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Evaluation of cellulolytic exogenous enzyme-containing microbial inoculants as feed additives for ruminant rations composed of low-quality roughage

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Animal Research Paper

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Abstract

The supplementation of ruminant diets with exogenous cellulolytic enzymes can improve their digestibility and feeding value. The objective of this study was to determine the effect of supplementing roughage (rice straw) and concentrate with inoculants containing four fungal strains (*Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Trichoderma reesei* and *Trichoderma viride*) and four bacterial strains (*Paenibacillus polymyxa*, *Bacillus megaterium*, *Bacillus circulans* and *Bacillus subtilis*), given separately or as a mixture, as a source of exogenous cellulolytic enzymes, on basic rumen parameters *in vitro*, including digestibility and methane production. A batch culture trial was used to select the best supplements, and a long-term rumen simulation technique (RUSITEC) was used to evaluate the effects of *P. chrysosporium*, *B. subtilis*, and a 1 : 1 mixture of these two on dietary component digestibility and fermentation parameters. In the batch culture evaluation, there were significant increases in the organic matter (OM) digestibility, the total gas production expressed as ml/g of dry matter, the OM, the neutral detergent fibre (NDF) and the acid detergent fibre (ADF) of the supplemented rations, as compared to the control, excluding the rations supplemented with *T. viride* and *B. circulans*. In the RUSITEC, the ration supplemented with mixed inoculants showed significantly higher digestibility of crude protein, ether extract, NDF and ADF than did the ration supplemented with the *P. chrysosporium* and *B. subtilis* inoculants. It can be concluded that the simultaneous use of fungal and bacterial exogenous cellulases on rice straw roughage improves its digestibility, without negative effects on other rumen parameters.

Introduction

Arid and semiarid regions present great challenges in providing livestock with feed that meets their nutritional requirements. Crop residues have been considered as a potential strategy to solve this problem, but these are usually low in nitrogen (N) and are poorly digested by animals (Romero *et al.*, 2013). This has encouraged researchers to attempt to improve the nutritive values of these residues so as to cover the nutrient requirements for production results (Klebaniuk *et al.*, 2019). To date, several approaches have been made to improve ruminant livestock forage quality, including physical treatment (such as heat, steam and pressure; Zayed 2018), chemical treatment (e.g. with acids, alkalis, NaOH, NH_3 and urea; Reddy *et al.*, 2016) and biological treatment (Zayed, 2018). These methods, particularly the biological approaches, may be more practical, more environmentally friendly and more cost-effective ways of enhancing the nutritive value of forage (Adesogan *et al.*, 2014).

Various studies have focused on using two biological treatments to improve the nutritive value of forage by improving nitrogen contents and digestibility by livestock. The first treatment is the direct-fed microbial method, which relies on the use of microbial inoculants (Seo *et al.*, 2010); the second treatment is the direct-fed enzyme method, which uses exogenous enzymes as additives (Adesogan *et al.*, 2014). A number of reports have confirmed that using microbial inoculants results in an improvement in the nutrient content of roughage, perhaps through the production of enzymes and phytohormones that stimulate the activities of microorganisms in the rumen (Zayed, 2018). Furthermore, other reports have emphasized the use of exogenous fibrolytic enzymes (EFE) as supplements for improving feed digestibility and animal performance. This is thought to improve ruminal fermentation, increase the digestibility

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of forage cell walls, reduce feed costs and support the production performance of ruminants (Adesogan *et al.*, 2014; Tirado-González *et al.*, 2018). In addition to these benefits, supplementation with EFE may have a positive effect on rumen microbial populations, bacterial attachment and colonization of roughage (Tirado-González *et al.*, 2018), resulting in improvements in the digestibility of the dry matter (DM; Yang *et al.*, 2000; Arriola *et al.*, 2017) and the neutral detergent fibre (NDF) (Peters *et al.*, 2015). The enzymes most commonly used as supplements in animal nutrition are cellulases, EFE capable of hydrolysing the fibre of the plant cell wall. These enzymes can be classified on the basis of the specific activity of three particular enzymes – endoglucanase, exoglucanase (CMCase) and D-glucosidase (cellobiases) – which work in sequence to hydrolyse the fibre of plant cell walls to cellooligosaccharides, cellobiose and glucose, respectively (Zhang and Lynd, 2004).

At present, there is little data in the literature on combinations of the two supplements – EFE and microbial inoculants – as food additives to improve animal performance (Jalč *et al.*, 2009). Addressing this, Jalč *et al.* (2009) and Reddy *et al.* (2016) have indicated that, although the use of EFE originating from fungi has been demonstrated to increase the nutritional value of poor-quality feed, the use of the fungi themselves, rather than their enzymes, would be easier and more economical. Moreover, fungi are increasingly often used to improve feed quality, and thus directly affect the quality of ruminant products (Varadyova *et al.*, 2018). Furthermore, Malik and Bandla (2010) concluded that supplementation with EFE and probiotics significantly improved ($P < 0.001$) organic matter (OM), NDF and acid detergent fibre (ADF) digestibility.

This study has two parts: the first involves the use of an appropriate method to produce dry-formulated exogenous cellulolytic enzymes in combination with their microbial inoculants, which have high cellulase activity during the storage period. The second part evaluates the efficiency of these inoculants on the fermentation and dietary component digestibility of rice straw, a low-quality roughage, using a short-term batch culture *in vitro* technique to select the best supplements for use in long-term *in vitro* rumen simulation technique (RUSITEC) experiments.

The research hypothesis is that the simultaneous use of inoculants containing fungal and bacterial exogenous cellulases on rice straw roughage improves its digestibility.

The objective of this study was to determine the effect of supplementing 40 : 60 roughage (rice straw) and concentrate feed mixture (CFM) with inoculants containing four fungal strains and four bacterial strains, given separately or as a mixture, as a source of exogenous cellulolytic enzymes on basic rumen parameters *in vitro*, including digestibility and methane production.

Materials and methods

Microorganisms used

We used four bacterial and four fungal strains. The fungal strains were *Pleurotus ostreatus* NRRL-2366, *Phanerochaete chrysosporium* NRRL-11460, *Trichoderma reesei* ATCC 28217 and *Trichoderma viride* F-416. The bacterial strains we employed were *Paenibacillus polymyxa* EMCCN 1108, *Bacillus megaterium* EMCCN 1028, *Bacillus circulans* EMCCN 1025 and *Bacillus subtilis* EMCCN 1152. The microbial inoculants were used as a source of exogenous cellulolytic enzymes for the ruminant livestock. All the strains obtained from the Microbial Inoculants Center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Nutrient agar medium (Jacobs and Gerstein, 1960) was used to cultivate, maintain and determine the densities of spore-forming bacteria, whereas potato dextrose agar medium was used to cultivate and maintain the fungal strains. Ten millilitres of each standard inoculum (ca. 10^8 CFU/ml for bacterial strains and 0.1 g ± 0.02 dry weight/10 ml for fungal strains) was used separately to inoculate 150 ml of the selective medium, which contained carboxymethyl cellulose (CMC; 10 g/l) as the sole source of carbon for each strain, in 250 ml Erlenmeyer flasks (De los Angeles Olvera-Treviño *et al.*, 1989). The inoculated media were incubated on a rotary shaker at 150 rpm at 25°C for 72 h in the case of the fungi and at 30°C for 48 h in the case of the bacteria.

Preparation of solid-state inoculant formulations of bacterial and fungi cultures using talcum powder as a carrier

The cultures for each bacterial strain were set up with a fixed cell load of about 10^9 CFU/ml; the calculation for the fungi worked with the gram dry weight per 10 ml as follows: *P. ostreatus*: 0.082 g dry weight/10 ml; *P. chrysosporium*: 0.084 g dry weight/10 ml; *T. viride*: 0.113 g dry weight/10 ml and *T. reesei*: 0.08 g dry weight/10 ml. A constant volume (400 ml) of all strains was added to the inoculant with a constant weight of talcum powder (200 g). The solid carrier-based cellulolytic exogenous enzymes and microbial inoculants are referred to as either biodual supplements or biodual formulations.

A mixture of bacterial strains was prepared by combining equal weights from the biodual formulations of all the bacterial strains together. The mixture of fungal strains was created by combining equal weights of the biodual formulations of all fungal strains together. Finally, a mixture of all the tested strains was obtained by mixing equal weights of all the bacterial and fungal tested strains together. The formulations were stored for 12 weeks at 25°C. Samples were taken from the prepared formulations every 2 weeks during the storage period to estimate cellulase and CMCase activity.

Enzyme assays in the formulations

Cellulase activities were determined in the supernatants and microbial formulations using the dinitrosalicylic acid (DNS) method, as reported by Miller (1959). Cellulase activities were determined at a pH of 5.0. A total of 1 ml of CMC substrate solution (1.0%, prewarmed to 40.0 ± 0.1°C for 5 min) was added to an equal volume of sample solution (prewarmed to 40.0 ± 0.1°C), mixed and transferred to a water-bath (at 40.0 ± 0.1°C) for 10 min, and then removed from the water bath; 4 ml of DNS solution was then added and mixed. The tubes were covered and placed in a boiling water bath for 15 min, and then cooled to room temperature. Insoluble substances were removed by centrifugation (3000 rpm, 10 min). The absorbance of samples was determined at 540 nm against a water blank. The glucose standard curve was prepared by adding 1 ml glucose standard solution (5, 10 and 15 µmol/l) instead of CMC substrate solution in the procedure described above, and the absorbance of the samples was determined at 540 nm to create a glucose standard curve. The glucose concentration in the sample was used to calculate the enzyme activity (U = µmol/min).

CMCase activity was recorded in both the supernatants and microbial formulations following the method reported by Mandels (1969). CMCase activities were determined at a pH of

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4.8. The test samples were prepared by adding 0.5 ml of sample to 0.5 ml CMC 1% solution in 0.05 M citrate buffer (pH 4.8) as substrate. The reaction was carried out at 50°C for 30 min. A total of 1 ml alkaline copper reagent was added to the mixture, then heated in a boiling bath for 10 min and cooled. The zero-time sample was prepared as described above. The blank sample was prepared by adding 1 ml alkaline copper reagent and 1 ml H_2O. The standard was prepared by mixing 0.5 ml standard glucose solution (3.5% w/v) with 1 ml alkaline copper reagent and 0.5 ml H_2O. Then 1 ml of chromogenic reagent was added to all samples and their final volumes were made up to 50 ml. The absorbance of all samples was measured at 660 nm. In the supernatants, one CMCase unit was defined as the amount of enzyme that reduces sugar at the rate of 1 µmol/min/µg protein. In the case of the microbial formulation, one CMCase unit was defined as the amount of enzyme that reduces sugar at the rate of 1 µmol/min/µg protein in 1 g of formula (U/g). The room temperature was 30 ± 2°C while the activities of the cellulose and CMCase enzymes were measured.

Determination of protein concentration in the formulations

The protein concentrations of the supernatants and microbial formulations samples were determined using the Bradford method (1976), using bovine serum albumin as a standard. Proteins eluted by column chromatography were assayed by their absorbency at 280 nm.

Batch culture evaluation

The short-term *in vitro* batch culture trial was carried out following a modified version of the protocol of Cieslak *et al.* (2016) to select the best biodual supplements for use in the long-term RUSITEC experiment. Briefly, a slaughterhouse provided fresh ruminal fluid from six Polish Whiteheaded mutton sheep (approximately 100 days old, body weight 28 ± 2 kg; two animals in each run). In the RUSITEC experiment, nine Polish Whiteheaded mutton sheep (three wethers for each run) of the same age and weight were used. The wethers were kept on commercial farm, and 20 days before slaughter, the animals were fed with 1 kg of commercial concentrate and wheat straw *ad libitum*. Either batch culture or RUSITEC experiments were repeated in three consecutive runs.

The collected rumen fluid was mixed and squeezed through a four-layered cheesecloth into a 5 litre bottle with an O_2-free headspace, and immediately transported to the laboratory in a thermal container containing a water bath at 39°C. A mixture of 40 : 60 roughage (rice straw) and CFM was used as the control group. The biodual supplements were added to the experimental groups as 1 g (contain one-unit cellulase activity) per kg dietary DM (1 kg inoculant per 1-tonne feed), in order to standardize the cellulase activity dosages in all inoculants.

The CFM consisted of 52.5 g yellow corn, 15 g soybean, 30 g wheat bran, 0.6 g salt, 0.5 g mineral mixture and 1.4 g limestone per 100 g. About 400 mg of the milled substrate (240 mg CFM and 160 mg rice straw) was added to incubation vessels with 120 ml capacity. The buffer solution (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 4 mg Na_2CO_3 and 600 mg cysteine hydrochloride per litre of double-distilled water) was mixed with rumen fluid in the 4 : 1 (v/v) ratio according to Cieslak *et al.* (2016). Each vessel was filled anaerobically with 40 ml of the blend of buffer solution and rumen fluid. The biodual supplements were added as one unit of cellulase activity per kg of DM, in order to standardize the cellulase activity dosages in all inoculants. Each treatment was tested in six replicates (six bottles), accompanied by control and blank vessels containing no substrate. The bottles were filled with CO_2, closed with a rubber stopper and sealed with an aluminium cap. Subsequently, the bottles were incubated for 24 h under anaerobic conditions with a pH of 6.5 and at 39°C. The bottles were agitated by hand every 120 min during incubation.

RUSITEC evaluation

This study employed the RUSITEC method, as described by Machmüller *et al.* (2002). The system was built from eight 1-litre fermenters. One trial was conducted to evaluate the effect of using the best fungal and bacterial biodual supplement, and of their mixture. In the RUSITEC system, clover hay (4.36 g) and CFM (6.54 g, control group); rice straw (4.36 g), CFM (6.54 g) plus *P. Chrysosporium*, rice straw (4.36 g), CFM (6.54 g) plus *B. subtilis* and rice straw (4.36 g), CFM (6.54 g) plus *P. Chrysosporium* mixed with *B. subtilis* (1 : 1) were used, and each substrate was incubated for 48 h. The experiment consisted of four treatments and was repeated three times. Rumen fluid and solid digesta were obtained in the same manner from sheep as in the batch culture experiment. The rumen materials were thoroughly mixed to provide a 5-litre sample of rumen fluid containing about 400 g of solid digesta. The rumen fluid was stored in an anaerobic environment and immediately transported to the laboratory in an incubated container at 39°C. Upon arrival at the laboratory, the rumen fluid was transferred to a water bath at 39°C until the RUSITEC fermentation chambers were ready to be filled.

On the first day of each experimental run, each of the 1 litre RUSITEC fermenters was initially filled with 100 ml of prewarmed synthetic saliva (Machmüller *et al.* (2002) and 820 ml rumen fluid strained through four layers of cheesecloth. Two nylon bags (70 × 140 mm, 100-µm pore size) were used for each fermenter. On the first day of each experiment, the first bag contained about 11 g DM of rumen digesta solids and the second bag contained an experimental diet (10.9 g of DM, 40 : 60 rice straw and CFM). After 24 h of incubation, on the second day of incubation, the fermenters were opened and the bag containing the rumen digesta solids was replaced with a nylon bag containing the experimental diet. Each bag with feed was therefore incubated for 48 h. Anaerobic conditions were reestablished in the fermenters by rinsing the system with gaseous N_2 for 3 min at a rate of 3 litres/min after the substrate had been replaced each day. Artificial saliva was supplied to each fermenter at a flow rate of 500 ml/day using a peristaltic pump (Gilson Minipuls 3, Middleton, WI, USA). The displaced effluent and fermentation gases were collected in an effluent vessel and gas-tight collection bag (Tecobag 81, Tesseraux Container, Bürstadt, Germany), respectively. To terminate fermentation in the effluent vessels, 10 ml of 10 mol/l H_2SO_4 was added to each of the effluent vessels. The samples for analysis were collected on days 7 through 10, after a 6-day adaptation period for the microbes. The number of samples was duplicated from four experimental days ($n = 8$), following Jalč *et al.* (2009). The substrates (clover, hay, rice straw and CFM) and their residues were collected after 48 h of incubation, dried at 70°C and subjected to chemical analysis.

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Measurements and sampling procedure for the incubated rumen fluid from the batch culture and RUSITEC systems

After 24 h of batch culture incubation, three of the six replicates were used to determine total gas production (GP), while the other three replicates were used to determine the basic parameters of the incubated rumen fermentation, the pH and the NH_3 concentration. The experiment was performed in three repetitions on three consecutive days. Total GP was determined at the end of the incubation period by the displacement of a syringe piston connected to the sample serum flasks. The total GP was expressed as ml per g DM, DM digestibility (dDM), OM, OM digestibility (dOM), incubated NDF and ADF. *In vitro* DM digestibility was analysed following the procedure described by Cieslak *et al.* (2016). Briefly, the residues of incubation obtained by filtering the incubated rumen fluid with the substrate through nylon with defined pore size (100 mm) were washed with 50 ml distilled water and dried at 105°C for 3 days. After oven drying, the samples were weighed and the percentage loss in the weight of the feed DM was calculated. The same bottles used for GP determination were then used for the analysis of DM digestibility. OM digestibility was determined by burning the residue DM in a muffle furnace at 550°C for 6 h. The pH of the incubated rumen fluid was measured using a pH meter (CP-104, Elmetron, Zabrze, Poland). Protozoa counts were determined following Cieslak *et al.* (2016) under a light microscope (Primo Star 5, Zeiss, Jena, Germany) using a drop of buffered rumen fluid of known volume (100 µl).

Quantitative analysis of the ammonia concentration was carried out using a modified version of Nessler's method, following Cieslak *et al.* (2016). Briefly, a sample of 3.6 ml of incubated rumen fluid was taken from the serum flasks and centrifuged (Hettich model 1615) at about 12,000 rpm, $G = 13{,}684$ g for 5 min. A 100 µl sample of the supernatant was transferred to a tube that contained 200 µl of 1% polyvinyl alcohol, 200 µl of 20% potassium sodium tartrate, 200 µl of Nessler reagent and 19.3 ml of ddH_2O, followed by 10 min incubation at room temperature. After incubation, the samples were examined with a spectrophotometer at 480 nm.

Metabolizable energy (ME) was estimated using the following equation from Menke *et al.* (1979):

$$\text{ME(MJ/kg DM)} = 2.20 + 0.136\text{GP} + 0.057\text{CP (\%)}\ [1\ \text{MJ ME} = 0.4185\ \text{Mcal ME}]$$

ME: metabolizable energy, GP: 24 h net gas production (ml/400 mg) and CP: crude protein.

Short-chain fatty acid (SCFA) concentration was calculated using an equation from Getachew *et al.* (2002):

$$\text{SCFA (mmol/200 mg DM)} = -0.00425 + 0.0222 \times \text{GP},$$

where GP is the net gas produced (ml) from a 200 mg DM sample after 24 h incubation. The samples of incubated rumen fluid from the RUSITEC system were collected 3 h before the addition of the bag with the feed. The pH, ammonia and protozoa counts were measured as described above for the incubated rumen fluid in the batch culture system. Total GP was determined by measuring the water displaced from a closed tube filled with a known volume of water after the bags of gas were connected and squeezed to release the gas.

The gases from the fermentation in the batch culture and RUSITEC systems were analysed for CH_4 concentration using a SRI310 gas chromatograph (SRI Instruments, Torrance, CA, USA) equipped with a thermal conductivity detector and a Carboxen-1000 column (mesh size 60/80; Sigma Aldrich, Poznań, Poland).

Samples of clover hay, rice straw and CFM, in both the *in vitro* batch culture and the RUSITEC experiments, were analysed using AOAC methods (2007) for DM (method no. 934.01) and for ash (method no. 942.05). Crude protein was determined using a VELP 129 analyzer (method no. 976.05) and the ether was extracted using a Soxhlet VELP SER-148 system (method no. 973.18). NDF (estimated using amylase and sodium sulphite and expressed with residual ash) and ADF were determined with the method of Van Soest *et al.* (1991). OM was calculated as the difference between DM and ash. Non-fibre carbohydrate (NFC) was calculated using an equation from NRC (2001):

$$\text{NFC\%} = 100 - (\text{NDF\%} + \text{CP\%} + \text{EE\%} + \text{Ash\%})$$

NFC: non-fibre carbohydrate, NDF: neutral detergent fibre, CP: crude protein and EE: ether extract.

Statistical analysis

The data from the *in vitro* batch culture were analysed along with RUSITEC digestibility and fermentation parameters using a general linear model following the User's Guide for the Statistical Analysis System (SAS Institute 1998). Separation of the group means was carried out using Duncan's new multiple range test. The following model was employed:

$$Y_{ij} = \mu + T_i + e_{ij},$$

where Y_{ij} is the observation of the model, μ is the general mean common element to all observation, T_i is the effect of treatment i and e_{ij} is the effect of errors. A P-value below 0.05 was considered to be statistically significant.

The enzyme activity data were analysed using PROC MIXED with POLYANOVA for the performance variables, where there were repeated measurements over time (cellulase and CMCase). The POLYANOVA model was used due to the triplicate repetitions for each variable, with the week taken as the repeat variable. Furthermore, the orthogonal response (both linear and quadratic) was tested in order to determine the difference in activity concerning the week variable.

Results

Cellulase and CMCase activities of the tested strains in culture media

The data in Fig. 1 show variations in cellulase activity and reducing glucose rate between all the tested strains. Of all the strains, *P. chrysosporium* showed the highest cellulase activity at 3.994 µmol/ml/min and the highest reducing glucose rate at 6000 µg/ml; this was followed by *B. megaterium* at 1.957 µmol/ml/min. The lowest cellulase activity and reducing glucose rate were found for the *T. viride* group, at 0.81 µmol/ml/min and 1000 µg/ml, respectively.

The data in Fig. 2 show variations in CMCase activity and total reducing glucose rate in all the strains. *P. chrysosporium* exhibited

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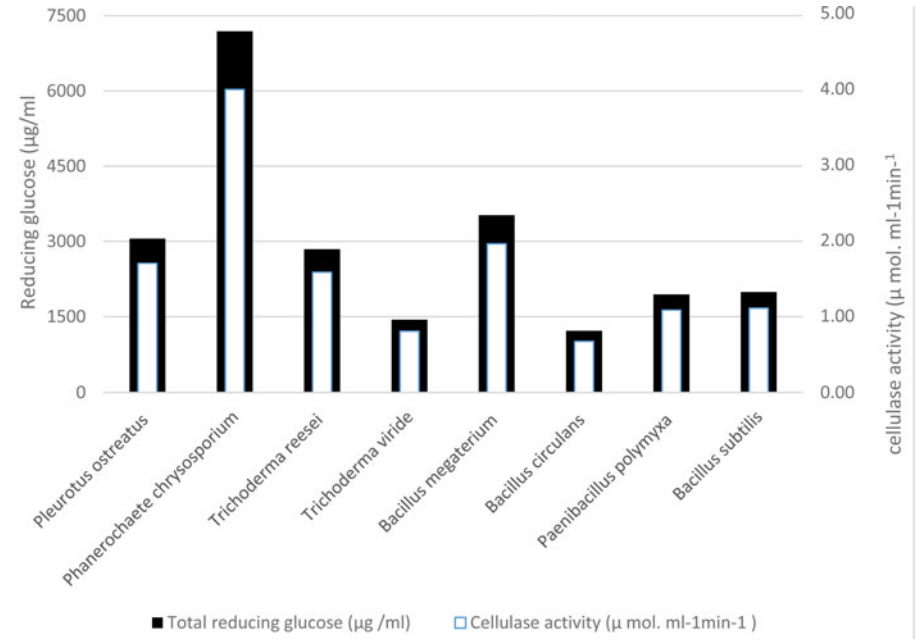

Fig. 1. Reducing glucose and cellulase activity rate of tested four fungal strains (*P. ostreatus*, *P. chrysosporium*, *T. reesei* and *T. viride*) and four bacterial strains (*P. polymyxa*, *B. megaterium*, *B. circulans* and *B. subtilis*) in culture media.

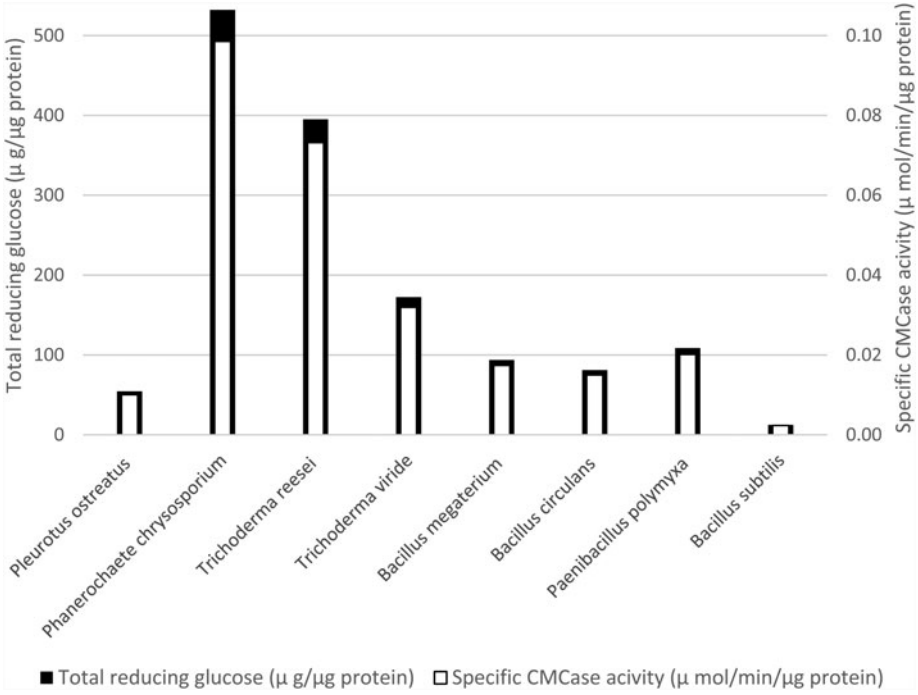

Fig. 2. Total reducing glucose and CMCase activity rate of tested four fungal strains (*P. ostreatus*, *P. chrysosporium*, *T. reesei* and *T. viride*) and four bacterial strains (*P. polymyxa*, *B. megaterium*, *B. circulans* and *B. subtilis*) in culture media.

the highest CMCase activity of all the strains, at 0.099 µmol/min/µg protein; *T. reesei* came next, at 0.073 µmol/min/µg protein. *B. subtilis* showed the lowest CMCase activity of all the tested groups, with a value of 0.002 µmol/min/µg protein. The same tendency was observed for the total reducing glucose rate (µg/µg protein).

Cellulase activity of biodual formulations during the 12-week storage period

The data in Fig. 3 show the variation in cellulase activity and between the tested biodual formulations. Generally, the cellulase activities decreased over time during the storage period. Two weeks after processing the biodual formulations, the cellulase activities of the individual strains ranged from 1.583 U/g in the case of *B. megaterium* to 0.493 U/g for *P. polymyxa*. Moreover, the mixed formulations showed a clear increase in cellulase activity over the individual formulations. The mixed biodual formulation, containing a combination of cellulases and microbial suspensions from all the strains, had the highest cellulase activity (1.566 U/g) of all the mixed formulations.

At the end of the 12-week storage period, all formulations showed clear decreases in cellulase activities compared to the initial storage stage. Among the single-strain formulations, the highest cellulase activities were recorded for *P. chrysosporium*,

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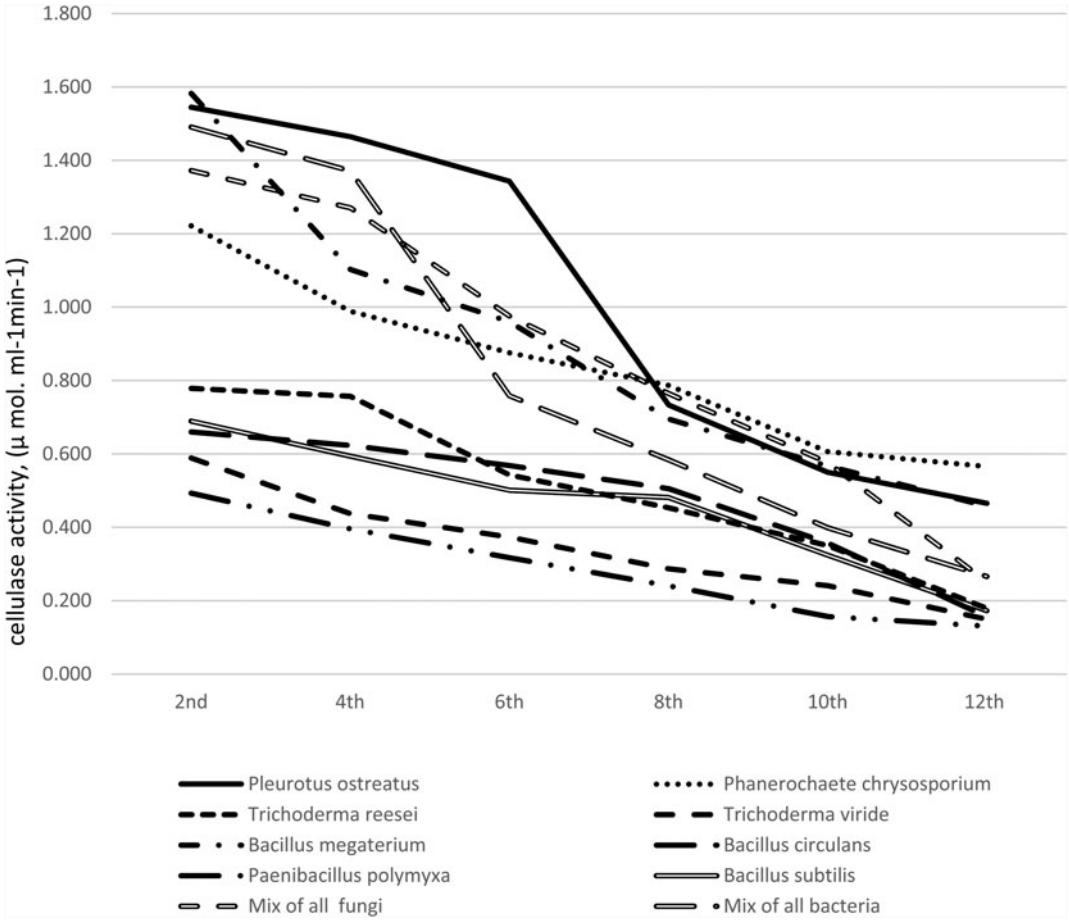

Fig. 3. Cellulase activities of four fungal strains (*P. ostreatus*, *P. chrysosporium*, *T. reesei* and *T. viride*), four bacterial strains (*P. polymyxa*, *B. megaterium*, *B. circulans* and *B. subtilis*), mix of all fungi and mix of all bacteria during 12 weeks of storage period.

P. ostreatus and *B. megaterium*, at 0.566, 0.465 and 0.458 U/g, respectively. The lowest cellulase activity was 0.131 U/g for *P. polymyxa*. The highest cellulase activity (0.603 U/g) among the mixed formulations was found for the biodual formulation containing the mixture of suspensions from all strains.

Table 1 shows the enzyme activity of different bacteria and fungi during culture. The highest cellulase activity was recorded for *P. ostreatus* at 1.017 µmol/ml/min, while the lowest was 0.298 µmol/ml/min for *P. polymyxa*. The fungi had higher average cellulase activities than the bacteria.

CMCase activities of the formulations during the 12-week storage period

The data in Fig. 4 show the variation in CMCase activities among the biodual formulations. Generally, CMCase activities decreased over time during the storage period. Two weeks after processing the biodual formulations, the single-strain formulations showed CMCase activities ranging from 0.099 U/g for *T. viride* to 0.023 U/g for *P. ostreatus*.

The mixed biodual formulations showed clear increases in CMCase activity over the single-strain biodual formulations. The mixture of all the strains had the highest CMCase activity of the mixed biodual formulations, at 0.08 U/g.

At the end of the 12-week storage period, all the biodual formulations showed a decrease in CMCase activity, compared to the initial storage stage. The highest CMCase activity among the single-strain biodual formulations was recorded for *B. subtilis*, at 0.017 U/g. Other strains recorded CMCase activities ranging from 0.006 to 0.0001 U/g. The highest CMCase activity among mixed biodual formulations, 0.007 U/g, was recorded for the formulation containing a mixture of fungal strains.

Batch culture evaluation

Dry matter (DM), organic matter (OM) digestibility and gas production

The data in Table 2 show no significant differences ($P = 0.723$) in terms of the DM digestibility of the rations with the addition of biodual supplements. However, statistically significant increases ($P = 0.004$) were found in the OM digestibility of the supplemented rations, compared to the control; this excludes rations supplemented with the *T. viride* and *B. circulans* supplements, which showed nonsignificant increases in OM digestibility (0.35 and 0.35, respectively).

Total GP expressed in ml/g of DM, OM, NDF and ADF of rations supplemented with various biodual formulations revealed statistically significant ($P \leq 0.004$) increases for the rations supplemented with biodual supplements, as compared to the control, but excluding the experimental biodual ration supplemented with both *T. viride* and *B. circulans*. However, no significant differences were seen between the different treatments in terms of total GP

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Table 1. Enzyme activities of different bacteria and fungi during culture

Source	Group	Cellulase activity (μmol/ml/min)	CMCase activity (μmol/ml/min)
	P. ostreatus	1.02[a]	0.008[f]
	P. chrysosporium	0.84[c]	0.023[c]
	T. reesei	0.51[d]	0.020[d]
	T. viride	0.35[f]	0.035[a]
∑Fungi mean		0.87	0.025
	B. circulans	0.48[de]	0.033[a]
	B. megaterium	0.89[b]	0.029[b]
	B. subtilis	0.46[e]	0.030[b]
	P. polymyxa	0.29[g]	0.014[e]
∑Bacteria mean		0.81	0.021
Between types of microbes			
	SE	0.028	0.0019
	P value		
	Group	<0.001	<0.001
	Group × Week	<0.001	<0.001
	Week		
	Linear	<0.001	<0.001
	Quadratic	<0.001	<0.001
Bacteria *vs* fungi			
	SE	0.073	0.0038
	P value		
	Source	0.106	0.023
	Source × Week	0.002	0.002
	Week		
	Linear	<0.001	<0.001
	Quadratic	<0.001	<0.001

SE, standard error of the mean.
[a,b,c,d,e,f]Different superscripts in the same column are statistically significant ($P < 0.05$).

per g of digestible DM (GP/dDM) or of digestible organic matter (GP/dOM).

The effects of the inoculant type (fungi or bacteria) were compared in terms of their performance in digestibility of DM and OM, as well as GP (ml/g). The mean effects of the fungi and bacteria were statistically compared to each other and to the control (Table 2). Generally, the fungal and bacterial biodual supplements showed significant ($P = 0.003$) increases in OM digestibility and GP as ml/g of DM, OM, NDF and ADF. However, no significant differences were found between the effects of fungal and bacterial biodual supplements in DM digestibility, GP/dDM or GP/dOM, compared to the control.

Methane production, ruminal fermentation and kinetic measurements

The data in Table 3 clearly show that no significant differences were recorded among the different experimental treatments in terms of total methane production (mmol/g DM), dDM, OM, NDF or ADF.

The total ammonia concentration obtained from the *in vitro* batch culture technique showed highly significant ($P < 0.001$) increases for all the supplemented rations, as compared to the control group. The greatest significant increase in ammonia was recorded for *B. subtilis*, at 124.2 mg/l. However, the pH values of the different supplemented rations showed widely varying decreases ($P = 0.001$) compared to the control ration. Moreover, no significant differences were recorded for the supplemented rations in the pH values of the incubated rumen fluids, except for in the case of those with *P. ostreatus*, where there was a significant ($P < 0.001$) decrease in the pH compared to those supplemented with *P. chrysosporium*, *B. megaterium* and *B. circulans*.

The data in Table 3 show significant increases in the calculated levels of SCFA (mmol/g DM; $P = 0.005$ for treatments and $P = 0.003$ for means) and ME (Mcal/kg DM; $P = 0.012$ for treatments and $P = 0.008$ for means) for most of the supplemented rations, with different biodual supplements, compared to the control; the exceptions are those supplemented with the biodual formula of *T. viride* and *B. circulans*, which showed no significant differences from the control.

The two enzyme sources (fungi or bacteria) showed no significantly different effects on methane production, ruminal fermentation and kinetic measurements compared to the control in any measurements. In all the tested groups, the enzyme source (regardless of origin and whether it was mixed or not) had no statistically significant effect on methane production, ruminal fermentation or kinetic measurements, compared to the control. Also, no significant differences were seen in fungal or bacterial inoculants (mixed biodual supplements) for methane production (mmol/g DM), dDM, OM, NDF or ADF compared to the control. However, the fungal and bacterial mixed biodual supplements showed significant decreases in pH and in mmol of CH_4 per g dOM compared to the control. In contrast, the biodual supplements recorded significant increases in ammonia concentration, SCFA mmol/g DM and ME (Mcal/kg DM) compared to the control.

RUSITEC evaluation

On the basis of the results shown in Figs 1 and 2 and in Tables 2 and 3, the best biodual fungal formulation was *P. chrysosporium*, which had the greatest capability of producing cellulase and CMCase enzymes, as well as higher DM and OM digestibility and total accumulated gas, of all the fungal biodual supplements. Furthermore, the best bacterial biodual supplement was *B. subtilis*, which showed the highest levels of OM digestibility and total accumulated gas of all the bacterial biodual supplements. These supplements were thus selected for comparison with a mixture of them in the RUSITEC experiment, where clover hay treatment was used to ensure proper fermentation quality. The rice straw and clover hay were minced in a domestic food mixer (Moulinette S, Moulinex, Paris, France), while the CFM was ground to a diameter of 1 mm.

Component digestibility and fermentation parameters

The data in Table 4 show that no significant differences were detected between the rations supplemented with *P. chrysosporium*, *B. subtilis* and their mixed inoculants (biodual supplements) in DM and OM digestibility ($P = 0.363$ and $P = 0.169$, respectively). However, the ration supplemented with *B. subtilis* and the mixed inoculants had significantly higher crude protein ($P < 0.001$) and ether extract digestibility ($P < 0.001$) than did the

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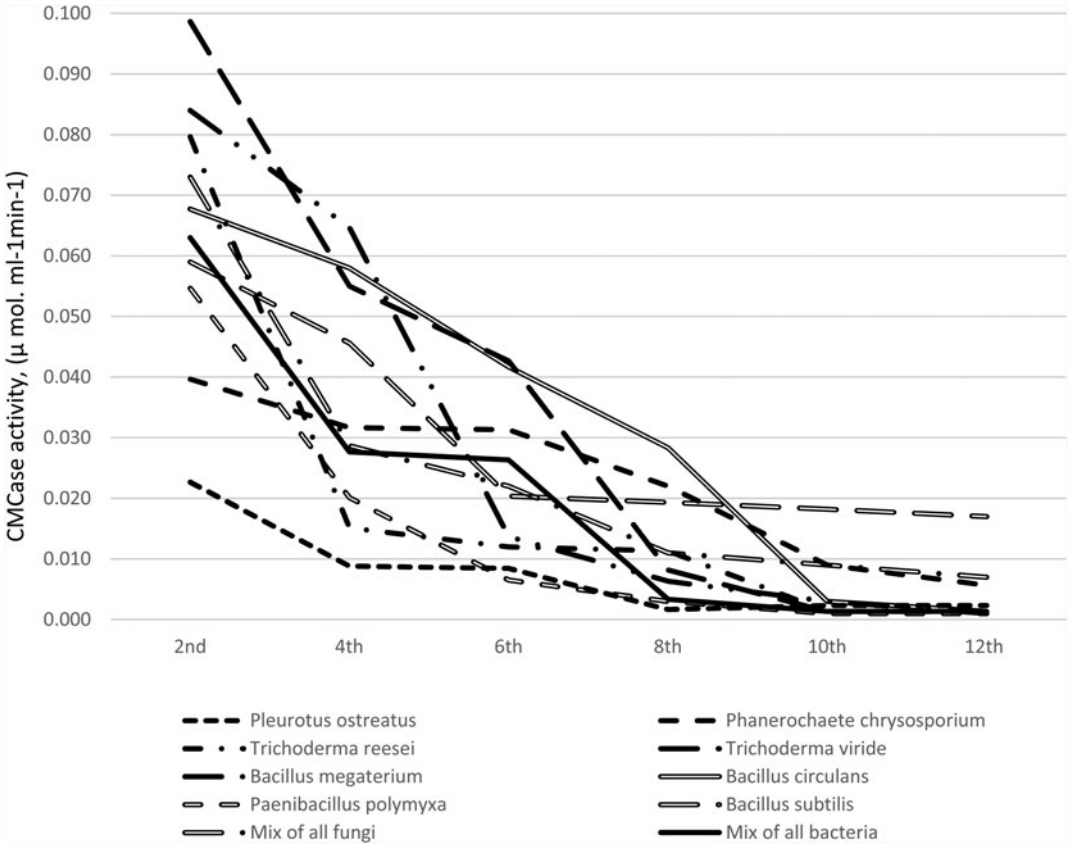

Fig. 4. CMCases activities of four fungal strains (*P. ostreatus*, *P. chrysosporium*, *T. reesei* and *T. viride*), four bacterial strains (*P. polymyxa*, *B. megaterium*, *B. circulans* and *B. subtilis*), mix of all fungi and mix of all bacteria during 12 weeks of storage period.

ration supplemented with the *P. chrysosporium* inoculant. Moreover, the ration supplemented with mixed inoculants showed higher NDF and ADF digestibility ($P < 0.001$) than did the ration supplemented with the *P. chrysosporium* and *B. subtilis* inoculants. The ration supplemented with *P. chrysosporium* inoculants showed significantly higher NFC digestibility levels ($P < 0.001$) than did the rations supplemented with *B. subtilis* and mixed inoculants.

The data in Table 4 show that there were no significant differences in ammonia concentration or pH values in the rations supplemented with different inoculants.

Total gas and methane production

The data in Table 5 clearly show that no significant differences were recorded in terms of the total GP ml per g of DM, dDM, OM or dOM for the rations supplemented with different inoculants. These results agree with those presented in Table 2, which indicate that there are no significant effects among the different experimental treatments in terms of DM digestibility. In addition, no significant differences were recorded between the rations supplemented with different inoculants in terms of total methane production mmol per g DM, dDM, OM or dOM.

Microbial counts

The total rumen protozoa and Entodiniomorpha counts from the RUSITEC samples did not show any statistically significant differences between groups ($P = 0.385$); however, the total Holotricha in the *P. chrysosporium* and *B. subtilis* inoculants showed statistical differences ($P = 0.029$) from the other groups.

Discussion

Cellulase and CMCase activities of the test strains in culture media and biodual formulations

Enzymes are protein compounds that are highly dependent on their structure to perform their catalytic activity (Sloth *et al.*, 2008). Cellulases are among the most important enzymes and possess a range of applications (Mai *et al.*, 2004). The cellulase and CMCase activities of the test strains were assessed for both the production media and formulated samples. The cellulases and CMCases produced by the strains in the production media showed distinguishable differences in their activities. These results are in line with those reported by Reddy *et al.* (2016) who stated that, although microorganisms with the ability to produce cellulases belong to very limited groups, the types and activities of the enzymes they produce are diverse, depending on the strain, the growth substrate and the culture. This diversity could be attributed to differences in their polypeptide characteristics, such as the capability of enzymes to adsorb to cellulose, their molecular weight, their isoelectric points, carbohydrate contents, catalytic activities, substrate specificities and amino acid compositions and sequences. Several studies have reported that the biosynthesis of cellulases increases when microorganisms are exposed to complex substrates (Wymelenberg *et al.*, 2002). Cellulases are thus

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Table 2. Effect of different biodual formulations on DM and OM digestibility and total GP after 24 h *in vitro* fermentation

	Digestibility		GP (ml/g)					
Treatments	DM	OM[1]	GP/DM[1]	GP/dDM	GP/OM[1]	GP/dOM	GP/NDF[1]	GP/ADF[1]
Control	0.28	0.34^{cC}	73^{cC}	259	80^{cC}	311	172^{cC}	258^{cC}
P. ostreatus	0.31	0.38^{a}	96^{a}	318	106^{a}	318	226^{a}	339^{a}
P. chrysosporium	0.32	0.37^{ab}	93^{ab}	295	103^{ab}	295	220^{ab}	330^{ab}
T. reesei	0.31	0.38^{a}	95^{a}	312	105^{a}	313	224^{a}	337^{a}
T. viride	0.31	0.35^{bc}	82^{bc}	266	90^{bc}	266	193^{bc}	290^{bc}
Fungal mean	0.32	0.37^{A}	91^{A}	289	100^{A}	289	213^{A}	320^{A}
B. megaterium	0.27	0.37^{ab}	90^{ab}	341	99^{ab}	342	211^{ab}	316^{ab}
B. circulans	0.26	0.35^{bc}	82^{bc}	325	90^{bc}	325	193^{bc}	290^{bc}
P. polymyxa	0.28	0.36^{ab}	87^{ab}	310	96^{ab}	310	205^{ab}	308^{ab}
B. subtilis	0.29	0.37^{ab}	94^{ab}	330	103^{ab}	330	219^{ab}	330^{ab}
Bacterial mean[1]	0.28	0.36^{B}	88^{B}	326	97^{B}	326	207^{B}	311^{B}
Treatments								
SE	0.03	0.06	3.6	33.1	56.0	32.3	8.5	12.7
P value	0.723	0.004	0.004	0.475	0.003	0.682	0.004	0.004
Means								
SE	0.12	0.03	1.9	13.1	2.1	13.5	4.5	6.7
P value	0.072	0.003	0.003	0.070	0.003	0.199	0.003	0.003

SE, standard error of the mean; GP/DM, gas production per g DM; GP/dDM, gas production per g degraded DM; GP/OM, gas production per g OM; GP/dOM, gas production per g degraded OM; GP/NDF, gas production per g NDF; GP/ADF, gas production per g ADF.
[1]a,b,c: Fungal and bacterial mean with different superscripts in the same column are statistically significant different ($P < 0.05$).
[1]A,B,C: Fungal and bacterial means with different superscripts in the same column are statistically significant different ($P < 0.05$).

known to constitute an inducible enzyme system (Lynd *et al.*, 2002), whereas glucose and other hydrolysis products repress the production of these enzymes (Lynd *et al.*, 2002; Reddy *et al.*, 2016).

In accordance with the inactivation profile of the formulations we tested, the results are suitable for predicting the potential stability and shelf life of the biodual formulations. These profiles showed changes in the activities of enzymes upon testing every 2 weeks over the 12-week storage period. The biodual formulations showed different decreased profiles in the activities of cellulases and CMCases, with a decrease generally discernible in cellulolytic enzyme activities in the first sample, as compared to their activities in media. This could be attributed to the decrease in the cellulases' water content, leading to the disorganization of the enzymes' protein conformation, and thus inactivation of the polypeptide characteristics of the cellulases during the formulation process (Wang *et al.*, 2018). This decrease could also be due to the loss of the native structures of the enzymes, as a result of them unfolding during the drying process (Sloth *et al.*, 2008). Enzyme inactivation during drying depends on the nature of the enzyme, the formulation of the ingredients and the process operating parameters; each formulation thus exhibits a different inactivation profile (Sloth *et al.*, 2008).

Studies have shown that cellulose has a synergistic reaction sequence (Lynd *et al.*, 2002), so the production of glucose from cellulose leads to a decrease in cellulase activity (Keshk and Sameshima, 2005). The formation of cellobiose as an intermediate metabolic product of cellulase hydrolysis reactions may also be a reason for the inactivation of the cellulase (Lynd *et al.*, 2002).

Batch culture evaluation of dry matter (DM), OM digestibility and gas production

The significant increase observed in OM digestibility and in millilitre of gas produced per g of DM, OM, NDF and ADF in the supplemented rations, as compared to the control ration, could be attributed to the effect of the fibrolytic exogenous enzyme found in the biodual supplements. These effects might be explained in turn by the addition of fibrolytic enzymes to roughage in the *in vitro* technique, which leads to the release of reducing sugars and other products that could both enhance rumen microbial population and increase the amount of feedstuff digested (Salem *et al.*, 2013; Mocherla and Kavitha, 2017; Sheikh *et al.*, 2017). Wang *et al.* (2018), in an *in vitro* study reported that the stimulation of rumen microbial population (nonfibrolytic and fibrolytic bacteria) by enzymes could result in a higher microbial biomass, which could then provide more total polysaccharidase activity to digest feedstuff. Blümmel and Ørskov (1993) reported that the fermentation of organic compounds produces gas as an end product, providing evidence for the strong correlation between OM digestibility and volume of gas produced. In this linear relationship, many studies have also shown that rations supplemented with fibrolytic enzymes led to increased *in vivo* and *in vitro* DM and OM digestibility, and particularly fibre digestibility (Lamid *et al.*, 2013). Jalilvand *et al.* (2008) also found a significant increase in DM digestibility resulting from the addition of fibrolytic enzyme to wheat straw, which would be expected to elevate fibre digestion by increasing the rate of ruminal digestion of the potentially

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Table 3. Effect of different biodual formulations on methane (CH_4) production, rumen fermentation and short chain fatty acids and metabolized energy after 24 h *in vitro* fermentation

Treatments		Methane[1] production, mmol/g						Rumen fermentation parameters[1]			
		CH_4/g DM	CH_4/g dDM	CH_4/g OM	CH_4/g[1] dOM	CH_4/g NDF	CH_4/g ADF	Ammonia[1] mg/l	pH[1]	SCFA[1] mmol/g DM	ME[1] Mcal/ kg DM
Control		0.92	3.3	1.0	4.0^{A}	2.16	3.4	74.9^{cB}	6.32^{aA}	0.32^{cB}	2.58^{cB}
P. ostreatus		0.92	3.0	1.0	3.0	2.15	3.3	101^{ab}	6.22^{c}	0.42^{a}	2.72^{a}
P. Chrysosporium		0.90	2.9	1.0	2.9	2.12	3.2	120^{ab}	6.27^{b}	0.41^{ab}	2.70^{ab}
T. reesei		0.90	2.9	1.0	3.0	2.12	3.2	127^{a}	6.25^{bc}	0.42^{a}	2.71^{a}
T. viride		0.89	2.9	1.0	2.9	2.09	3.2	120^{ab}	6.26^{bc}	0.36^{bc}	2.63^{c}
Fungal mean		0.90	2.9	1.0	2.9^{B}	2.12	3.2	118^{A}	6.25^{B}	0.40^{A}	2.68^{A}
B. megaterium		0.90	3.4	1.0	3.4	2.11	3.2	119^{ab}	6.27^{b}	0.39^{ab}	2.67^{ab}
B. circulans		0.82	3.3	0.9	3.3	1.92	3.2	119^{ab}	6.27^{b}	0.36^{bc}	2.63^{bc}
P. polymyxa		0.88	3.1	1.0	3.1	2.07	2.9	117^{ab}	6.25^{bc}	0.38^{ab}	2.66^{ab}
B. subtilis		0.95	3.4	1.0	3.4	2.23	3.1	124^{a}	6.24^{bc}	0.41^{ab}	2.70^{ab}
Bacterial mean		0.89	3.3	1.0	3.3^{B}	2.08	3.1	120^{A}	6.26^{B}	0.39^{A}	2.66^{A}
Treatments	SE	0.036	0.33	0.40	0.37	0.083	0.13	6.1	0.012	0.016	0.025
	P value	0.551	0.788	0.539	0.439	0.530	0.545	0.001	0.001	0.005	0.012
Means	SE	0.015	0.130	0.017	0.150	0.038	0.056	2.9	0.001	0.008	0.011
	P value	0.685	0.087	0.650	0.010	0.658	0.668	0.001	0.001	0.003	0.008

SE, standard error of the mean; CH_4, methane, SCFA, short-chain fatty acids; ME, metabolizable energy.
[1]a,b,c: Means with different superscripts in the same column are statistically significant different ($P < 0.05$).
[1]A,B: Fungal and bacterial means with different superscripts in the same column are statistically significant different ($P < 0.05$).

Table 4. Effect of *P. chrysosporium*, *B. subtilis* and their mixture (1 : 1) on dietary component digestibility and fermentation parameters after 48 h of RUSITEC fermentation

	Treatments[1]				
	P. chrysosporium	*B. subtilis*	*P. Chrysosporium* + *B. subtilis*	SE	*P* value
Component digestibility					
DM	0.61	0.60	0.61	0.071	0.363
OM	0.60	0.60	0.62	0.077	0.169
Crude protein	0.67^{b}	0.71^{a}	0.71^{a}	0.068	0.001
Ether extract	0.63^{b}	0.74^{a}	0.73^{a}	0.069	0.001
NDF	0.23^{b}	0.24^{b}	0.33^{a}	0.012	0.001
ADF	0.16^{b}	0.16^{b}	0.42^{a}	0.013	0.001
NFC	0.98^{a}	0.91^{b}	0.92^{b}	0.017	0.001
Fermentation parameters					
Ammonia (mg/l)	59	59	63	2.9	0.588
pH	6.78	6.78	6.77	0.020	0.492

SE, standard error of the mean.
[1]a,b: Means with different superscripts in the same row are statistically significant ($P < 0.05$); Clover hay was the primary substrate used in conjunction with bacterial and fungal enzymes.

digestible NDF fraction (Yang *et al.*, 1999). Tang *et al.* (2008) found that the digestibility of dietary components (DM, OM and NDF) and GP in an *in vitro* fermentation technique was more pronounced when different crop residuals were supplemented by fibrolytic enzymes, mixed with either probiotic bacteria or yeast, as compared to the control.

The insignificant differences recorded for the different treatments in terms of DM and OM digestibility and total GP – as

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Table 5. Effect of *P. chrysosporium*, *B. subtilis* and their mixture (1 : 1) on gas and methane production and total counts of protozoa after 48 h fermentation in rumen stimulation technique (RUSITEC)

	Treatments[1]				
	P. Chrysosporium	*B. subtilis*	*P. Chrysosporium* + *B. subtilis*	SE	*P* value
Gas production					
GP/DM, ml/g	432	459	435	21.9	0.214
GP/dDM, ml/g	492	516	484	24.6	0.259
GP/OM, ml/g	705	763	707	33.7	0.214
GP/dOM, ml/g	815	862	783	38.6	0.131
Methane production					
CH_4/DM, mmol/g	7.2	7.7	8.3	0.34	0.158
CH_4/dDM, mmol/g	8.2	8.6	9.2	0.39	0.255
CH_4/OM, mmol/g	11.7	12.8	13.5	0.57	0.161
CH_4/dOM, mmol/g	13.6	14.4	14.9	0.65	0.404
Protozoa counts					
Holotricha ($\times 10^2$)	12^a	12^a	4^b	2.1	0.029
Entodiniomorpha ($\times 10^3$)	4.1	3.2	3.6	0.73	0.714
Protozoa ($\times 10^3$)	5.3	4.4	4.0	0.70	0.385

SE, standard error of the mean; GP/DM, gas production per g DM; GP/dDM, gas production per g degraded DM; GP/OM, gas production per g OM; GP/dOM, gas production per g degraded OM; CH_4, methane.
[1]a,b: Means with different superscripts in the same row are statistically significant ($P < 0.05$); Clover hay was the primary substrate used in conjunction with bacterial and fungal enzymes.

well as the insignificant differences between the two sources of inoculants – may be attributed to the addition of a constant concentration of cellulase activity (1 U cellulase per kg DM) to all the treatments in each formulation.

Methane production, ruminal fermentation and kinetic measurements

In ruminants, CH_4 is produced either by a process of microbial methanogenesis from hydrogen and CO_2 (from hydrolysed carbohydrates such as cellulose and hemicellulose), or by microbial fermentation of amino acids. Our results show that the addition of biodual supplements does not significantly affect the methane production process. Furthermore, there was no correlation between digestibility and methane production. Supplementing the rations with biodual supplements did not have a significant effect on the energy lost through methane production, which will help in lowering environmental emissions (Bhatta *et al.*, 2007). In this connection, Demarchi *et al.* (2003) reported a correlation between methane production and fibre digestibility, since forage with a high content of effectively degraded fibre promotes greater CH_4 production. Our results also disagree with those of Kurihara (1995), who observed that CH_4 production in less digestible forage was lower than that in highly digestible forage.

Ammonia concentration generally acts as an indicator of protein digestibility (Peripolli *et al.*, 2017). The average ammonia concentration of all treatments ranged from 100.7 to 126.7 mg/day, which is within the normal range required for optimal fermentation and rumen microbial growth (Anantasook and Wanapat, 2012). The significantly higher ammonia concentration in the ration supplemented with biodual supplements than in the control may be due to the effect of the cellulolytic activity of the biodual supplements, which improves OM digestibility (Table 1). From these results, it could be suggested that biodual supplements increase the digestion of carbohydrate directly and of nitrogen indirectly, consequently increasing ammonia concentration. These results are in line with those reported by Peripolli *et al.* (2017), who noted that fibrolytic enzymes tended to increase ammonia concentration in *in vitro* fermentation studies.

pH is an important indicator for rumen fermentation status (Gunun *et al.*, 2013), as it regulates the relationship between microorganisms and substrates in the rumen. Thus, pH values near neutral increase bacterial adhesion to the fibre (Allen and Mertens, 1988). In this study, pH values ranged from 6.22 to 6.32, which is considered optimal for normal rumen microbial fermentation (Anantasook and Wanapat, 2012). In response to the higher digestibility rate for the rations supplemented with biodual supplements, our results indicate a significant reduction in the pH value for the supplemented rations, as compared to the control. These results are in accordance with those of Sheikh *et al.* (2017), who suggested that using fibrolytic enzymes in combination with probiotics had a significant positive effect on the pH of the *in vitro* medium. In contrast, some researchers have observed either no effect, or even a reduction in rumen pH, upon supplementation with yeast (Ganai *et al.*, 2015). These variations may be attributed to different experimental conditions related to rumen liquor sources, diet composition, experimental design, enzyme dosages and sources, and types of microorganisms used (in terms of strain, dose, combination and viability).

Fibrolytic enzymes are capable of digestibility lignocellulosic materials to produce SCFA (Kumar *et al.*, 2009). In the current study, biodual supplements significantly increased GP and organic matter digestibility, leading to greater SCFA production and metabolized energy in all the supplemented rations than in the control; the only exceptions were the groups containing *T. viride* and *B. circulans*. These results suggest that increased SCFA

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and ME values could be a response to increased digestibility and GP (Table 2), which could be attributed to the addition of biodual supplements. Research by López *et al.* (2013) suggests that fibrolytic enzymes have the ability to alter the diversity of rumen microbial communities, which in turn affect SCFA values. These results are in line with those obtained by Salem *et al.* (2013), who reported that fibrolytic enzymes can increase total SCFA concentrations. Similar notable increases were found in the same parameters by Selçuk *et al.* (2016), thus providing more evidence for positive correlations between *in vitro* OMD, the amount of gas released and ME during fermentation. However, López *et al.* (2013) and Beauchemin *et al.* (2003) reported that suitable fibrolytic enzymes can improve fermentation efficiency, thus increasing metabolized energy, particularly when the substrate is made of fibre-rich material and is limited in nutrient energy.

RUSITEC evaluation

The main goal of this trial was to compare the compatibility of the best fungal and bacterial biodual supplements, and their combinations, in a long-term *in vitro* study of the ruminal ecosystem. The nonsignificant differences documented in terms of DM and OM digestibility, total gas and methane production, ammonia concentration and pH values among the different biodual supplements may be due to the additional constant concentration of cellulase activity (1 U cellulase/kg DM) to each formula in the experimental ration. In this regard, Beauchemin *et al.* (2003) reported that exogenous enzymes have the potential to increase ruminal forage digestibility. However, the supplementation rate that give the optimal enzyme level appears to be dependent on the diet type, with responses to incremental levels of enzymes typically being nonlinear (Kung *et al.*, 2000).

The significant increase in crude protein and ether extract digestibility for the ration supplemented with *B. subtilis* and mixed inoculants, compared to that with the *P. chrysosporium* inoculant, may be due to the effect of *B. subtilis* spore cells in these inoculants, which act as probiotics and improve dietary component digestibility, especially for proteins. Qiao *et al.* (2009) found that using inoculants containing *B. subtilis* and *B. licheniformis* as feed additives increased rumen digestibility in lactating Holstein Friesian dairy cows.

The significant increase in NDF and ADF digestibility for the ration supplemented with mixed inoculants, compared to *B. subtilis* and *P. chrysosporium* inoculants given separately, may be due to the two inoculant sources having a synergistic effect.

The simultaneous use of fungal and bacterial exogenous cellulases on rice straw roughage improves its digestibility without producing a negative effect on other rumen parameters. It can thus be concluded that the use of a mixed culture of fungal and bacterial biodual formula in low-quality forage treatment may be a means of producing improved feed for ruminants in semiarid and arid regions.

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Conflict of interest. We declare that we have no conflicts of interest.

Ethical standards. Not applicable; rumen fluid was obtained from sheep after slaughterhouse processing.

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