least square means for in vitro fermentation parameters after 120 h of incubation.

Items	OMD (%)	OMCV (mL/g)	T_{max} (h)	R_{max} (mL/h)
		Inoculum effect		
Buffalo	70.6	241	6.69	6.59
Bovine	67.4	251	4.06	8.15
p value	<0.001	<0.001	<0.001	<0.001
		Substrate effect		
p value	0.0070	<0.001	<0.001	<0.001
		Interaction Substrate x *Inoculum*		
p value	0.0080	<0.001	<0.001	<0.001
MSE	1.24	5.67	0.14	0.09

OMD: Organic Matter Disappearance; OMVC: Cumulative Volume of gas related to incubating Organic Matter; R_{max}: maximum fermentation rate; T_{max}: time at which R_{max} occurs. MSE: mean square error.

3.2. End-Product Measurement

The results with bovine rumen liquor obtained by the Dunnett test are reported in Table 6. In comparing experimental diets with the control, EXP 4 showed significant differences for several parameters and, in particular, for total VFA and propionic acid. EXP 4 showed a lower (p<0.01) value of pH and, together with EXP 1 and 3, higher (p<0.01) levels of volatile fatty acids (VFA). EXP 3 had a higher (p<0.01) value of Acetate, while EXP 2, 3, and 4 reported a lower (p<0.01) level of Propionate. Experimental diets 3 and 4 were lower (p<0.05) and higher (p<0.01) in Butyrate than the control diet. EXP 4 had a higher (p<0.05) percentage of BCFA compared to the control diet.

Table 6. In vitro fermentation end-products: control vs. experimental diets with bovine *inoculum.*

							CTR vs.			
Items	**Units**	**CTR**	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**MSE**
pH		6.54	6.53	6.52	6.51	6.49	NS	NS	NS	**	3e-3
VFA	mmol/l	98.7	109	99.2	112	108	***	NS	***	***	1.29
Ace	%VFA	59.1	60.1	60.1	61.3	58.8	NS	NS	**	NS	0.51
Prop	%VFA	19.4	19.5	18.3	17.7	17.7	NS	***	***	***	0.09
Iso-But	%VFA	1.68	1.40	2.12	1.56	1.51	*	**	NS	NS	0.01
But	%VFA	14.2	13.9	14.5	13.6	15.6	NS	NS	*	***	0.05
Iso-Val	%VFA	2.99	2.57	3.11	3.62	4.20	NS	NS	NS	**	0.11
Val	%VFA	2.61	2.39	2.13	2.07	2.36	NS	**	**	NS	0.03
BCFA	%VFA	4.66	3.98	5.01	5.18	5.51	NS	NS	NS	*	0.16
A/P		3.05	3.08	3.29	3.48	3.11	NS	NS	**	NS	0.02

CTR: control diet without liquid feeds; EXP 1: experimental diet 1 containing liquid feed 1; EXP 2: experimental diet 2 containing liquid feed 2; EXP 3: experimental diet 3 containing liquid feed 3; EXP 4: experimental diet 4 containing liquid feed 4. VFA: total volatile fatty acids; Ace, acetate; Prop: propionate; Iso-But: Iso-Butyrate; But: Butyrate; Iso-Val: Iso-Valerate; Val: Valerate; BCFA: branched-chain fatty acids; Ace/Prop: Acetate to Propionate ratio. *: p<0.05; **: p<0.01; *** p<0.001; NS: not significant; MSE mean square error.

The results with buffalo rumen liquor obtained by the Dunnett test are reported in Table 7. As a whole, experimental diet 1 showed fewer differences compared to the control diet from the other diets. Among volatile fatty acids, only the total ones, acetate and iso-valerate, highly differ. In particular, EXP 1, 3, and 4 showed lower (p<0.01) pH values than the control diet. Moreover, EXP 3 and 4 reported a lower amount of VFA (p<0.01), whereas EXP 2 showed a higher (p<0.001) amount of VFA compared to CTR. Acetate production was lower (p<0.01) in EXP 2 than the CTR diet, whereas it was higher in EXP 3 and 4 (p<0.01 and p<0.05, respectively). EXP 2 and 3 reported slightly higher (p<0.05) levels of propionate percentage with respect to the control diet. Butyric acid was higher in EXP 1 (p<0.01) and EXP 2 (p<0.05). All experimental diets showed a lower (p<0.01) percentage of BCFA than the CTR diet.

Table 7. In vitro fermentation end-products: control vs. experimental diets with buffalo *inoculum.*

							CTR vs.			
Items	**Units**	**CTR**	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**EXP1**	**EXP2**	**EXP3**	**EXP4**	**MSE**
pH		6.52	6.48	6.51	6.49	6.50	***	NS	**	**	4.2×10^{-4}
VFA	mmol/l	107	108	111	95.5	98.9	NS	**	***	***	0.74
Ace	%VFA	62.5	62.6	60.8	64.7	63.5	NS	***	***	*	0.12
Prop	%VFA	19.4	19.4	20.3	20.2	19.4	NS	*	*	NS	0.09
Iso-But	%VFA	1.12	1.15	1.13	1.04	0.94	NS	NS	NS	*	0.005
But	%VFA	12.4	14.07	13.5	11.6	12.5	**	*	NS	NS	0.32
Iso-Val	%VFA	2.99	2.06	2.22	2.05	1.90	***	***	***	***	0.002
Val	%VFA	1.73	1.70	1.53	1.56	1.99	NS	NS	NS	NS	0.01
BCFA	%VFA	3.96	3.18	3.22	2.98	2.82	***	***	***	***	0.03
A/P		3.25	3.17	2.97	3.23	3.30	NS	**	NS	NS	0.009

CTR: control diet without liquid feeds; EXP 1: experimental diet 1 containing liquid feed 1; EXP 2: experimental diet 2 containing liquid feed 3; EXP 3: experimental diet 3 containing liquid feed 3; EXP 4: experimental diet 4 containing liquid feed 4. VFA: total volatile fatty acids; Ace, acetate; Prop: propionate; Iso-But: Iso-Butyrate; But: Butyrate; Iso-Val: Iso-Valerate; Val: Valerate; BCFA: branched-chain fatty acids; Ace/Prop: Acetate to Propionate ratio. *: p<0.05; **: p<0.01; *** p<0.001; NS: not significant; MSE mean square error.

In Table 8, the least square means for the in vitro fermentation end-products evaluated after 120 h of incubation, considering the five substrates incubated with both *inocula*, are reported. Most parameters were significant (at least p<0.001) for *inoculum*, substrate, and interaction effects. Concerning the comparison between *inocula*, buffalo *inoculum* resulted in higher values for Acetate, Propionate, and Acetate/Propionate ratio. Otherwise, bovine *inoculum* showed a significantly higher amount of pH value, total volatile fatty acid production, and branched-chain fatty proportion.

Table 8. Least square means for in vitro fermentation end products (% VFA) evaluated after 120 h of incubation.

	pH	**VFA**	**Ace**	**Prop**	**Iso-But**	**But**	**Iso-Val**	**Val**	**BCFA**	**A/P**
					Inoculum effect					
Buffalo	6.50	104	62.8	19.7	1.06	12.8	2.24	1.70	3.23	3.20
Bovine	6.52	105	59.9	18.5	1.65	14.4	3.30	2.31	4.87	3.18
p value	0.0007	0.0015	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.710
					Substrate effect					
p value	0.0008	<0.001	<0.001	0.0002	<0.001	<0.001	0.0014	0.0006	0.0008	0.0040
					Interaction Substrate x *Inoculum*					

P value	0.0142	<0.001	0.0001	<0.001	<0.001	<0.001	<0.001	0.0890	<0.001	<0.001
MSE	2e-4	1.00	0.34	0.09	0.008	0.21	0.08	0.027	0.09	0.014

VFA: total volatile fatty acids; Ace, acetate; Prop: propionate; Iso-But: Iso-Butyrate; But: Butyrate; Iso-Val: Iso-Valerate; Val: Valerate; BCFA: branched-chain fatty acids; Ace/Prop: Acetate to Propionate ratio. MSE: mean square error.

4. Discussion

4.1. Liquid Feed Effects on In Vitro Fermentation

In the following study, four liquid feeds were added to a total mixed ratio, formulated to satisfy lactating animals' requirements, to evaluate the effect on in vitro microbial activity, in terms of fermentation parameters and kinetics, using the microbial population collected by rumen of two donor species (bovine and buffalo). The liquid feeds, slightly different for some ingredients, include fermentable compounds (i.e., a simple sugar, acids, etc.) that could modulate rumen microbial activity, underlining the differences between the species. Similarly, the tested liquid feeds differ for crude protein content. In particular, Liquid Feed_3 showed a high level of crude protein, probably due to the presence of soluble condensed molasses, which are rich in nonprotein nitrogen compounds, such as free amino acids. Furthermore, the content of non-structural carbohydrates (as an energy source) and protein amount must be considered to better understand the fermentation results. Indeed, the four liquid feeds differ for crude protein and non-structural carbohydrate ratio (CP/NSC): 0.20, 0.18, 0.39, and 0.16 for LF_1, LF_2, LF_3, and LF_4, respectively.

Considering the ingredients, cane molasses is the only raw material present in LF_1; the other liquid feeds mainly differ for citrus molasses (LF_2), soluble condensed molasses (LF_3), and glycerol (LF_4). Cane molasses is used as an energy supplement in ruminant nutrition also to stimulate feed consumption [20] and significantly affected gas production and fermentation kinetics with both *inocula*, particularly with bovine rumen liquor, favouring a higher gas production and faster fermentation kinetics. Few differences were found in fermentation end-products. Palmonari et al. [12] reported that cane molasses contains mainly soluble sugars and, consequently, it is rapidly fermented into the rumen. The experimental diet containing citrus molasses (EXP 2) showed significant differences in terms of OM degradability and Propionate production (lower value) using bovine *inoculum* compared to the control diet. Otherwise, with EXP 2, significant differences in terms of gas production and total volatile fatty acids, especially branched chain (higher value), were observed using buffalo rumen liquor. Regarding fermentation kinetics, citrus molasses favours the fermentation with both animal species. Highly degradable citrus carbohydrates could be used as an alternative to cereal rich in starch to cover the energy requirements of ewes without risk, as an even higher ruminal pH was maintained throughout the day [21]. On the other hand, Wing et al. [22] reported depression in rumen parameters (pH, CP, and AFD digestibility, and production of acetic and propionic acids) when more than 6% citrus molasses soluble was *in vivo* added to diets for lactating cow.

When soluble condensed molasses (LF_3) was added to TMR, differences emerged in bovine and buffalo *inoculum* compared to the control diet. Considering bovine *inoculum*, slight differences appear for degradability and kinetics. However, the total VFA production was higher than the control diet, mainly due to acetic and propionic acid. While using buffalo rumen liquor, higher gas production, kinetics, and lower total and branched fatty acids have been reported. Our results contrast with Ravelo et al. [23], who observed a decrease in total VFA concentration in both cattle and buffaloes when molasses were included in the diet. However, Jian et al. [24] reported that higher content of carbohydrates could improve the activity of starch decomposition bacteria, promoting the synthesis of propionate and butyrate.

The experimental diet enriched with LF_4 resulted in significantly lower gas production, degradability, and fermentation kinetics, but higher total VFA production (mainly due to Butyrate and Iso-Valerate) and lower pH value than the other experimental diets

with bovine *inoculum.* As reported by McDonald et al. [6], low pH values inhibit microbial cellulolytic activity and reduce fibre digestibility. On the contrary, with buffalo *inoculum,* the values of maximum fermentation rate and gas production were higher and VFA lower compared to the other experimental diets. These results may be due to the presence of glycerol in the liquid feed. In this regard, Khattab [25] reported that, in vitro, the supplementation of glycerol could inhibit the growth and activity of cellulolytic bacteria in the rumen, which are responsible for gas production [26].

In vivo investigation reports that diets supplemented with sugar-based liquid feeds improve the palatability and rumen digestibility; moreover, the higher volatile fatty acid production registered is probably due to rapid *in vivo* degradation of molasses into the rumen [9]. Petri et al. [27] reported that replacing corn with sugar beet pulp molasses improved the rumen and hindgut conditions and fibre digestibility by promoting the physiological pH and bacterial diversity.

4.2. Buffalo vs. Bovine

The differences recorded in this study between bovine and buffalo reflect the differences in metabolism between these two ruminant species [28]. Mediterranean buffalo shows a higher capacity to digest protein and fibre compared with cattle and sheep [29]. Moreover, Bartocci et al. [29] highlighted that Mediterranean buffaloes have a slow passage rate of solid particles, hence the feed is retained longer in the rumen of buffalo than cattle. As consequence, buffaloes are able to degrade nutrients better than bovines.

In this regard, in our investigation, buffalo rumen liquor reported higher OM degradability than bovine *inoculum*. Moreover, when incubated with buffalo rumen liquor, all diets reported higher fermentation kinetics. Despite lower levels of volatile fatty acids compared to bovine *inoculum*, buffalo rumen liquor produced a higher amount of Acetate and Propionate. Considering that acetic acids are the main end-products of structural carbohydrate fermentation [30], the addition of liquid feeds to TMR seems to improve the carbohydrate availability for the microorganisms in the rumen. It is probable also that the different proportion between nitrogen and energy (CP/NSC ratio) in the liquid feed added to the TMR, influencing these results. It seems that the rumen fermentation in bovine is favoured by a higher CP/NSC ratio, whereas in buffalo, a lower CP/NSC ratio can also be used (0.39 and 0.18 in LF_3 and LF 2, respectively). These results agree with previous studies carried out in vitro [31], where it was observed that diets having the same energy content, but less protein can be fed to buffalo, since they seem to have lower protein requirements than cattle. As known, buffalo shows an efficient capacity in recycling blood urea throughout the rumen wall and saliva compared to bovine [32]. Furthermore, Tong et al. [33] evidenced that buffalo and cattle showed significant differences in the rumen microbiota. In particular, the authors observed that buffalo and cattle showed significant differences in Firmicutes and Bacteroidota and, consequently, the Firmicutes/Bacteroidata (F/B) ratio. Specifically, Bacteroidota species, in the dominant genus of Prevotella, are capable of degrading non-cellulose plant fibres [34]. Therefore, the higher abundances of Fibrobacter, which are responsible for cellulolytic plant fibre digestion, suggested that buffalo is better adapted to rough forage than cattle [33].

5. Conclusions

The results obtained from the in vitro trial showed that supplementing liquid feeds to a standard diet appears to improve fermentations by leading to increased kinetics and production of volatile fatty acids, a source of energy for ruminants. Moreover, no detrimental effects were observed on in vitro fermentation at the dosage utilised (0.025 g).

The differences in fermentation parameters registered in vitro between bovine and buffalo ruminal liquid showed that the buffalo can ferment roughage forage, with low protein requirements, better than the bovine. Further studies are needed to understand whether increasing the dose of liquid feeds in the diet can show additional beneficial

effects on ruminal fermentation. It will also be interesting to evaluate the effects of rumen fermentation adding the liquid feeds to different types of diets.

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