

Effect of salt concentrations on *in vitro* rumen fermentation of cellulose, starch, and protein

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Abstract

The aim of this study was to evaluate the effects of various concentrations of three salts (sodium chloride (NaCl), magnesium chloride (MgCl₂), and calcium chloride (CaCl₂)) on the *in vitro* rumen fermentation of cellulose, starch, and protein substrates. Six salt concentrations were tested, separately, namely 0, 100, 200, 400, 800, and 1600 mg/dL. The experiment was conducted using the completely randomized design in a 6 × 3 × 3 factorial arrangement with main effects of salt concentration and salt type (six levels of three salts (NaCl, MgCl₂, or CaCl₂) (0, 100, 200, 400, 800, and 1600 mg/dL) into three substrates [starch, cellulose, and glucose]) with three replicates. Cellulose- and glucose-fermenting bacteria were sensitive to NaCl concentrations greater than 400 mg/dL (17.48 decisiemens per metre (dS/m)) and 800 mg/dL (20.55 dS/m) in the media, respectively. In contrast, starch-fermenting bacteria continued to grow in NaCl concentrations up to 1600 mg/dL (29.09 dS/m). Thus, it was concluded that starch-fermenting microorganisms tolerated higher concentrations of NaCl compared with the other microbial groups. Cellulose-fermenting microorganisms are less tolerant to MgCl₂ in relation to the other microbial groups. Starch, cellulose-, and glucose-fermenting bacteria from cattle tolerate CaCl₂ concentrations of up to 1600 mg/dL (12.26 dS/m). These results suggest that brackish water may be used for ruminants. However, it is important perform an analysis of that water and then to adjust diets to minimize the effects of types of salt and concentrations of salt on rumen microorganisms.

Keywords: brackish water, dissolved salts, rumen microbes, water quality

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Introduction

Water is becoming increasingly scarce in the world as a result of the population growth and of its improper management and use (El-Keblawy & Al-Shamsi, 2008). It is estimated that less than half of the world population has access to adequate drinking water. Water is a necessary nutritional component in all stages of animal production. Minimizing its importance, in addition to generating health problems, compromises animal production (Araujo *et al.*, 2010).

The supply of brackish water to ruminants can pose health risks, depending on the concentration and composition of the dissolved salts. Animals can tolerate different salinity contents according to their species, age, water intake level, and physiological conditions (NRC, 2007; Alves *et al.*, 2017). Additionally, the semi-arid regions of the planet commonly present a large seasonal variation in rainfall distribution over the year, culminating in irregular availability and quality of water for animal consumption (Descheemaeker *et al.*, 2010). Besides, many coastal regions have a high content of salt (mainly NaCl) in their water resulting from the mixture of fresh and sea water, which also makes it more difficult to feed all animals in this region with plentiful quality water (Visscher *et al.*, 2013).

Limited information is available in the literature regarding the tolerance of rumen microorganisms to different levels of minerals in the water (El Shaer, 2010). This knowledge is of paramount importance for

semi-arid and coastal regions, given the varied population of ruminants in these areas and the competition among these animals for the scarce drinking water that is already used by the human population. A large portion of brackish waters have a high concentrations of Chlorine (Cl), Magnesium (Mg), Calcium (Ca), and Sodium (Na) ions, mainly in the form of NaCl, MgCl₂, and CaCl₂ salts (Manera *et al.*, 2016), and constitute important sources to these animals. Thus, the aim of the present study was to evaluate the effect of different concentrations of NaCl, MgCl₂, and CaCl₂ on the *in vitro* rumen fermentation of cellulose, starch, and protein.

Materials and Methods

This study was undertaken in accordance with the technical norms of biosafety and ethics, on approval by the Ethics Committee in the Use of Animals of the Centre for Biotechnology (CBiotec) of the Federal University of Paraíba (UFPB) (document no. 022/2012). *In vitro* trials were carried out at the Laboratory of Forage Crops at the Department of Animal Science of UFPB, Areia - PB, Brazil, from October 2013 to August 2014. The experiment involved a 3-year-old rumen-cannulated bullock that was housed at the Unit for Research with Large Ruminants at the Department of Animal Science of UFPB. It was fed with a mixed ration composed of 80% fresh grass and 20% concentrate (ground corn and soybean meal).

Rumen fluid was collected manually two hours before feeding to obtain inoculant containing active microbial population, which was transported to the laboratory. At the laboratory, the fluid was saturated with carbon dioxide, and left to rest at 39 °C. After the formation of liquid interfaces, the intermediate fluid was collected and centrifuged at 500 × g for 10 min, while the supernatant was discarded to obtain an inoculant that contained active microbial populations (Oliveira *et al.*, 2012). The residue from centrifugation (pellet), which contained predominant bacteria in the rumen fluid, was re-suspended in McDougall's buffer (9.80 g NaHCO₃; 4.65 g Na₂HPO₄·2H₂O; 0.57 g KCl; 0.12 g MgSO₄·7H₂O; and 0.04 g CaCl₂, diluted in distilled water up to the volume of 1000 mL).

The effects of NaCl, MgCl₂, and CaCl₂ in various concentrations in the culture media were evaluated in isolation. Six concentrations of the three salts were tested, namely 0, 100, 200, 400, 800, and 1600 mg/dL. Each treatment was replicated three times. This experiment was conducted as a completely randomized design in a 6 × 3 × 3 factorial arrangement consisting of six concentrations of NaCl, MgCl₂, and CaCl₂ (0, 100, 200, 400, 800, and 1600 mg/dL) in three substrates (starch, cellulose, and glucose), with three replicates.

Each incubation bottle was filled with 20 mL of growth medium (16 mL McDougall's buffer and 4 mL inoculant), 150 mg substrate, and the concentrations of the salt. Starch, cellulose, and glucose were used as substrates for the rumen microorganisms. Treatments were composed as shown in Table 1.

Table 1 Distribution of treatments according to substrate, salt type and the salt concentration

Substrate		Salt concentration				
		NaCl				
Starch	0	100	200	400	800	1600
Cellulose	0	100	200	400	800	1600
Glucose	0	100	200	400	800	1600
		MgCl ₂				
Starch	0	100	200	400	800	1600
Cellulose	0	100	200	400	800	1600
Glucose	0	100	200	400	800	1600
		CaCl ₂				
Starch	0	100	200	400	800	1600
Cellulose	0	100	200	400	800	1600
Glucose	0	100	200	400	800	1600

NaCl: sodium chloride, MgCl₂: Magnesium chloride, CaCl₂: Calcium chloride

All bottles were saturated with carbon dioxide, closed with special lids, and sealed with adhesive tape to prevent contact with the external medium. Next, they were incubated at 39 °C in a water bath under

agitation (35 rpm) for 48 hours. During this period, the incubation times of 0, 6, 12, 24, and 48 hours were evaluated by collecting the fluid with syringes to prevent the entry of oxygen. All gas produced was removed with a needle, which remained for 10 seconds at each of the incubation times. The pH was measured with a digital potentiometer at incubation times of 0 and 48 hours. At zero time, the medium was saturated with carbon dioxide to maintain the pH between 6.00 and 7.00, in all treatments. Each time a 2.0 mL sample was collected from the culture medium of each experimental unit and placed in Eppendorf® tubes, which were then centrifuged in a microcentrifuge at $1200 \times g$ for 10 min. The supernatant was frozen for analyses of ammonia nitrogen ($\text{NH}_3\text{-N}$) and volatile fatty acids (VFA). The pellet that was formed, which contained the predominating microorganisms in the rumen fluid, was re-suspended in 0.9% saline solution, homogenized, and re-centrifuged at $1200 \times g$ for 10 min. The supernatant was discarded. The pellet that was formed during the last centrifugation was re-suspended in 0.9% saline solution and frozen at -10°C for later analysis of microbial protein, which was performed according to the methodology proposed by Makkar *et al.* (1982).

For the analysis of VFA, 500 μL of supernatant was collected, to which 500 μL 25% metaphosphoric acid was added and placed in Eppendorf® tubes. The material was analysed in a high-performance liquid chromatograph (HPLC) coupled to an ultraviolet (UV) detector, at a wavelength of 210 nm. Ammonia nitrogen analyses were performed by the colorimetric method, while protein was quantified by the method of Bradford (1976). Electric conductivity (dS/m) was measured at zero time and at 48 hours of incubation, using a conductivity meter.

The experimental design was completely randomized, with six concentrations of salt, three types of salt and three substrates, according to the statistical model below:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_k + (\alpha\beta_{ij}) + (\alpha\delta_{ik}) + (\beta\delta_{jk}) + (\alpha\beta\delta_{ijk}) + B_z(\beta_j) + \varepsilon_{ijkl},$$

Where: Y_{ijkl} = the l^{th} observation of a dependent variable;

μ = overall mean;

α_i = fixed effect of i^{th} concentrations of salt;

β_j = fixed effect of j^{th} substrate;

δ_k fixed effect of k^{th} salt type;

$\alpha\beta_{ij}$ = interaction between levels of salt and substrate;

$\alpha\delta_{ik}$ = interaction between levels of salt and salt type;

$\alpha\beta\delta_{ijk}$ = interaction between levels of salt, substrate and salt type;

$B_z(\beta_j)$ = effect of z^{th} blocks within the substrate; and

ε_{ijkl} = error associated with observation Y_{ijkl} .

All statistical analyses were performed in SAS® System for Windows™ 9.4 (SAS Institute Inc., Cary, NC, USA, 2015). Null hypotheses were rejected at the significance level 0.05. Data were subjected to univariate analysis of variance via the GLM (general linear models) procedure and to regression analysis.

Results

Sodium chloride, MgCl_2 and CaCl_2 levels had an increasing linear effect ($P < 0.05$) on the conductivity of the medium (Table 2). The substrates had no differences in conductivity when compared with the same NaCl concentrations. However, when compared within the same MgCl_2 and CaCl_2 concentrations, cellulose was the substrate with the highest conductivity values.

There was a quadratic ($P < 0.05$) effect of NaCl content on microbial protein concentration after 48 hours of incubation in the medium that contained glucose (Table 3). According to the regression equation, maximum microbial growth was estimated at NaCl concentration of 880 mg/dL when the substrate was glucose, with microbial protein production of 1899.04 mg/dL.

The media that contained cellulose did not differ ($P < 0.05$) from that containing starch up to NaCl concentrations of 400 mg/dL in terms of microbial protein concentration (Table 3). Cellulolytic microorganisms were sensitive to NaCl, reducing their microbial production linearly ($P < 0.05$) as the concentration of this salt in the media was elevated.

The medium that contained starch had the highest ($P < 0.05$) concentration of microbial protein when compared with cellulose and glucose in the presence of MgCl_2 (Table 3). Magnesium chloride had a quadratic effect ($P < 0.05$) on the growth of cellulolytic bacteria. The regression equation indicated greatest microbial growth at MgCl_2 concentration of 751 mg/dL (Table 2).

Media that contained starch and glucose did not differ ($P < 0.05$) in microbial protein values at the CaCl_2 levels (Table 3). However, lower concentrations of microbial protein were produced when the media included the substrate cellulose, indicating that the microorganisms that use it as a source of energy are sensitive to high levels of calcium chloride (Table 3). There was a quadratic effect on the concentration of

microbial protein in the medium that contained cellulose, with the lowest growth of these microorganisms estimated at the CaCl_2 concentration of 1.175 mg/dL (Table 3). Calcium chloride salt concentrations caused a decreasing linear effect on microbial protein production in glucose and starch substrates, so it was inferred that increased CaCl_2 salt concentrations possibly affected microbial activity (Table 3).

Table 2 Mean values of conductivity (dS/m) of growth medium *in vitro* at different concentrations of sodium chloride magnesium chloride and calcium chloride

Substrate	Concentrations of salt (mg/dL)						P-value		
	0	100	200	400	800	1600	SE	L	Q
NaCl									
Starch	10.23 ^a	11.31 ^a	12.38 ^a	14.57 ^a	21.03 ^a	33.84 ^a	1.45	<0.0001	0.1621
Cellulose	9.89 ^a	10.62 ^a	11.45 ^a	13.98 ^a	20.24 ^a	33.20 ^a		<0.0001	0.1003
Glucose	10.39 ^a	11.38 ^a	11.94 ^a	14.10 ^a	16.71 ^b	32.59 ^a		<0.0001	<0.0001
MgCl ₂									
Starch	11.70 ^b	12.04 ^b	12.51 ^b	13.25 ^b	15.26 ^b	18.49 ^b	0.14	<0.0001	0.4061
Cellulose	13.51 ^a	13.88 ^a	14.11 ^a	14.92 ^a	16.46 ^a	22.61 ^a		<0.0001	<0.0001
Glucose	11.44 ^b	11.85 ^b	12.24 ^b	13.05 ^b	14.61 ^c	18.33 ^b		<0.0001	0.0102
CaCl ₂									
Starch	10.86 ^c	11.43 ^b	11.70 ^b	12.50 ^b	15.54 ^b	28.49 ^b	0.34	<0.0001	<0.0001
Cellulose	13.69 ^a	14.12 ^a	14.43 ^a	14.98 ^a	17.55 ^a	31.01 ^a		<0.0001	<0.0001
Glucose	11.62 ^b	12.01 ^b	11.97 ^b	12.53 ^b	16.03 ^b	28.29 ^b		<0.0001	<0.0001

Within salt, column means with different superscripts differ significantly at $P < 0.05$

NaCl: sodium chloride, MgCl₂: Magnesium chloride, CaCl₂: Calcium chloride

Table 3 Mean values of microbial protein (mg/dl), after 48 hours of *in vitro* incubation at different concentrations of sodium chloride, magnesium chloride and calcium chloride

Substrate	Concentrations of salt (mg/dL)						SE	<i>P</i> -value	
	0	100	200	400	800	1600		L	Q
NaCl									
Starch	792.1 ^b	872.4 ^b	831.3 ^b	765.8 ^b	876.6 ^b	675.8 ^b	140.7	0.1930	0.3330
Cellulose	635.8 ^b	781.3 ^b	826.6 ^b	848.8 ^b	572.9 ^c	526.0 ^b		0.0139	0.4530
Glucose	1536.1 ^a	1876.1 ^a	1848.7 ^a	1683.5 ^a	1899.0 ^a	1257.6 ^a		0.0002	<0.0001
MgCl ₂									
Starch	3612.5 ^a	3407.5 ^a	3665.8 ^a	3459.2 ^a	4635.8 ^a	2005.8 ^c	184.7	0.1113	0.4298
Cellulose	2279.1 ^c	2580.8 ^b	2764.1 ^b	2844.2 ^b	3150.8 ^b	2702.5 ^b		0.6445	<0.0001
Glucose	3130.8 ^b	3640.8 ^a	4077.5 ^a	2829.2 ^b	2777.5 ^b	3237.5 ^a		<0.0001	<0.0001
CaCl ₂									
Starch	888.9 ^a	871.1 ^a	740.0 ^{ab}	757.8 ^a	798.9 ^a	894.4 ^a	237.7	0.0081	0.0004
Cellulose	317.8 ^b	502.2 ^a	425.6 ^b	534.4 ^a	281.11 ^b	421.1 ^b		0.1746	0.0039
Glucose	911.1 ^a	841.1 ^a	903.3 ^a	883.3 ^a	674.44 ^a	555.6 ^{ab}		0.0005	0.0009

Different superscript letters within the column differ significantly at $P < 0.05$

NaCl: sodium chloride, MgCl₂: Magnesium chloride, CaCl₂: Calcium chloride

There was an increasing linear effect ($P < 0.05$) of the NaCl and CaCl_2 concentrations in the medium that contained cellulose for $\text{NH}_3\text{-N}$ production (Table 4). No effect of NaCl salt concentrations was detected ($P < 0.05$) on $\text{NH}_3\text{-N}$ production in the medium that contained glucose (Table 4). Sodium chloride salt levels promoted a quadratic negative effect on $\text{NH}_3\text{-N}$ production in medium that contained starch (Table 4).

Magnesium chloride levels in the medium that contained cellulose promoted a quadratic negative effect on $\text{NH}_3\text{-N}$ production (Table 4). No effect of MgCl_2 was observed ($P < 0.05$) on ammonia nitrogen production in the medium with starch and glucose (Table 4). In the medium that contained starch, a lower $\text{NH}_3\text{-N}$ content was detected in relation to cellulose and glucose at the CaCl_2 levels of 800 and 1600 mg/dL (Table 4).

Table 4 Mean values of ammonia nitrogen content after 48 hours of *in vitro* incubation at different concentrations of sodium chloride, magnesium chloride and calcium chloride

Substrate	Concentrations of salt (mg/dL)						P-value		
	0	100	200	400	800	1600	SE	L	Q
NaCl									
Starch	9.11 ^a	5.25 ^a	7.86 ^{ab}	5.06 ^a	5.61 ^b	10.88 ^b		0.1710	0.0402
Cellulose	3.93 ^a	6.23 ^a	6.29 ^b	10.60 ^a	22.46 ^a	26.91 ^a	3.13	<.0001	0.0116
Glucose	7.58 ^a	5.95 ^a	12.83 ^a	8.58 ^a	6.26 ^b	8.26 ^b		0.7648	0.8670
MgCl_2									
Starch	4.61 ^b	4.94 ^b	5.46 ^b	2.31 ^b	4.18 ^b	5.25 ^b		0.7430	0.1387
Cellulose	13.87 ^a	14.71 ^a	9.47 ^a	7.49 ^a	10.51 ^a	16.35 ^a	1.67	0.0101	<.0001
Glucose	6.14 ^b	4.75 ^b	4.51 ^b	6.19 ^a	4.71 ^b	4.50 ^b		0.4041	0.9867
CaCl_2									
Starch	1.93 ^a	2.15 ^a	1.84 ^b	5.23 ^a	1.29 ^c	0.37 ^c		0.0527	0.1075
Cellulose	3.60 ^a	4.03 ^a	5.22 ^a	4.29 ^a	10.72 ^a	17.70 ^a	1.49	<.0001	0.2842
Glucose	2.88 ^a	4.46 ^a	2.84 ^{ab}	4.52 ^a	4.30 ^b	4.25 ^b		0.3725	0.4336

Different superscript letters within the column differ significantly at $P < 0.05$

NaCl: sodium chloride, MgCl_2 : Magnesium chloride, CaCl_2 : Calcium chloride

There was a decreasing linear effect ($P < 0.05$) of the NaCl concentration in the media that contained starch, cellulose, and glucose after 48 hours of incubation on the concentration of acetate (Table 5). There was no difference ($P < 0.05$) between the substrates in the NaCl salt concentration from 200 to 1600 mg/dL.

The NaCl concentrations had a quadratic effect ($P < 0.05$) on propionate concentration when the substrates starch and cellulose were used (Table 5). The propionate content varied ($P < 0.05$) in the medium that contained starch, with lowest concentrations estimated at 660 mg/dL NaCl. In the medium that contained cellulose, the propionate concentration decreased linearly ($P < 0.05$) with NaCl concentration. However, at the NaCl concentration of 1600 mg/dL, the medium that contained starch had higher propionate contents than the media that included cellulose and glucose (Table 5).

There was a quadratic effect ($P < 0.05$) of NaCl concentrations in the medium with starch and a decreasing linear effect in the media that contained either cellulose or glucose on butyrate concentrations after 48 hours of incubation (Table 5). In the medium that contained starch, the highest butyrate concentration was estimated at the NaCl concentration of 120 mg/dL, with production of approximately 13.74 mM (Table 5).

No effect of substrate was detected ($P < 0.05$) on the concentration of acetate in the media that contain either MgCl_2 or CaCl_2 after 48 hours of incubation (Table 6). By contrast, there was a negative quadratic effect ($P < 0.05$) of MgCl_2 levels after 48 hours of incubation, with the lowest acetate concentration being found at 130 mg/dL. Thus, no effect of the substrates was observed ($P < 0.05$) in the media with MgCl_2 and CaCl_2 on acetate propionate and butyrate contents. There was a negative quadratic effect ($P < 0.05$) for MgCl_2 , with lowest propionate concentration (16.76 mM) estimated at 200 mg MgCl_2 /dL. There was no effect ($P < 0.05$) of CaCl_2 on propionate and butyrate production which averaged 16.23 and 8.73 mM, respectively (Table 6).

Table 5 Mean values of acetate, propionate and butyrate content (mM) after 48 hours of *in vitro* incubation at different concentrations of sodium chloride

Substrate	NaCl Concentrations (mg/dL)						P-value		
	0	100	200	400	800	1600	SE	L	Q
Acetate (mM)									
Starch	34.00 ^{ab}	41.24 ^a	37.83 ^a	31.49 ^a	28.59 ^a	32.23 ^a		0.0200	0.0300
Cellulose	38.99 ^a	35.35 ^{ab}	37.74 ^a	25.96 ^a	22.31 ^a	25.21 ^a	3.90	<0.0001	0.0002
Glucose	26.77 ^b	31.71 ^b	30.91 ^a	26.52 ^a	25.66 ^a	24.43 ^a		0.0488	0.7552
Propionate (mM)									
Starch	22.78 ^{ab}	25.98 ^a	26.70 ^a	21.67 ^a	18.13 ^a	25.29 ^a		0.6689	0.0079
Cellulose	25.67 ^a	22.43 ^a	26.02 ^{ab}	16.58 ^a	12.30 ^a	15.04 ^b	3.10	<0.0001	0.0003
Glucose	18.31 ^b	20.68 ^a	20.25 ^b	16.83 ^a	16.64 ^a	14.91 ^b		0.0265	0.7176
Butyrate (mM)									
Starch	11.60 ^{ab}	13.74 ^a	13.53 ^a	11.22 ^a	9.55 ^a	13.41 ^a		0.9536	0.0090
Cellulose	13.57 ^a	10.89 ^a	13.83 ^a	8.83 ^a	6.38 ^a	7.95 ^b	1.64	<0.0001	0.0004
Glucose	9.41 ^b	11.31 ^a	11.00 ^a	8.84 ^a	8.72 ^a	8.00 ^b		0.0263	0.6687

Different superscript letters within the column differ significantly at $P < 0.05$

NaCl: sodium chloride

Table 6 Mean values of acetate, propionate, and butyrate content (mM), after 48 hours of *in vitro* incubation at different concentrations of salt

Salt	Concentrations of salt (mg/dl)						P-value		
	0	100	200	400	800	1600	SE	L	Q
Acetate (mM)									
MgCl ₂	26.06	23.65	25.34	22.98	22.42	24.63	2.24	0.3344	0.0018
CaCl ₂	24.56	24.22	24.07	24.35	25.37	19.81	5.10	0.0521	0.1527
Propionate (mM)									
MgCl ₂	17.71	15.64	16.76	14.86	14.75	16.65	2.04	0.5953	0.0025
CaCl ₂	17.26	15.84	15.47	17.29	16.72	14.98	3.32	0.3326	0.4579
Butyrate (mM)									
CaCl ₂	8.82	8.21	9.01	9.21	9.05	8.12	1.70	0.4780	0.2187

MgCl₂: Magnesium chloride, CaCl₂: Calcium chloride

The MgCl₂ concentrations in the medium that contained cellulose had no effects ($P < 0.05$) after 48 hours of incubation on the butyrate concentration (Table 7). However, MgCl₂ in the media that contained either starch or glucose had a quadratic effect ($P < 0.05$) on butyrate production, with the lowest values estimated at 240 and 180 mg/dL, respectively. This quadratic effect may be associated with the change in microbial population at MgCl₂ concentrations higher than 240 and 180 mg/dL in the media with starch and glucose, respectively.

Table 7 Mean values of butyrate content (mM), after 48 hours of *in vitro* incubation at different concentrations of magnesium chloride

Substrate	MgCl ₂ Concentrations (mg/dL)						P-value		
	0	100	200	400	800	1600	SE	L	Q
Starch	9.37	8.07	7.62	6.86	8.01	8.78		0.6955	0.0470
Cellulose	8.24	8.50	9.15	9.55	7.85	8.46	1.17	0.6475	0.8541
Glucose	10.43	7.92	8.95	7.51	7.67	8.94		0.5563	0.0093

MgCl₂: Magnesium chloride

Discussion

For animals, as excess total dissolved solids in water (TDS) are increased, the water quality declines, leading to decreased intake and poor performance (Umar *et al.*, 2014). According to these authors, water with salinity levels that are greater than 700 mg/dL poses a great risk to young, pregnant, and lactating animals. Saline water can thus negatively affect rumen microbial growth. The presence of foreign solutes reduces the ability of the water to dissolve additional solutes, which may influence the osmolarity of the cell plasma. Solutes exert an inadequate attraction on the water through the membranes, making the water movement an inhibitor of microbial activity (Al-Khalasi *et al.*, 2010). However, various strains of ruminal microorganisms can tolerate amounts greater than 700 mg/dL of NaCl or MgCl₂, or CaCl₂ (Table 3). The presence of NaCl in the media up to 880 mg/dL and CaCl₂ up to 1175 mg/dL did not interfere with the growth of cellulolytic microorganisms. The microorganisms grown on media that contained starch did not have their growth affected by salt concentrations up to 1600 mg/dL, demonstrating that amylolytic microorganisms are more tolerant of high salinity levels.

The substrate that provided the greatest microbial growth at all concentrations of NaCl, MgCl₂, and CaCl₂ was glucose (Table 3). Glucose is the monosaccharide that composes fibrous (cellulose and hemicellulose) and non-fibrous (starch, amylopectin, and others) carbohydrates. Glucose is freed from plant polymers (fibrous carbohydrates) and starch through enzymatic systems associated with membranes from bacteria that hydrolyse β bonds (endocellulase and exocellulases) and α bonds (amylases and endoglycosidases), releasing glucose in the media (Oliveira *et al.*, 2008). It can then be used as an energy source by fibrous and non-fibrous carbohydrate-fermenting microorganisms, possibly stimulating growth in the culture media of both fermenter groups. Thus, in the media that contained glucose, the presence of salts that inhibited only one group of microorganisms favoured the growth of another group that was not sensitive (Tables 3 and 4). Only when the salt inhibited the growth of the two groups (amylolytic or cellulolytic) or drastically inhibited a group was a reduction observed in the concentration of microbial protein in the media. This could be observed when there were high NaCl concentrations in the media (Table 3).

Valtorta *et al.* (2008) did not find any effect of water salinity on the rumen parameters (microorganism count, pH, and VFA) of dairy cows. However, the microbial growth of cellulolytic bacteria declined as the presence of TDS was increased from 100 to 1000 mg/dL. Thomas *et al.* (2007) also observed a decrease in bacteria when the rumen NaCl concentration was increased, demonstrating compromised rumen function. Alves *et al.* (2017) observed a reduction of neutral detergent fibre digestibility when cattle consumed saline water with a high NaCl content, which the current results corroborate, with a linear decreasing effect for the acetate contents as there were increases in the concentration of NaCl (Table 5).

According to Oliveira *et al.* (2008), structural carbohydrate-fermenting bacteria that degrade cellulose and hemicellulose from the plant cell wall have a relatively slower growth rate and depend on ammonia and branched-chain fatty acids (isovalerate, isobutyrate, and 2-methylbutyrate) for protein synthesis. The growth of amylolytic bacteria is faster when compared with that of cellulolytic bacteria, which provide a higher concentration of microorganisms and consequently greater microbial protein production in the media that contained starch and glucose. This low growth speed of cellulolytic microorganisms is described in Table 2, which shows that the microorganisms had a higher microbial protein concentration with the substrates of glucose (1899.04 mg/dL with NaCl and 903.3 mg/dL with CaCl₂) and starch (4635.8 mg/dL with MgCl₂ and 894.4 mg/dL with CaCl₂) than with cellulose (572.9 mg/dL with NaCl, 3150.8 mg/dL with MgCl₂, and 421.19 mg/dL with CaCl₂).

Valtorta *et al.* (2008) also observed a gradual increase in ammonia concentration in the rumen environment as they elevated the water salinity level to 100, 500, and 1000 mg/dL TDS, in dairy cows. When the NaCl levels were increased, there was a linear decrease in microbial protein content and an increase in

the concentration of ammonia in the medium that contained cellulose (Tables 3 and 4). Magnesium chloride and CaCl_2 did not interfere with the cellulose digestion. Ammonia is essential for the growth of structural carbohydrate-degrading bacteria (Figueiras *et al.*, 2010). Therefore, ammonia accumulation in the media may be a consequence of inhibition of cellulolytic microorganisms in the media by NaCl.

Microorganisms that ferment cellulose have the acetate pathway as the main NADH re-oxidation route (Oliveira *et al.* 2008). In this way, the lower concentration of this VFA in the media may be associated with lower microbial growth and increasing concentration of NaCl. In this study, when the CaCl_2 levels in the media were increased, acetate production decreased. The microorganisms that use the acetate pathway for re-oxidation of NADH_2 are probably sensitive to the CaCl_2 levels in the media or change their NADH_2 re-oxidation route.

When the media is not conducive to the re-oxidation of NADH via the acetate pathway, some bacterial species use the propionate pathway for this purpose, because the metabolism of microorganisms can be changed without modifying their species (Nussio *et al.*, 2011). The lack of effect of CaCl_2 on propionate production demonstrates that microorganisms that used the acetate pathway did not change the NADH re-oxidation route to propionate, indicating that the inhibition of acetate production was linked to a reduction of bacterial species that use the acetate pathway for the re-oxidation of NAD^+ (Table 7). This can be verified by the reduced concentration of microbial protein in the media with cellulose (Table 3).

Acetyl CoA is a precursor of both acetate and butyrate (Valadares & Pina, 2011). Many bacteria utilize the butyrate pathway for the re-oxidation of NADH. However, some bacterial species present this route only for this purpose, irrespective of the hydrogen pressure in the media (Oliveira *et al.* 2008). In this way, the butyrate-producing bacteria are sensitive to the NaCl in the media. Potassium is the main intracellular cation; sodium is the main extracellular cation; and chloride is the main extracellular anion. The relative concentrations of these three elements create an electrochemical gradient through the cell membranes that is essential for nutrient transport and generation of energy, and they contribute indirectly to the maintenance of pH balance. Imbalances among these elements result in a number of disorders that may culminate in death of microbial cells (Umar *et al.*, 2014).

In the media that contain starch as substrate, a lower effect of NaCl salt was observed on microorganisms, even when there were elevated concentrations of this salt in the media, demonstrating that amylolytic microorganisms are less sensitive to NaCl. On the other hand, in the medium that contained cellulose, there was a decreasing linear effect of this salt in the media, likely due to the inhibition of cellulolytic microorganisms. This response is observed by the lower concentrations of microbial protein, acetate and propionate in the media and higher ammonia production when NaCl was increased. Cellulolytic microorganisms are likely sensitive to NaCl in the medium.

In the medium that contained glucose, there was a decline in microbial growth and VFA production at the NaCl concentration of 1600 mg/dL and an increase in the levels of ammonia in the medium. Rumen microorganisms seem to be sensitive to NaCl concentrations greater than 1600 mg/dL or 29.09 dS/m in the medium. Therefore, when cattle consume water with high levels of NaCl, microbial fermentation in the rumen may be affected, especially when the diet has larger proportions of fibrous carbohydrates.

In the medium that had starch as substrate, a lower effect of CaCl_2 was observed on microorganisms, even when high concentrations of this salt were present, suggesting that amylolytic microorganisms are less sensitive to CaCl_2 . By contrast, in the medium with cellulose and glucose, salt levels greater than 1600 mg/dL inhibited microorganism growth. This response is verified by the lower concentrations of microbial protein and volatile fatty acids and greater ammonia concentration when 1600 mg CaCl_2 /dL were present in the medium. Cellulolytic and glycolytic microorganisms seem to be sensitive to CaCl_2 concentrations greater than 1600 mg/dL or 12.26 dS/m in the medium.

In the media where starch and glucose were used as substrate, a lower effect of the MgCl_2 salt was observed on the present microorganisms, even when there were elevated concentrations of this salt in the medium, indicating that amylolytic microorganisms are less sensitive to MgCl_2 . In the medium that contained cellulose, however, beyond the salt level of 400 mg/dL, microorganism growth was inhibited. This response is verified by the lower concentrations of microbial protein and volatile fatty acids and larger ammonia production when the medium contained 400 mg MgCl_2 /dL.

Conclusions

Starch-fermenting microorganisms tolerate higher concentrations of NaCl when compared with the other microbial groups. However, cellulose-fermenting microorganisms are less tolerant to MgCl_2 . In the present work, starch-, cellulose-, and glucose-fermenting bacteria that originated from cattle tolerated CaCl_2 concentrations of up to 1600 mg/dL (12.26 dS/m). The results obtained in this study indicate that brackish water may be used to ruminants. However, it is important to perform an analysis of that water and, then, according to this analysis, to adjust diets to minimize the effects of these salts on rumen microorganisms.

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Authors' Contributions

The idea for the study was conceived by EMS, who secured the funding and supervised GGLA, JSO, EMS and AMZ. ECBC was recruited as the student to work on the project and received her MSc cum laude. ECBC, LTH and GAP performed the experiment. AFP and RMAP were responsible for statistical analysis and structuring the manuscript formatting. EMS was the supervisor at the university and gave guidance on the study. All co-authors participated in management and discussion of the results, statistical analysis and writing, and corrected the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Declaration

The authors declare there are no conflicts of interest.

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