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Effects of organic and inorganic nitrogen sources on in vitro degradability of citrus byproduct and milk thistle

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Abstract: In order to evaluate rumen microbial activity, the effects of two nitrogen sources (organic and inorganic) were analyzed in vitro by inoculating dairy cow rumen fluid and using citrus byproduct (CBP) and milk thistle (*Silybum marianum*, MT) as energy source substrates. The experimental design was 2 × 3 where we used three different nitrogen sources (N1: no nitrogen added, N2: ammonium bicarbonate, N3: sodium glutamate) with a basic concentration of nitrogen (100 mg/1 L). Culture pH, dry matter degradability %, ammonia-N (NH_3-N), total volatile fatty acids (TVFAs), individual volatile fatty acid proportions, and acetate propionate ratio (A:P) were measured after 24 h of fermentation. Culture pH values were in the optimal range with CBP and MT in all media. Supplementing nitrogen sources had similar tendencies to increase dry matter degradability ($P < 0.01$) and NH_3-N production in N2 and N3 with MT and CBP. Glutamate significantly ($P < 0.01$) increased TVFAs, which were twice as high than in N1 and N2 with MT. These results show that N supplementation had minor effects on in vitro rumen microbiota activity compared to N1; however, it could be proposed to improve microbial biomass production in mixed rations of agricultural byproducts with local forages.

Key words: Ammonia-N, citrus byproducts, milk thistle, nitrogen sources, volatile fatty acids

1. Introduction

Many countries in North Africa are trying to find a balanced diet in the ruminant nutrition field by considering animal needs. The lack of good quality forage resources is a major constraint to the development of livestock production. The use of agroindustrial byproducts or even herbaceous plants as complementary feedstuffs could be a sustainable solution. Algeria has many semiarid and arid lands, which affects and limits the livestock food. Moreover, there are some herbaceous plants like milk thistle (MT, *Silybum marianum* L.) Gaertn., which belongs to the family Asteraceae and has nutritional properties worthy of attention (1). It is distributed in the high steppe plateau, south of the Saharan Atlas, and in sandy pastures and low wet areas in Algeria (2). It is naturalized in the western United States, South Africa, and Australia (3), while in Italy it is mostly found in the south of the Tuscan region and Liguria, and sporadically in the Po valley (4).

Citrus byproduct (CBP) contains a high amount of energy, mainly as pectin, which is rapidly degradable (5), and it has been introduced in ruminant feed to replace starch and corn. It is also known for its easily digestible neutral detergent fiber (NDF) fraction (6). One of the benefits of this byproduct as reported (7) is that there is no risk of acidosis, as acetic acid is the main end product from pectin fermentation rather than lactic acid (8).

In order to evaluate feedstuff degradability for ruminants, in vitro incubation is a widely recommended method, being cheaper and faster than in vivo methods (9). One major aspect in these techniques is the choice of the medium that plays the role of artificial saliva to provide the right environment for ruminal microorganism growth and metabolism. Ammonia is the most important nitrogen source used by rumen bacteria (10). Glutamine and glutamate are the most abundant amino acids in milk and play important roles in neonatal development and growth. It was reported that the minimum nitrogen concentration in the medium is 80 mg N/L according to Dryhurst and Wood (11). Starting from this, our experimental approach focused on the in vitro supplementation of ammonium bicarbonate and Na-glutamate as nitrogen sources for rumen microbiota to assimilate NH_3-N when fed with two

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substrates differing in their energy source contents: CBP and MT. The aim was to evaluate the effects of nitrogen sources on in vitro ruminal degradation of CBP and MT as energy sources.

2. Materials and methods

2.1. Plant material, inoculum, and nitrogen sources

The first substrate used was agricultural CBP (from orange juice production), mainly as an energy source. The second material tested was MT (*Silybum marianum*), a fibrous Mediterranean plant also used as energy source. Samples were ground to pass through a 1-mm screen (standard model 4; Arthur H. Thomas Co., Swedesboro, NJ, USA). The rumen fluid was taken from two Holstein cows surgically fitted with rumen cannulae in the morning of the incubation day. The pH was immediately measured and then the rumen fluid was put into prewarmed flasks and transported to the ruminant nutrition laboratory. Three different media were set up, containing two nitrogen sources: medium N2 (NH_4HCO_3, ammonium bicarbonate, a mineral nitrogen source), medium N3 (Na-glutamate, an organic nitrogen source), and medium N1 (blank control with no nitrogen source).

2.2. Mixed rumen fermentation

The ground and dried substrates were weighed (700 mg of each) in ANKOM bags previously washed with acetone and dried and then sealed with a heat sealer and put into 100-mL rubber-capped bottles. The substrates were incubated for 24 h with 60 mL of incubation medium N1 consisting of 2.4 mL of rumen fluid, a reducing solution ($Na_2S \times 10$ H_2O NaOH), resazurin, and sodium bicarbonate buffer (57.6 mL); moreover, medium N2 contained hydrogen carbonate ammonium (NH_4HCO_3) and medium N3 contained Na-glutamate as nitrogen sources in addition to what was mentioned for N1 as content. On the day of inoculation the reducing solution was prepared and 8 mL of 1 N NaOH was added to every medium. The two rumen fluid sources were mixed, filtered through 2 layers of cheese cloth, and added to each reduced medium under continuous flow of CO_2. Then the mixture (60 mL) was transferred to bottles containing the substrates, which were placed in an oven at 39 °C for 24 h.

2.3. Chemical analysis

Following the AOAC methods (12), all feeds were analyzed in triplicate for dry matter (DM), organic matter (OM), ether extract (EE), and ash. Crude protein (CP) was determined by a macro-Kjeldahl method analyzer (Kjel-Foss Automatic, Model 16210, Foss Food Technology Corp., Hilleroed, Denmark). NDF content of feeds was determined according to Mertens (13). The amylase-treated NDF (aNDF) was measured by means of an ANKOM220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). Acid detergent fiber (ADF), inclusive of residual ash, was analyzed after NDF and finally acid detergent lignin (ADL) was measured by the method described by Robertson (14). Nonfiber carbohydrates (NFCs) were computed as 100 – CP – NDF – EE – crude ash. After incubation, ANKOM bags were dried in an oven at 55 °C for 24 h and the weight loss was calculated. NH_3-N ammonia was measured using the phenol/hypochlorite method of Weatherburn (15) utilizing a Dynatech MRX Microplate reader. To each sample, 1 mL of 1% H_2SO_4 was added, and then samples were stored at –40 °C. After thawing the samples at room temperature, they were diluted at the 1:6 ratio that is typical for rumen fluid. Volatile fatty acids (VFAs) were quantified, which included acetic, propionic, isobutyric, butyric, iso-valeric, valeric, and caproic acids, using a gas chromatograph (Model 5890, Hewlett-Packard, Palo Alto, CA, USA) with a capillary column (30 m × 0.32 mm i.d., 1 µm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA) and flame-ionization detection. The oven temperature was 170 °C, held for 4 min, which was then increased by 5 °C/min to 185 °C and then by 3 °C/min to 220 °C and held at this temperature for 1 min. The injector temperature was 225 °C, the detector temperature was 225 °C, and the carrier gas was helium (16). To all samples, 1 mL of 25% m-HPO_3 was added, and they were subsequently stored at –40 °C.

2.4. Statistical analysis

The chemical composition of substrates was indicated by standard deviation. One-way ANOVA was used to compare the means and the classification of fermentation parameters was done by Student–Newman–Keuls post hoc test for $P < 0.05$ using SAS 9.4 (17).

3. Results

Table 1 shows the composition of MT and CBP (% DM basis). MT was higher in NDF and ADF (68.79% compared to 15.67% and 52.37% compared to 11.72% DM, respectively), while it was lower in CP and NFC in comparison with the CBP (4.32 versus 5.75 and 13.25 versus 75.33 g/kg DM). Total sugars content was higher in CBP (48.26% DM) than in MT (8.5% DM).

The results obtained from the batch culture of CBP are shown in Table 2. The N3 medium significantly ($P < 0.01$) decreased the pH after 24 h of incubation compared to the control. DM degradability and total VFA concentrations increased significantly ($P < 0.01$) in the N3 medium compared to the control, N1. The concentration of NH_3-N was consistently higher ($P < 0.05$) for the organic nitrogen-supplemented medium than the inorganic nitrogen-supplemented one (13.6 vs 5.23 mg/100 mL). The three main VFAs, acetate, propionate, and butyrate, followed the same tendency with all media: the acetate proportion was

Table 1. Chemical composition (% DM) of the substrates (n = 2).

Item[1]	Milk thistle	Citrus byproduct
DM, %	88.1 ± 0.12	89.0 ± 0.21
OM	87.0 ± 0.02	97.4 ± 0.04
CP	4.32 ± 0.318	5.75 ± 0.153
NDF	68.8 ± 0.78	15.7 ± 0.09
ADF	52.4 ± 0.02	11.7 ± 0.13
ADL	8.56 ± 0.086	ND[3]
AIA	0.19 ± 0.015	ND
EE	0.62 ± 0.002	0.60 ± 0.007
CF	45.4 ± 0.29	10.4 ± 0.35
NFC[2]	13.25 ± 1.13	75.33 ± 0.28

[1]DM = Dry matter; NDF = neutral detergent fiber; CP = crude protein; OM = organic matter; ADF = acid detergent fiber; ADL = acid detergent lignin; AIA = acid insoluble ash; EE = ether extract; CF = crude fiber.
[2]Nonfiber carbohydrates = 100 – CP – NDF – EE – crude ash.
[3]Not determined.

Table 2. Ruminal fermentation characteristics in batch culture with citrus byproduct (24 h).

Item	Nitrogen sources[1]			SEM	P-value
	N1	N2	N3		
Culture pH	6.61^{a}	6.57^{a}	6.47^{b}	0.021	<0.01
DM degradability, %	59.8^{b}	64.5^{a}	64.4^{a}	0.83	<0.01
NH_3-N,[2] mg/100 mL	0.003^{c}	5.23^{b}	13.6^{a}	1.720	<0.01
Total VFAs, mM	49.6^{b}	40.2^{b}	66.7^{a}	4.21	0.01
Individual VFAs, mol/100 mol					
Acetate (A)	56.7^{a}	40.0^{a}	48.0^{ab}	2.60	0.01
Propionate (P)	27.4^{b}	35.0^{a}	28.1^{b}	1.34	0.02
Butyrate	11.5^{b}	17.9^{a}	16.5^{a}	1.01	<0.01
Valerate	2.72^{b}	4.73^{a}	3.42^{b}	0.316	0.01
Isobutyrate (IB)	0.61^{c}	0.84^{b}	1.16^{a}	0.079	<0.01
Isovalerate (IV)	0.96^{b}	1.26^{b}	2.29^{a}	0.182	<0.01
IB + IV	1.57^{b}	2.10^{b}	3.45^{a}	0.258	<0.01
A:P	2.07^{a}	1.14^{b}	1.81^{a}	0.161	0.03

[a–c]Means within a row with different superscripts differ significantly (P < 0.05).
[1]N1 = No nitrogen added (control); N2 = ammonium bicarbonate; N3 = Na-glutamate.
[2]NH_3-N = ammonia-N.

always higher than that of propionate, followed by butyrate. Interestingly, the propionate proportion was significantly ($P < 0.02$) higher in the N2 medium compared to N1 and N3.

Table 3 provides the results obtained from the ruminal fermentation experiment in vitro with MT after 24 h. The present study found that TVFA, DM degradability, and propionate followed the same trend with both N-supplemented media; they significantly ($P < 0.01$) increased compared to the control, N1. The ammonia nitrogen produced showed statistically different results ($P < 0.01$). Another important finding was that the acetate propionate ratio decreased significantly ($P < 0.01$) when supplementing nitrogen sources.

As Table 4 shows, the two substrates were significantly different in all metabolic parameters when the nitrogen source was ammonium bicarbonate (N2).

4. Discussion

In our study, we incubated CBP and MT as energy and nitrogen sources in supplemented media with inorganic nitrogen and organic nitrogen sources. A recent study by Peixoto et al. (18) reported higher values for CP (6.0), NDF (18.3), and ADF (13.7% DM) in citrus pulp, whereas the OM content of our CBP was higher than that reported by Miron et al. (6), 91.2% DM. In earlier research both the NRC (19) and Miron et al. (6) found different contents of CP (6.9%, 6.70%), NDF (24%, 21.6%), and ADF (22.2%, 21.2%) in % DM basis. CBP contains 25.0% DM in pectin content, which is higher than reported by Bampidis and Robinson (7) at 22.3% and Miron et al. (6) at 20.7% DM. Taglipietra et al. (20) reported that the nitrate-N content in MT was at a safe level as a forage (305 mg/kg DM), while CBP had no nitrate-N. The different compositions of CBP in previous reports could be explained by the environmental and climatic conditions of the citrus harvest. On the other hand, MT and CBP are two distinct substrates; while CBP is rich in soluble sugars and pectin, MT is a poor quality forage rich in fiber.

In Tables 2 and 3, pH values are in the optimal range of 6 to 7 according to Thivend et al. (21). As can be seen from Table 2, supplementation of an organic nitrogen source led to a significant ($P < 0.01$) increase in DM degradability and TVFAs while pH decreased significantly ($P < 0.01$) with N3 compared to N1, which may be explained by the improved rumen bacterial activity (cellulolytic and proteolytic ammonia-producing bacteria and VFAs) in the presence of ammonia (besides VFAs and peptides as growth factors).

Table 3. Ruminal fermentation characteristics in batch culture with milk thistle (24 h).

Item	Nitrogen sources[1]			SEM	P-value
	N1	N2	N3		
Culture pH	7.03[a]	6.89[b]	6.82[c]	0.027	<0.01
DM degradability, %	24.7[c]	30.6[b]	34.9[a]	1.35	<0.01
NH_3-N,[2] mg/100 mL	3.07[c]	9.88[b]	18.6[a]	1.963	<0.01
Total VFAs, mM	29.8[b]	32.7[b]	66.9[a]	5.12	<0.01
Individual VFAs, mol/100 mol					
Acetate (A)	71.1[a]	68.2[b]	63.7[c]	0.97	<0.01
Propionate (P)	13.0[b]	17.6[a]	17.9[a]	0.72	<0.01
Butyrate	8.29	8.95	12.2	0.53	0.32
Valerate	2.04	1.75	1.70	0.094	0.32
Isobutyrate (IB)	1.80[a]	1.07[c]	1.40[b]	0.103	<0.01
Isovalerate (IV)	3.05[a]	2.07[b]	2.76[a]	0.148	<0.01
IB + IV	4.84[a]	3.14[b]	4.16[a]	0.245	<0.01
A:P	5.51[a]	3.88[b]	3.55[b]	0.271	<0.01

[a–c]Means within a row with different superscripts differ significantly ($P < 0.05$).
[1]N1 = No nitrogen added (control); N2 = ammonium bicarbonate; N3 = Na-glutamate.
[2]NH_3-N = ammonia-N.

Table 4. Comparison of metabolic characteristics between milk thistle and citrus byproduct.

Item	Nitrogen sources[1]		
	N1	N2	N3
	P-value	P-value	P-value
Culture pH	<0.0001	<0.0001	<0.0001
DM degradability, %	<0.0001	<0.0001	<0.0001
NH_3-N,[2] mg/100 mL	<0.0001	<0.0001	0.02
Total VFA, mM	<0.0001	0.0136	0.98
Individual VFA, mol/100 mol			
Acetate (A)	0.0001	<0.0001	0.02
Propionate (P)	<0.0001	<0.0001	0.01
Butyrate	0.02	<0.0001	0.04
Valerate	0.27	<0.0001	0.004
Isobutyrate (IB)	0.0005	<0.0001	0.10
Isovalerate (IV)	0.0002	<0.0001	0.04
IB + IV	0.0002	<0.0001	0.06
A:P	<0.0001	<0.0001	0.003

[1]N1 = No nitrogen added (control); N2 = ammonium bicarbonate; N3 = Na-glutamate.
[2]NH_3-N = ammonia-N.

The tendency of increased NH_3-N was presumably linked to the degradation of proteins by proteolytic enzymes and the deamination of amino acids into extracellular NH_3 plus VFAs and gas production (22). Blümmel and Lebzien (23) reported that supplementing N in the fermentation medium increased SCFA production with all diets tested (even or equal parts of roughage and concentrates). As mentioned earlier, supplementation with ammonia enhances the activity of cellulolytic bacteria, which after fermentation of cell wall constituents produces VFAs and gases (mainly CO_2 and CH_4) (22). Grings et al. (24) reported similar results for acetate percentage of total SCFAs, which increased in the N-rich medium (ammonium bicarbonate), and, as concluded by Blümmel et al. (25), molar production of VFAs depends on the nature of the feed. With the N2 medium, propionate production was associated with the buffering of SCFAs with ammonium bicarbonate (in addition to sodium bicarbonate) to release CO_2 (26).

Turning now to the experiment with MT in Table 3, these findings further support the idea of Maeng et al. (27), who reported that replacement of a source of nonprotein nitrogen (NPN, in our experiment replacing N2 by N3) like urea with 18 amino acids increased substrate disappearance. Moreover, they noted an increase in microbial dry matter, RNA, and DNA, which was a sign of higher fermentation rates to increase intake of low energy rations, like in this study, where MT could be used as potential feedstuff (13.25 NFC% DM) for the ruminant.

With the presence of the NPN source in the N2 medium (NH_4HCO_3), only 9.88 mg/100 mL of NH_3-N was released with little VFA production at 32.7 mM compared to the control, N1, at 29.8 mM. On the other hand, double the NH_3-N was released with the N3 medium (organic nitrogen) (18.6 mg/100 mL). These findings could be explained by the limited microbial growth by N, which resulted in an energetic uncoupling (28) where there was an increase in TVFA production in N2.

The highest acetate proportion remained in the control medium (71.1 mol/100 mol) and was significantly different ($P < 0.01$) from N2 and N3 (68.2 and 63.7 mol/100 mol, respectively). The acetate production came from the degradation of the cell wall (NDF fraction in MT) (29), which the rumen bacteria used as the main source of energy and protein in the absence of other supplemented nitrogen sources.

This findings of the present study were consistent with those of Kajikawa et al, (30), who found that incubating individual amino acids caused a positive effect on the growth rate of mixed ruminal bacteria with glutamic acid and glutamine ($P < 0.01$) compared to the control, where ammonium sulfate was added as a nitrogen source.

Comparing the two substrates in Table 4, the DM degradability of CBP was significantly higher ($P < 0.001$) than that of MT, along with pH, TVFAs, propionate, and valerate proportions. With the N1 and N2 media all results were different, which was likely due to the chemical composition of the two substrates, a concentrate feedstuff and a fibrous plant. The latter has low DM degradability (29% approximately) compared to CBP (62% approximately).

In conclusion, the present study was designed to determine the effects of organic and inorganic nitrogen sources on the in vitro degradation of MT and CBP through the analysis of fermentation parameters. There was stable pH in the average optimal physiological conditions in the rumen even though CBP is high in soluble carbohydrates. Using feeds of this composition, there would be no risk of acidosis in spite of the fact that TVFA production increased significantly with both substrates, CBP and MT. However, NH_3-N increased above the optimal values. This is an important issue for future research, suggesting that the same experiments should be performed with mixtures of conventional feedstuff like oats and barley in all forms, which are the main forage corps in Algeria, plus CBP, with caution for the CP optimal concentration to maximize microbial biomass production and consequently microbial protein synthesis. Strong evidence is presented that MT degradability is an asset for future trials with this endemic forage in ruminant diets.

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