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In vitro aflatoxins recovery after changing buffer or protozoa concentrations in the rumen fermentation fluid

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Abstract

This study simulates in vitro the effects of (i) rumen acidity and (ii) change in rumen protozoa numbers on the recovery of aflatoxins (AFs). Two 24-h fermentation experiments were carried out using the same batch in vitro fermentation systems and substrate (dried corn meal) containing 11.42, 2.42, 7.65 and 1.70 μg/kg of AFB1, AFB2, AFG1 and AFG2 respectively. In Experiment 1, two buffer concentrations (normal salts dosage or lowered to 25%) were tested. Buffer reduction decreased gas production (730 vs. 1101 mL, $p < 0.05$), volatile fatty acids (VFA) and NH_3 concentrations in the fermentation liquid (39.8 vs. 46.3 mmol/L, and 31.7 vs. 46.5 mg/dL respectively, $p < 0.01$). Recovery of all four AFs types was higher ($p < 0.01$) in the reduced buffer fermentation fluid, both as a percentage of total AF incubated (73.6% vs. 62.5%, 45.9% vs. 38.1%, 33.6% vs. 17.9% and 18.9% vs. 6.24% for AFB1, AFB2, AFG1 and AFG2 respectively) and as amounts relative to VFA production (163.4 vs. 123.5, 22.1 vs. 15.7, 48.8 vs. 22.5 and 6.16 vs. 1.86 ng/100 mmol of VFA, for AFB1, AFB2, AFG1 and AFG2 respectively). In Experiment 2, *Stevia rebaudiana* Bertoni extracts (S) or a Camphor essential oil (Cam) were added to fermenters and compared to the control (no additives, C). S and Cam addition resulted in a 25% reduction ($p < 0.05$) and a 15% increase ($p < 0.05$) in protozoa counts respectively, when compared to C. Both plant additives slightly reduced ($p < 0.05$) AFB1 recovery as a percentage of total AFB1 incubated (68.5% and 67.7% vs. 74.9% for S, Cam and C respectively). Recoveries of all other AFs were unaffected by the additives. In conclusion, the rumen in vitro AFB1 recovery (63%–75%) was higher than other AFs (3%–46%) and the acidic fermentation environment increased it. In our conditions, changes in protozoa numbers did not affect AFs recovery.

KEYWORDS
feed, nutrition, ruminants

1 | INTRODUCTION

Mycotoxins are secondary metabolites produced by several fungal species that are toxic to plants, animals, and humans. Aflatoxins (AFs) are a class of mycotoxins mainly produced by two *Aspergillus* species (*A. flavus* and *A. parasiticus*) and include a variety of compounds that are highly toxic and carcinogenic to animals (Gallo et al., 2015; Jiang et al., 2021).

The most commonly found AFs are AFB1 and AFG1 and their dihydro derivatives AFB2 and AFG2. If AFB1-contaminated feed is used for cattle nutrition, the toxin is partially hydroxylated in the liver to AFM1, which is secreted in milk. In this regard, the amount of AFM1 in dairy milk may represent from 0.3% to 6.2% of the AFB1 ingested, depending on several animal-related factors (species, age, ingestion level, the health status of animals and so on) (Masoero et al., 2007). Because AFM1 is a group 1 human carcinogenic chemical, this event poses a serious threat to food safety (IARC, 2002).

Feeding dairy cows with noncontaminated forages and concentrates is a significant challenge for animal welfare, milk production, and dairy products safety. Farmers can use different agronomic strategies to reduce the contamination of plant products in the field (e.g., crop rotation, soil management, genetic choice of crops, appropriate fungicide use, set up of harvester equipment) but in years with particularly adverse meteorological conditions (e.g., wet and high temperatures), contamination prevention is difficult and detoxification actions are required. Several postharvest remediation or detoxification strategies, based on chemical, physical and biological approaches (detoxifying microbes and catabolizing enzymes) have been proposed (Jiang et al., 2021; Liu et al., 2022; Zhu et al., 2016), but many of these treatments have proved to be too expensive, ineffective or not applicable in full scale.

Ruminants are thought to be more resistant to AFs contamination than monogastric due to the rumen fermentative process and toxin degradation by microbiota (Min et al., 2021). However, an exact estimate of AFB1 disappearance in the rumen is not available, and in vitro fermentation tests provided very variable results. Kiessling et al. (1984) conducted short-term rumen fermentation tests on several mycotoxins and concluded that AFs are barely degraded in the rumen environment. Jiang et al. (2012), on the other hand, discovered very high rumen in vitro AFB1 degradation (80%–90%). Both, Gallo and Masoero (2010) and Upadhaya et al. (2009) have reported intermediate AFB1 degradation values (12%–20% and 35% respectively).

The batch in vitro systems are rapid and economic methods to study rumen fermentation and metabolism and they are largely used for different purposes (Spanghero et al., 2022; Yáñez-Ruiz et al., 2016). Particularly in the case of studies that involve toxic substances fed to animals (such as mycotoxins), these methods are an important alternative to in vivo experiments which are risky for animal welfare and health.

The examined literature gives variable information about the entity of AFs disappearance in the rumen, especially regarding the impact of rumen acidity. Furthermore, it is unclear what role different rumen microbiota components (such as protozoa) have in the AFB1 degradation capacity, and little is known about the rumen disappearance of the other AFs compounds (i.e., AFG1, AFB2 and AFG2).

The aim of this in vitro study is to provide more information about AFs rumen disappearance and to simulate the effects of (i) rumen acidity (throughout the reduction of buffer in the fermentation solution) and (ii) the change in rumen protozoa numbers (adding appropriate natural extracts) on the recovery of AFB1 and other AFs.

2 | MATERIALS AND METHODS

A series of preliminary in vitro tests (5 different fermentation runs for a total of 47 replicates) were carried out to validate the procedure for collecting, manipulating, and analyzing the fermentation liquid residue from in vitro fermentation. Then, two 24-hour fermentation experiments (Experiment 1 and Experiment 2) were carried out using the same apparatus, in vitro method and substrate. All of the rumen inoculum used in each fermentation run of both experiments was measured for AFs content to ensure the absence of an external contribution.

2.1 | Substrates and dietary treatments

In the preliminary experiments, a late-season corn hybrid (Pioneer Hi-Bred International) was grown and inoculated with a toxigenic strain of *A. flavus* (ITEM 8069, 1×10^5 spores/mL, Gallo et al., 2021), and the corresponding corn silage contaminated with AFB1 (20 μg/kg DM [dry matter]) was used as substrate. In the following fermentation experiments (Experiment 1 and Experiment 2), a certified test material (F2061/CM; Testveritas) represented by cornmeal with 11.42, 2.42, 7.65 and 1.70 μg/kg of AFB1, AFB2, AFG1 and AFG2 respectively, was used as substrate.

In Experiment 1, filtered rumen fluid was mixed with the Menke et al. (1979) buffer (ratio 1:2, vol/vol), which was prepared using either the required salts concentrations or 25% of the amount needed for all salts (named 'normal' and 'reduced' in tables respectively). Four fermentation bottles were used for each buffer type, and within each type of buffer, the bottles were inoculated with two different rumen fluids (two bottles for each combination of buffer type and rumen inoculum). The fermentation was repeated in four subsequent repetitions (runs). In Experiment 2, filtered rumen fluid was mixed with the Menke et al. (1979) buffer (ratio 1:2, vol/vol), and a control treatment (no extracts added, C) was compared to a substrate added with an extract (in ethanol solution) from dried and milled leaves of *Stevia rebaudiana* Bertoni (1.5% incubated DM, S) or with the Camphor essential oil (1.5% incubated DM, Cam). Two fermentation bottles were used for each dietary treatment and the fermentation was repeated in four subsequent repetitions (runs).

In both experiments, substrates were weighed and introduced into each bottle as ground and dry material (3670 mg, 90% of DM), and then bottles were hermetically closed and immersed in a water bath at 39°C for 24 h. The total amount of AFs incubated in each

fermenter was 41.91, 8.89, 28.12 and 6.24 ng for AFB1, AFB2, AFG1 and AFG2 respectively.

2.2 | Rumen inoculum and apparatus used for in vitro experiments

The rumen fluid for all experiments was collected in the same slaughterhouse in controlled conditions within 20 min of slaughter from two culled dairy cows fed with productive total mixed rations based on corn silage: in Experiment 1, rumen liquids were maintained separately, while in Experiment 2, they were mixed. All the animals were transported from farms within 50 km of the slaughterhouse, and they were in good health and had not been slaughtered in an emergency state. The fluid was delivered, within half an hour of it being collected, to the laboratory in airtight glass bottles refluxed with CO_2 and maintained at 39°C.

We recently described (Braidot et al., 2022) the in vitro apparatus used in this work. Briefly, it is composed of fermentation glass bottles (100 mm external diameter; 150 mm high and 750 mL total available capacity) closed with an airtight cap in which an electric engine is inserted that rotates an internal mixing paddle. Each bottle has on top, above the fermentation liquid level, an outlet connected with a flexible plastic tube (4 mm id) where the gas flowing from the fermentation bottle enters a gas counter (Ritter Apparatebau GmbH & Co. KG) suitable to measure low gas flow rate (from 1 mL/h to 1 L/h; measuring accuracy 3%).

2.3 | Sampling of fermentation fluid and ammonia, volatile fatty acids (VFA), AF analysis and protozoa counts

At the end of the incubation, pH was directly measured (GLP 22; Crison Instruments, SA), while samples of fermentation liquid for NH_3 (5 mL) and VFA (5 mL were added with 5 mL of H_2SO_4 0.01 N) determinations were collected and stored at −20°C until the analyses. Samples for ammonia determination were thawed at room temperature and analyzed by using an Ammonia Gas Sensing Combination Electrode (Hach Company).

Samples for VFA analysis were thawed at room temperature, centrifuged at 20,000*g* for 20 min at 4°C (centrifuge model 6K15; Sigma), and filtered through syringe filters (RC 0.45 µm, 25 mm; DTO Servizi S.r.l.). The filtrate was transferred into the autosampler vials and 20 µL was injected into high-performance liquid chromatography (HPLC). The system included an LC-20AT pump, a vacuum degasser, a Prominence SPD-M20A photodiode-array detector, a Prominence SIL-20AC HT autosampler (20 µL loop) and a Prominence CTO-20AC column oven set at 40°C (Shimadzu Corporation). The HPLC separations were achieved using an Aminex HPX-87H column (300 mm × 7.8 mm) with a precolumn (Bio-Rad). Sulphuric acid 0.008 N was used as the mobile phase at a flow rate of 0.6 mL/min. Full spectra were recorded in the range of 190–400 nm and the optimum wavelength detection for all VFA was found to be 220 nm. Peaks of analytes were compared with the retention times of a standard mixture and quantification was based on the external standard method. VFA standards of acetic, propionic, butyric, isobutyric, isovaleric, valeric and lactic acids were obtained from Merck.

At the end of the incubation, samples of fermentation fluid for protozoa count were collected and added to an 18.5% formaldehyde solution (50:50 ratio). Protozoa were counted as described by Dehority (2003).

The AFs determination was performed on all the samples of rumen fluid used as inoculum and on the fermentation fluid remaining from each bottle (500 mL). The liquids were centrifuged at 4600*g* for 20 min. The pellet fraction obtained was resuspended in 100 mL of acetone:water (70:30, vol/vol) solution. The sample was maintained under constant agitation for 45 min for the toxin extraction and after the incubation period, it was centrifuged at 4600*g* for 20 min. The supernatant was collected and stored at −20°C until the analysis. Five milliliters ofacetone extract was diluted with 45 mL of distilled water and then purified with EASY EXTRACT columns (R-Biopharm). The column was washed with 20 mL of Milli-Q water and then eluted with methanol:water (1.25:0.75, vol/vol) solution. The purified extract was filtered with 0.45 µm nylon filters and subsequently, 50 µL were injected into an HPLC system composed of an isocratic pump, an autosampler, and a fluorescence detector (models PU 2020 Plus, FP 2020 Plus and AS 2055 Plus respectively, Jasco Corporation). The excitation wavelength was set to 350 nm while the emission wavelength was 450 nm. The HPLC separations were achieved using a Luna C18 column at room temperature (150 × 4.6 mm, 3 µm particle size; Phenomenex) eluted with methanol:acetonitrile:water (22.5:22.5:55) at a flow rate of 0.6 mL/min. The AFs were derivatized before the detection with Kobracell (R-Biopharm).

2.4 | Sampling of fermentation fluid, DNA extraction, sequencing and bioinformatics analyses

At the end of fermentation from each fermentation bottle, 2 mL of fluid was collected and stored at −20°C for bacterial DNA extraction (DNeasy PowerSoil Pro Kit; Qiagen) and analysis. The DNA extraction was performed according to the manufacturer's protocol. For bacterial community identification, the V1–V3 region of the 16s gene was amplified as previously described by Takahashi et al. (2014). For the DNA amplification, the primers set Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) were used. The amplifications were performed using 5 µL of the extracted DNA using high fidelity Platinum Taq DNA polymerase (Thermo Fisher Scientific) in a final reaction volume of 25 µL. The subsequent protocol was adopted: initial denaturation at 94°C for 1 min, 25 cycles of 94°C for 30 s as denaturation, 55°C for 30 s as annealing and 68°C for 45 s as extension followed by a final extension at 68°C for 7 min.

The libraries were purified with Beads Amplure XP 0.8X, amplified with Indexes Nextera XT Illumina, normalized, mixed and loaded on Miseq with 2 × 300 bp (paired-end) approach to generate a minimum of 50,000 sequences (±20%). The raw sequences were trimmed by the primers and then filtered by quality and length by the

Qiime2 software (v 2021.4). The amplicon sequence variants (features) obtained were compared to Greengenes v13-8 and Silva v.138 databases and corresponding taxonomy were assigned.

2.5 | Statistical analysis

The fermentation runs were completed in sequenced periods (weeks) and data from two fermentation bottles within each run were averaged and used as statistical units (replicates among runs). Statistical analyses were performed with the general linear model (GLM) procedure of SAS Software (Version 9.4; SAS Institute).

In Experiment 1, data were statistically analyzed as a completely randomized design (CRD) with a factorial arrangement of treatments, using the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_{j(k)} + \gamma_k + \varepsilon_{ijk},$$

where y_{ijk} is the experimental data, μ is the overall mean and α_i is the fixed effect of the amount of buffer in the fermentation fluid ($i = 1, 2$); β_j is the random effect (block) of rumen inoculum nested within the fermentation run ($j = 1, 2$); γ_k is the random effect (block) of the fermentation run ($k = 1, 4$) and ε_{ijk} is the residual error.

In Experiment 2, data were statistically analyzed as a CRD with a factorial arrangement of treatments, using the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where y_{ij} is the experimental data, μ is the overall mean and α_i is the fixed effect of the dietary treatment ($i = 1, 3$); β_j is the random effect (block) of the fermentation run ($j = 1, 4$) and ε_{ij} is the residual error.

Multiple comparisons of means among dietary treatments were conducted using the PDIFF option of the GLM procedure. The metagenomics data were analyzed with the R environment (v. 4.1.3). The Shannon index was calculated using the R package vegan (version 2.5-7) (Oksanen et al., 2015).

3 | RESULTS

3.1 | Preliminary results

The preliminary tests carried out to validate the procedure for collecting, manipulating and analyzing the fermentation liquid residue revealed that the AFB1 was mainly present in the pellet fraction (95.4 ± 1.6%). Moreover, the analytical procedure showed a good degree of precision with a 5% variability coefficient in repeatability. Finally, none of the inoculum used showed AFs presence (data not shown).

3.2 | Results of Experiments 1 and 2

Tables 1 and 2 show the results of Experiment 1 on the effects of a 75% buffer reduction in in vitro artificial saliva on fermentative parameters, AFs recovery and composition of the rumen microbial community.

TABLE 1 Effect of reduction of the buffer in the fermentation fluid (normal vs. reduced) on the in vitro fermentation parameters and the recovery of aflatoxins[1] (AFs).

	Buffer in the fermentation fluid[2]		
	Normal	Reduced*	RMSE
pH			
Initial	6.76^{A}	6.31^{B}	0.067
Final	6.61^{A}	5.87^{B}	0.126
Difference	0.15^{A}	0.44^{B}	0.105
Gas production, mL	1101^{a}	730^{b}	234
NH_3, mg/dL	46.5^{A}	31.7^{B}	7.17
Protozoa, 10^3 cells/mL			
Entodinia	218	207	26.7
Holotricha	11.0	8.0	10.4
Total	229	215	33.7
Total VFA, mmol/L	46.3^{A}	39.8^{B}	2.81
% Total VFA			
Acetate (A)	67.6	65.6	2.51
Propionate (P)	14.4	14.7	0.33
Isobutyrate	1.08	1.53	0.94
Butyrate	12.7^{b}	13.6^{a}	0.54
Isovalerate	2.57^{b}	2.88^{a}	0.19
Valerate	1.28	1.97	1.01
A:P	4.76	4.57	0.21
Recovery, %			
AFB1	62.5^{B}	73.6^{A}	5.53
AFB2	38.1^{B}	45.9^{A}	3.96
AFG1	17.9^{B}	33.6^{A}	5.26
AFG2	6.24^{B}	18.9^{A}	6.96
Recovery, ng/100 mmol of VFA			
AFB1	123^{B}	163^{A}	18.6
AFB2	15.7^{B}	22.1^{A}	2.60
AFG1	22.5^{B}	48.8^{A}	6.28
AFG2	1.86^{B}	6.16^{A}	1.74

Abbreviations: RMSE, residual mean square error; VFA, volatile fatty acid.
[1]The total amount of AFs incubated in each fermenter with the contaminated corn substrate is 41.91, 8.89, 28.12 and 6.24 ng for AFB1, AFB2, AFG1 and AFG2 respectively.
[2]Means with different superscripts differ (a–b: $p < 0.05$, A–B: $p < 0.01$).
*Buffer salts lowered to 25% of the normal dose.

As buffers were reduced, the initial and final pH was lowered (6.31 vs. 6.76 and 5.87 vs. 6.61 respectively, $p < 0.01$), and the difference between the initial and final pH increased (0.44 vs. 0.15, $p < 0.01$). The reduced buffer fermentation fluid produced less gas (730 vs. 1101 mL, $p < 0.05$) and lowered VFA and NH_3 concentrations in the fermentation

liquid (39.8 vs. 46.3 mmol/L, and 31.7 vs. 46.5 mg/dL respectively, $p < 0.01$). The proportions of the primary VFA (acetate and propionate) were not affected by the buffer reduction whereas butyrate and isovalerate were increased when the buffer concentration was reduced. Recovery of all four types of AFs was higher ($p < 0.01$) in the reduced buffer fermentation fluid, both as a percentage of total AFs incubated (73.6% vs. 62.5%, 45.9% vs. 38.1%, 33.6% vs. 17.9% and 18.9% vs. 6.24%, for AFB1, AFB2, AFG1 and AFG2 respectively) and as amounts relative to VFA production (163.4 vs. 123.5, 22.1 vs. 15.7, 48.8 vs. 22.5 and 6.16 vs. 1.86 ng/100 mmol of VFA respectively, for AFB1, AFB2, AFG1 and AFG2).

In Experiment 1, as shown in Figure 1a, the Shannon index, which represents a measure of within-sample diversities, did not differ between treatments ($p > 0.1$). Table 2 reports the main phyla obtained from metagenomics analysis. No statistically significant differences between treatments were found in *Bacteroidota* (36.6%–41.6%) and *Firmicutes* (39.4%–39.0%) which represent the main phyla while the buffer reduction determined a significant decrease ($p < 0.05$) in the relative abundance of *Fibrobacterota*, *Spirochaetota* and *Thermoplasmatota*.

Tables 3 and 4 show the results of Experiment 2 about the effects of changes in the protozoa population induced by the addition of S and Cam on fermentative parameters, AFs recovery and composition of the rumen microbial community.

The S extract reduced the protozoa count when compared to the control (171 vs. 233×10^3 cells/mL, $p < 0.01$), whereas Cam increased the protozoa count (267×10^3 cells/mL). The addition of natural substances did not affect the fermentation parameters studied (pH, gas production, VFA yield and composition and NH_3). A difference was founded in isobutyrate, where Cam concentrations were lower than S (1.53% vs. 1.77% total VFA, $p < 0.05$) and C values were in the middle (1.67% total VFAs), but not statistically different from other treatments.

The addition of S and Cam slightly reduced ($p < 0.05$) AFB1 recovery as a percentage of total AFB1 incubated (68.5% and 67.7% vs. 74.9% for S, Cam and C respectively), but the absolute recoveries of other AFs and all recoveries expressed per VFA were unaffected by the additions.

The Shannon index did not differ between treatments (Figure 1b, $p > 0.10$). The main phyla in both treatments were *Bacteroidota* (34.0%–38.2%) and *Firmicutes* (33.4%–36.9%) and there were no notable variations in the relative abundance of the major phyla in this experiment.

4 | DISCUSSION

The results in both experiments of this study demonstrated that AFB1 has a significantly higher in vitro recovery (63%–75%) than other AFs (3%–46%). As a result, AFB1, the most pervasive and toxic

TABLE 2 Effect of reduction of the buffer in the fermentation fluid (normal vs. reduced buffer) on the relative abundance of main bacterial phyla[1] in Experiment 1.

Phyla, %	Buffer in the fermentation fluid[2]		RMSE
	Normal	Reduced*	
Bacteroidota	36.6	41.6	6.21
Bdellovibrionota	0.66	0.47	0.75
Cyanobacteria	0.56	0.54	0.66
Elusimicrobiota	0.78	0.77	0.67
Euryarchaeota	4.31	4.48	0.95
Fibrobacterota	4.99[a]	2.96[b]	1.09
Firmicutes	39.4	39.0	3.88
Patescibacteria	4.53	5.08	1.51
Proteobacteria	1.14	1.12	0.37
Spirochaetota	2.43[a]	1.44[b]	0.52
Thermoplasmatota	3.30[a]	1.56[b]	1.42

Abbreviation: RMSE, residual mean square error.

[1]Only phyla with relative abundance greater than 0.50% from 16S rRNA sequencing are reported.

[2]Within rows mean with different superscripts differ (a–b: $p < 0.05$).

*Buffer salts lowered to 25% of the normal dose.

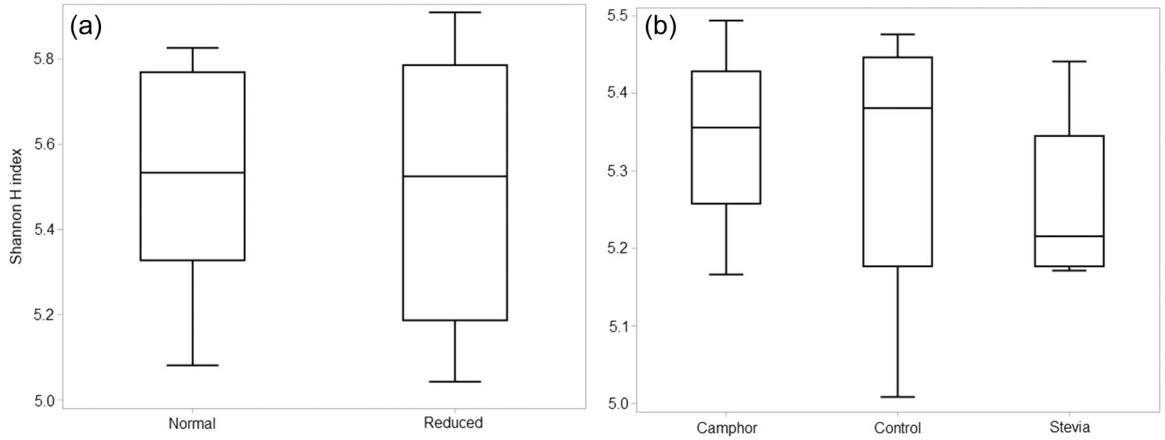

FIGURE 1 Box plot of the ruminal bacterial diversity (Shannon index) divided by treatments of Experiment 1 (a) and Experiment 2 (b).

TABLE 3 Effect of protozoa population changes induced in vitro by the plant extract (essential oil from Camphor and extract from the *Stevia rebaudiana*) addition on the in vitro fermentation parameters and on the recovery of aflatoxins[1] (AFs) (Experiment 2).

		Plant extracts[2]		
	Control	*Stevia rebaudiana*	Camphor	RMSE
pH	6.67	6.68	6.68	0.01
Gas production, mL	1080	1159	1112	95.6
NH_3, mg/dL	28.9	29.8	30.3	1.29
Protozoa, 10 cells/mL				
Entodinia	233[b]	171[c]	267[a]	6.45
Holotricha	8.8	6.9	7.7	1.33
Total	242[b]	178[c]	275[a]	6.90
Total VFA mmol/L	56.1	55.2	53.1	2.45
% Total VFA				
Acetate (A)	70.5	70.7	70.9	0.29
Propionate (P)	12.4	12.3	12.3	0.10
Isobutyrate	1.67[ab]	1.77[a]	1.53[b]	0.11
Butyrate	9.07	8.85	9.01	0.15
Isovalerate	4.47	4.42	4.39	0.92
Valerate	1.87	1.89	1.83	0.10
A:P	5.70	5.74	5.78	0.07
Recovery, %				
AFB1	74.9[a]	68.5[b]	67.7[b]	2.78
AFB2	37.7	39.6	39.8	5.55
AFG1	17.9	17.6	17.1	2.06
AFG2	4.16	3.20	3.19	1.10
Recovery, ng/100 mmol of VFA				
AFB1	113	105	109	5.42
AFB2	12.16	12.89	13.49	1.74
AFG1	18.7	18.1	19.0	2.28
AFG2	1.01	0.81	0.76	0.26

Abbreviations: RMSE, residual mean square error; VFA, volatile fatty acid.
[1]The total amount of AFs incubated in each fermenter with the contaminated corn substrate is 41.91, 8.89, 28.12 and 6.24 ng for AFB1, AFB2, AFG1 and AFG2 respectively.
[2]Within rows mean with different superscripts differ (a–b–c: $p < 0.05$).

TABLE 4 Effect of protozoa population changes induced in vitro by the plant's extract (essential oil from Camphor and extract from the *Stevia rebaudiana*) addition on the relative abundance of main bacterial phyla[a] in Experiment 2.

		Plant extracts		
Phyla, %	Control	*Stevia rebaudiana*	Camphor	RMSE
Bacteroidota	34.4	36.9	33.4	8.6
Bdellovibrionota	1.30	1.10	0.83	0.26
Cyanobacteria	1.33	1.10	0.92	0.24
Elusimicrobiota	1.51	1.69	1.08	0.37
Euryarchaeota	3.16	3.32	3.31	0.64
Fibrobacterota	6.21	5.19	6.99	1.65
Firmicutes	34.4	34.0	38.2	3.9
Patescibacteria	5.85	5.00	4.30	2.66
Proteobacteria	3.76	3.50	3.28	1.08
Spirochaetota	1.56	1.90	2.15	0.69
Thermoplasmatota	5.03	5.23	4.23	0.55

Abbreviations: RMSE, residual mean square error; rRNA, ribosomal RNA.
[a]Only phyla with relative abundance greater than 0.50% from 16S rRNA sequencing are reported.

AFs, also seems to have the highest level of resistance to the rumen microbiome.

Existing literature shows an adverse effect of AFB1 on rumen fermentation (Jiang et al., 2012; Westlake et al., 1989), but as reported by Jiang et al. (2021) in many in vitro experiments the concentrations of AFB1 in the fermentation liquid (from 100 to 10,000 μg/L) is much higher than those occurring in the rumen. Jiang et al. (2020) found that a low concentration (0.75 μg/L) did not affect in vitro rumen fermentation (e.g., VFA yield after 24 h). In our experiments, the expected AFB1 concentration in the fermenters was even much lower (0.080–0.085 μg/L) and therefore a possible adverse effect on fermentation was assumed limited.

4.1 | Effect of buffer concentration in fermentation fluid

In Experiment 1, the reduction of buffer in fermentation fluid caused a greater drop in the final pH (−0.44 vs. −0.15). This was associated with a total gas reduction (from 1101 to 730 mL) and VFA accumulation (from 46.5 to 31.7 mmol/L) in the fermentation fluid, without a relevant modification of the proportions of different acids. This is consistent with the findings of Judd and Kohn (2018) who tested different buffering conditions by varying the inoculum/buffer solution ratios and found reductions in gas production, VFA yield and pH. Contrary to our findings, there was also a change in the VFA composition (e.g., reduction of acetate:propionate ratio). Recently, in a Rusitec system (Guo et al., 2022), the pH was reduced from 6.8 to 5.5 by lowering the buffers to 20% of the normal level and, as in the present study, the effect was a reduction of degradability and total concentration of VFA.

AFs molecules are known to form bonds with micro-organism walls (e.g., bacteria and yeasts) or feed substrates (e.g., fibres) and to be highly reactive with proteins and DNA molecules. Sequestering

agents, which are used as feed additives to minimize the toxicity of contaminated feeds, are just based on this great AFs' ability to bind (Gallo & Masoero, 2010; Jiang et al., 2021). Moreover, AFs can be transformed by the microbiota of the digestive tract into other compounds (Guerre, 2020; Min et al., 2021). As a result, the term 'degradation' often does not accurately reflect the AFs balance in biological systems, such as the rumen environment, and might skew comparisons between studies due to the use of various matrices, additives, or systems in different investigations.

In the present study, the AFB1 average recovery was 69%, while the other AFs showed a greater aptitude to disappear in the rumen with a recovery of 40%, 21% and 7% respectively, for AFB2, AFG1 and AFG2. Kiessling et al. (1984) investigated the disappearance of six mycotoxins in short-term (up to 3 h) rumen fermentation tests and concluded that AFs are barely degraded in the rumen environment. On the contrary, Jiang et al. (2012) showed a very high rumen in vitro degradation of AFB1, ranging from 83% to 90% after 72 hours of fermentation. Upadhaya et al. (2009) utilized different inoculums and found low values of AFB1 degradation which ranged from 12% to 20% (steer or goat respectively) after 12 h of incubation, whereas Gallo and Masoero (2010) measured rumen degradability around 35% following 2 h incubation.

The reduction of buffer and the consequent depression of fermentation resulted in a substantial increase in AFB1 and AFB2 recoveries (+18 and +20% respectively), which was highly intense for AFG1 (+88%) and threefold for AFG2. Part of these increments of the toxins has to be attributable to an overall reduction of degradation when the buffer is reduced. Therefore, the recovery was expressed per unit of VFA produced (ng of toxin/100 mmol of VFA) to account for differences in fermentation intensity, and the fermenters with a lower buffering capacity still had a much higher recovery. When the rumen environment changes to acidic conditions, this observation shows that the microbial population's AF degradation capacity is lowered. These findings support previous results (Upadhaya et al., 2009) where in vitro AF degradation was more intense when rumen fluid from animals fed with a high forage:concentrate ratio was used (80:20 vs. 60:40). However, dietary conditions were tested in separate trials and this represented a limitation of that experiment. Recently, Pantaya et al. (2016) compared two diets with different starch contents and demonstrated that rumen acidic conditions caused a higher urinary secretion of AFB1 compared to faecal output. The authors concluded that AFB1 is more bioavailable in starchy diets and hypothesized that low rumen pH increases the absorption of AFB1 due to toxin molecule ionization conditions. In contrast with our results, these authors assumed a constant biodegrading capacity of the ruminal microbiota irrespective of starch content based on the unchanged rumen degradability of other toxins (e.g., ochratoxins and trichothecenes). Other research (Debevere et al., 2020) concurs with our findings, indicating that when cattle are fed starch-rich diets, mycotoxin breakdown by rumen microbes is inhibited.

The lack of effects on protozoa counts, phylogenetic diversity, and the expected high relative abundance of the main rumen bacteria phylum (*Bacteroidota* and *Firmicutes*) indicate that variations in pH conditions and the subsequent effect on AF degradation have relatively minor impacts on rumen microbiota under the current study conditions. The relative abundance of bacteria phylum *Fibrobacterota*, which is one of the most common cellulolytic bacteria in the rumen (Stewart et al., 1997), decreased when the buffer concentration was reduced. This change reflects the more prohibitive acidic conditions in the low-buffered fermenters for cellulolytic bacteria and is associated with a lower disappearance of AF toxins.

4.2 | Effect of protozoa counts in fermentation fluid

Based on an earlier study (Kiessling et al., 1984) showing that protozoa had a higher degradation capacity towards some toxins than bacteria (e.g., ochratoxin, zearalenone, trichothecenes), the possible degradative action of protozoa against AFs was investigated. Protozoa are highly sensitive to in vitro conditions, and their population declines during fermentations (Muetzel et al., 2009). To ensure that a sufficiently numerous and metabolically active protozoa population could be maintained, we chose a short time frame of 24 h to test different protozoa counts in terms of AFs degradation. In previous experiments, it was observed that some natural substances change the in vitro protozoa counts (Sarnataro et al., 2020; Sarnataro & Spanghero, 2020). Specifically, *Stevia* leaf extract was responsible for a reduction of approximately 50% after 24 h of fermentation using the same in vitro apparatus and dosage as in the current study (Sarnataro & Spanghero, 2020). A class of iminosugars (2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine), which operate as glycosidase inhibitors, are chemical compounds contained in this plant that may be responsible for the suppression of protozoa (Ramos-Morales et al., 2017). These substances were detected in our extract at a concentration of 0.1%–0.2% DM (R. Nash, personal communication, 2022). Contrarily, the Camphor essential oil resulted in a protozoa increase of about 50% in another experiment, where both batch and continuous fermentation systems were used (Colombini et al., 2021). In the current study, a 25% reduction and a 15% increase in protozoa counts compared to a control treatment were achieved by adding S and Cam respectively.

In terms of diet fermentability, VFA yield, or microbiota no differences between treatments were found. These findings support the results of our recent meta-analysis, where protozoa count from in vitro rumen batch experiments were not related to modifications in the main fermentative parameters (Spanghero et al., 2022). The variation in protozoa counts did also not result in a change in AFs recovery and we conclude that protozoa have no substantial impact on these toxins' degradation. It was not possible to find any in vitro experiments to compare our results. Recently, an in vivo study (Thukral et al., 2022) investigated the relationships between fermentative rumen parameters and variation in AFM1 excretion in milk from lactating dairy cows and buffaloes: there was no statistical correlation between rumen protozoa counts and AFM1 in milk.

Anyway, the recovery values for AFB1 found in the previous experiment (Experiment 1) were confirmed (74% vs. 62%) as well as

the higher aptitude of other AFs types to be degradable at the rumen level. This result indicates that our experimental conditions are sufficiently reproducible.

5 | CONCLUSION

According to the current data, AFB1 has an in vitro recovery (63%–75%) higher than other AFs (3%–46%). In vitro, increasing rumen acidic conditions by lowering the buffer solution concentration, significantly increases AFs recovery and this result, when translated into in vivo conditions, suggests that animals suffering from rumen acidosis may have a reduced capacity for degradation. In our conditions, changes in protozoa populations did not affect AFs recovery.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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