In vitro growth and starch digestion by *Entodinium exiguum* as influenced by the presence or absence of live bacteria

M. Fondevila and B. A. Dehority

Department of Animal Sciences, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster 44691-4096

ABSTRACT: In a preliminary study, the addition of antibiotics was shown to reduce bacterial concentrations in *Entodinium exiguum* cultures by more than 99% in 4 h, whereas the protozoal population was apparently unaffected. Using this procedure, the growth and amylolytic capability of *Entodinium exiguum*, in the presence or absence of live bacteria, was studied in vitro. Treatments for Trial 1 were protozoa plus antibiotics (PA), PA plus autoclaved bacteria (PAB), protozoa plus living bacteria (PLB), and only bacteria (BAC). Autoclaved or non-autoclaved cornstarch was used as an energy source. Treatment main effects were as follows: higher concentration of *E. exiguum* in PLB than in PA or PAB at 24 and 48 h (*P* < 0.01); PA and PAB were not different (*P* > 0.05); concentrations of *E. exiguum* higher in autoclaved cornstarch at 12 h (*P* < 0.05) but lower than in non-autoclaved cornstarch at 24 and 48 h (*P* < 0.01); and starch digestion in PLB was higher than in PA and PAB at all time periods, but only greater than BAC up to 24 h (*P* < 0.01). In Trial 2, only treatments PA, PLB, and BAC were tested. Rice starch and cornstarch were used as substrates. With rice starch, growth was higher in PLB than in PA at 24 and 48 h (*P* < 0.05). Starch digestion started earlier in PLB with rice starch (*P* < 0.05) but was complete for both substrates after 24 h. Up to 12 h (autoclaved cornstarch and rice starch) and 24 h (non-autoclaved cornstarch and cornstarch), the sum of digestion by bacteria and protozoa did not equal the extent of digestion in PLB, suggesting some kind of synergism. Total extent of digestion with protozoa was similar between the two sources; however, bacteria digested rice starch faster and to a greater extent than cornstarch. Approximate lag times with rice starch, autoclaved cornstarch, and non-autoclaved cornstarch were 6, 3, and 12 h for bacteria and < 6, 3, and 9 h for protozoa, respectively. Rate of digestion for non-autoclaved cornstarch was similar for bacteria and protozoa, whereas the rate of bacterial digestion was much faster with the other two substrates (autoclaved cornstarch and rice starch).

Key Words: *Entodinium exiguum*, In Vitro Growth, Rumen Protozoa, Starch Digestion

Introduction

Species of *Entodinium* generally comprise over 90% of the total rumen protozoa in animals fed high-concentrate diets (Williams and Withers, 1993; Franzolin and Dehority, 1998). *E. exiguum*, one of the smaller rumen protozoa, can be found in many host species (Ogimoto and Imai, 1981); however, little is known about its generation time and role in starch digestion. Even though the specific amylolytic activity of the genus *Entodinium* seems to be lower than that of larger protozoa (Coleman, 1986; Jouany and Ushida, 1994), referring enzymatic activity to microbial mass shows their important contribution to the overall process in the rumen. Strategies of starch digestion vary between protozoa and bacteria. In bacteria, enzymatic activity is exocellular, whereas protozoa ingest large amounts of particulate starch very rapidly and then digest it intracellularly (Coleman, 1992). Protozoa also ingest large amounts of bacteria and thus may affect total numbers and bacterial contribution to starch digestion. Whereas Coleman (1989) suggested that amylolytic bacterial numbers may decrease with high numbers of *Entodinium* spp., Williams and Withers (1993) did not observe differences in amylolytic bacterial concentrations between faunated (96% *Entodinium*) and defaunated sheep.

Study of starch digestion by a given protozoal species requires isolation from other protozoa and from amylolytic bacteria. Because the protozoa require bacteria for...
their survival (Onodera and Henderson, 1980), it is necessary to determine whether cultivation without bacteria allows protozoa to behave normally during the experiments.

The main objectives of this work were 1) to study growth and starch digestion by *E. exiguum*, alone or in co-culture with live bacteria; 2) to compare this activity with bacterial starch digestion; 3) to determine the potential role of *E. exiguum* in overall rumen starch digestion; and 4) to study the effect of the type of starch on the above-mentioned parameters.

**Materials and Methods**

Isolation and *In Vitro Maintenance of Entodinium Exiguum Cultures*. Rumen contents, obtained from a sheep fed alfalfa hay located at the Ohio Agricultural Research and Development Center, Wooster, were filtered through cheesecloth and 1 mL was added anaerobically to 9 mL of anaerobic dilution solution (Bryant and Burkey, 1953) in a 16- × 150-mm culture tube. The tube was placed in a 39°C water bath and continuously flushed with CO₂. Samples were removed from the tube and placed as droplets on a glass slide and one or more *E. exiguum* cells were drawn into a capillary pipette under the microscope. After checking to verify that the pipette contained only *E. exiguum* cells, the cells were inoculated into a 16- × 150-mm culture tube containing 10 mL of medium M (Dehority, 1998). The tube was closed and incubated at 39°C at an angle of 10°. All inoculations, transfers, feeding procedures, and experiments were carried out anaerobically under O₂-free CO₂. The culture tubes were opened daily and the protozoa were fed with 0.1 mL of a suspension containing 1.5% ground wheat and 1.0% orchardgrass hay (Dehority, 1998).

The tube was observed under a microscope at 30× magnification to verify the establishment and growth of the protozoal culture. Purity was determined in sub-samples examined at 450× and 1,000× magnifications. For routine maintenance, 5 mL of the culture was transferred every 4 d with the help of a wide-mouth (2-mm opening) pipette to another tube containing 5 mL of fresh medium plus 0.1 mL of substrate suspension. Average size of the *E. exiguum* cells was 37 (30 to 48) μm in length and 22 (15 to 33) μm in width. Before starting the experiments, the culture was allowed to stabilize for 1 mo.

**Experimental Procedures.** The dependence of *E. exiguum* on live bacteria for growth was studied in two trials, using starch with two different particle sizes, corn (large) or rice (small), as substrates. In addition, the effect of autoclaving the starch was investigated. An initial experiment was conducted to determine the effect of antibiotics on the bacteria present in the protozoal culture and their possible effect on *E. exiguum* itself. Nine test tubes filled with 5-mL volumes of medium M were autoclaved. Immediately before inoculation, 1 mL of antibiotic solution and 0.1 mL of feed suspension were added. The antibiotic solution contained 12,000 U penicillin G and 780 U streptomycin per milliliter, dissolved in CO₂-gassed distilled water and filtered through a 0.2-μm Acrodisc membrane filter (Gelman Sciences, Ann Arbor, MI). Tubes were inoculated with 2.5 mL of *E. exiguum* culture and incubated at 39°C at an angle of 10° for 4, 8, or 24 h (three tubes at each time period). A 1-mL subsample was taken from both the inoculum and all the tubes and mixed with 1 mL of 18.5% formaldehyde for protozoal counts. A second 1-mL subsample was taken from the inoculum and from the tubes incubated for 4 or 8 h for total bacterial counts.

In Trial 1, 6.6 mL of phosphate buffer (3.3 mL of 0.63 M NaH₂PO₄ and 3.3 mL of 0.43 M K₂HPO₄) and 0.1 g of trypticase were added per 100 mL to medium M. Cornstarch, 0.35 g/100 mL, was added either before tubing and autoclaving or to the autoclaved medium just prior to dispensing it into sterile tubes. Both media were tubed anaerobically in 8-mL volumes into 16- × 150-mm culture tubes. The treatments were protozoa only (PA), protozoa plus dead bacteria (PAB), protozoa plus live bacteria (PLB), and live bacteria only (BAC). All treatments were run in duplicate. In addition to the 8 mL of basal medium, the following additions were made for the different treatments: PA, 1.5 mL of antibiotic solution and 4.0 mL of protozoal culture as inoculum; PAB, 1.5 mL of solution containing dead bacteria and antibiotics plus 4.0 mL of protozoal culture; PLB, 1.5 mL of sterile distilled water plus 4.0 mL of protozoal culture; BAC, 1.5 mL of sterile distilled water plus 4.0 mL of protozoa-free medium obtained from the protozoal culture. The antibiotic solution was the same as that used in the initial experiment. The solution of dead bacteria was prepared as follows: 100 mL of supernate, obtained by centrifuging strained rumen fluid at 1,000 × g for 10 min, was centrifuged at 21,000 × g for 7 min; the supernate was decanted; the pellet was dried at 100°C; the pellet was dissolved in 40.0 mL of CO₂-gassed distilled water; and the pellet was autoclaved, after which antibiotics were added to obtain the same concentration as in the antibiotic solution above. The living bacteria (treatments PLB and BAC) were those already present in the protozoal culture, and the inoculum for the BAC treatment was obtained by filtering the protozoal culture through a membrane filter (5-μm pore size). In order to kill the bacteria before inoculation in treatments PA and PAB, the protozoal culture was pretreated by adding antibiotic solution to the inoculum (4 mL of culture plus 1 mL of antibiotic solution) 4 h before the start of the experiment. Tubes were incubated as before for 3, 6, 9, 12, 24, or 48 h. After each incubation period, tubes for treatments PA, PAB, and PLB were sampled (1 mL) for protozoal counts (except at the 9-h incubation period) as described previously. Another 1-mL sample was taken from tubes for all treatments and frozen immediately for subsequent starch analysis. Zero-time samples were taken from each tube for the determination of initial starch concen-
In vitro culture of Entodinium exiguum

The inocula for treatments PA, PAB, and PLB were sampled for determination of protozoal concentrations. The inoculum for treatment BAC and two tubes from all treatments after 24 h of incubation were sampled for determination of bacterial concentrations.

In Trial 2, experimental culture media were prepared as described for non-autoclaved cornstarch in Trial 1. However, two starch sources were used because of their differences in particle size: cornstarch, which ranges from 9 to 19 µm in diameter, and rice starch, which ranges from 3 to 8 µm in diameter. Treatments were PA, PLB, and BAC for each source of starch and were prepared as in Trial 1. Samples for both protozoal counts and starch analysis were taken after 6, 12, 24, 36, and 48 h. Each experimental tube was sampled immediately after inoculation to determine initial protozoal and bacterial concentrations.

Microbiological and Chemical Analyses. Samples for determination of protozoal concentration (1 mL of culture plus 1 mL of formaldehyde) were diluted with 2 mL of 30% glycerol, stained with two drops of brilliant green dye, and allowed to stand overnight. One milliliter was pipetted into a Sedgewick-Rafter chamber and Entodinium exiguum were counted under the microscope at 100× magnification, as described by Dehority (1984). Protozoal concentration was estimated as a percentage of initial inoculum concentration. Generation time (h) for each time of incubation was estimated from the equation proposed by Creager et al. (1990) for exponential growth: 

\[
\text{Generation time} = \frac{\text{transfer interval}}{\log \text{final concentration} - \log \text{initial concentration}} / \log 2
\]

Bacterial concentrations were determined by serially diluting 1-mL samples in 9 mL of anaerobic dilution solution and inoculating triplicate MPN tubes with \(10^4\) to \(10^9\) dilutions (Dehority et al., 1989). Growth was estimated after 7 d by pH differences.

Samples for starch analysis (stored less than 7 d at −4°C in glass tubes) were thawed, autoclaved (121°C, 20 min), and allowed to cool at room temperature. Two 5-mm glass beads were added to each tube and the tubes were incubated with 1 mL of enzyme solution in a water bath at 60°C for 3 h. Each tube was mixed immediately after adding the enzyme solution and then stirred occasionally (first, after 10 min, and then every 20 min). The enzyme solution was prepared with 1.5 mL of a heat-stable α-amylase (No. A3403, Sigma Chemical, St. Louis, MO) and 50 mg amylglucosidase (No. A7255, Sigma) in 100 mL of 0.1 M acetate buffer, pH 4.6, which gave a final concentration of 392 U α-amylase and 10 U amylglucosidase per milliliter. After incubation, glucose released was measured with a glucose oxidase assay (Trinder; No. 315-100, Sigma). Triplicate 10-µL samples were incubated at 37°C for 10 min in ELISA plates with 250 µL of prewarmed reagent solution and optical density was read immediately at 492 nm wavelength. Starch concentration was determined by multiplying glucose concentration by 0.9 and correcting for dilution.

Statistical Analysis. The results were subjected to ANOVA using the Statistix software package (Analytical Software, Tallahassee, FL). Data were analyzed by time of incubation, considering type of starch, treatment, and their interaction as sources of variation. Means were contrasted by the least significant difference procedure at \(P < 0.05\).

Results

Results of the initial experiment showed that the mixture and level of antibiotics used were very effective against rumen bacteria (Table 1) and reduced bacterial concentrations to less than 0.3% after 4 h and to near 0.0% after 8 h. Growth of Entodinium exiguum, estimated as a percentage of the starting concentration, doubled in 24 h with a generation time of 23.3 h, indicating that the antibiotics had no major effects on protozoal metabolism.

Trial 1. Bacterial concentrations determined for the inoculum preincubated with antibiotic solution for 4 h, and in tubes from treatments PA and PAB after 24 h, were less than 10 bacteria/mL. Therefore, in Trials 1 and 2, we assumed that treatment PA and PAB essentially contained no living bacteria. Bacterial concentrations in the inoculum for treatments PLB and BAC were 21 and 93 × 10^5/mL, respectively. After 24 h, bacterial concentrations in these treatments were 13 and 59 × 10^6/mL for PLB and BAC, respectively, a sixfold increase in both cases.

Figure 1 shows protozoal growth when Entodinium exiguum was cultured without bacteria (PA), with dead bacteria

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Protozoal growth, %</th>
<th>Generation time, h</th>
<th>Bacterial conc., (\times 10^9)/mL</th>
<th>Ratio of bacterial conc.: protozoal conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>—</td>
<td>24,000</td>
<td>1,408.5</td>
</tr>
<tr>
<td>4</td>
<td>107 ± 5.6</td>
<td>23.5 ± 1.41</td>
<td>61 ± 44.6</td>
<td>10.9 ± 7.76</td>
</tr>
<tr>
<td>8</td>
<td>122 ± 5.2</td>
<td>30.1 ± 5.35</td>
<td>0.2 ± 0.12</td>
<td>0.0 ± 0.03</td>
</tr>
<tr>
<td>24</td>
<td>208 ± 14.4</td>
<td>23.3 ± 1.97</td>
<td>—</td>
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</tbody>
</table>

*aEach value is the mean of three fermentation tubes. Concentrations at 0 h are based on counts in the inoculum.
In vitro growth of *E. exiguum* without bacteria (○,●), with autoclaved bacteria (■,▲), or with live bacteria (△,▲), incubated in culture media with autoclaved (open symbols) or non-autoclaved (closed symbols) cornstarch. Lower bars show standard error of means (n = 2).

Digestion was higher in BAC at incubation times greater than 12 h (P < 0.05). The BAC digestion reached 92% after 48 h of incubation, whereas at that time it was only 77 to 78% in PA and PAB.

With non-autoclaved starch (Figure 3), the beginning of digestion was delayed up to 9 h in PLB (23%) and it was not completed until 48 h. Digestion began after 12 to 24 h in both PA and PAB (21 to 22% after 24 h) and reached 84 to 86% after 48 h. In BAC, digestion also began after 24 h (25%) but was complete at 48 h.

It is worth noting that the sum of starch digested in either PA or PAB and BAC was less than in PLB at 3, 6, and 9 h in autoclaved cornstarch and at 9, 12, and 24 h in non-autoclaved cornstarch. For example, after 24 h of incubation, the digestion of non-autoclaved cornstarch by PA or PAB and BAC was only 46 to 47%, whereas with PLB digestion it was 85%.

**Trial 2.** In vitro growth of *E. exiguum* with corn or rice starch as a substrate is shown in Figure 4, and generation times are presented in Table 3. Growth of *E. exiguum* did not differ with the source of starch, but generation time at 36 and 48 h tended (P < 0.10) to be lower with corn than with rice (18.2 vs 22.1 and 20.5 vs 27.7 h with corn and rice at 36 and 48 h, respectively). Protozoal concentrations and generation time were not

**Table 2.** Estimated generation time of *Entodinium exiguum* when cultured with autoclaved or non-autoclaved cornstarch, without bacteria (PA), with autoclaved bacteria (PAB), or with live bacteria (PLB)

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Autoclaved</th>
<th></th>
<th>Non-autoclaved</th>
<th></th>
<th></th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>34.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>30.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>19.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>14.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>41.9&lt;sup&gt;p&lt;/sup&gt;</td>
<td>36.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>22.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>23.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>26.3&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>14.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each value is the mean of two fermentation tubes.

<sup>b,c,d</sup>Means in the same row followed by different superscripts differ (P < 0.05).
In vitro culture of *Entodinium exiguum*

Figure 3. Percentage of non-autoclaved cornstarch digestion in vitro by *E. exiguum* without bacteria (●), with autoclaved bacteria (■), with live bacteria (▲), and by bacteria alone (▲). Upper bars show standard error of means (n = 2).

Different with corn as a substrate. However, with rice starch, *E. exiguum* concentrations were higher and generation times lower from 24 to 48 h in the presence of live bacteria (PLB) (P < 0.05).

Protozoal digestion of corn and rice starch with or without live bacteria is presented in Figure 5. Starch digestion started earlier with rice than with corn (P < 0.05 at 6, 12, and 24 h). With corn, digestion in PLB was almost complete after 24 h and was higher than that in PA at 24 h (P < 0.05) and BAC at 24, 36, and 48 h (P < 0.05). Digestion of rice starch reached 99 and 93% in PLB and BAC, respectively, after 24 h. In contrast, digestion proceeded more slowly in PA and was lower than in the other two treatments at 12, 24, and 36 h (P < 0.05). The sum of PA and BAC starch digestion at 12 h for rice and 24 h for corn were both less than that for PLB at the same time period.

Discussion

The combination of antibiotics selected to inhibit rumen bacteria was effective and supports the previous results of Obispo and Dehority (1992). Onodera and Henderson (1980) and Morgavi et al. (1994) removed rumen bacteria from protozoal suspensions in vitro using mixtures of penicillin, streptomycin, and chloramphenicol. Although Onodera and Henderson (1980) reported a rapid reduction (2 to 4 d) of *Entodinium caudatum* numbers when those antibiotics were included at concentrations from 50 to 150 μg each per milliliter of culture, Morgavi et al. (1994) were able to maintain concentrations of antibiotic-treated protozoa for about 3 d in vitro. In our work, the absence of any effect of the penicillin-streptomycin mixture on *E. exiguum* in short-period incubations (Table 1) is apparent from the similarity of growth and generation time in the subsequent trials without antibiotics and a separate study with incubations up to 72 h (Fondevila and Dehority, 2000). In our initial study, the addition of the antibiotic mixture lowered the bacteria:protozoa ratio from approximately 1,400:1 to 11:1 after 4 h of incubation. After 8 h, the bacteria had essentially disappeared, indicating that the bacteria probably do not contribute much to overall starch digestion in the protozoa plus antibiotic treatments. However, as a precautionary measure in our further experiments on starch digestion rate, we decided to preincubate the inoculum in the presence of antibiotics to avoid any possible bacterial interference in the results of the bacteria-free treatments.

Onodera and Henderson (1980) demonstrated the need for living bacteria by *E. caudatum*. The protozoa

Figure 5. In vitro corn (open symbols) and rice (closed symbols) starch digestion by *E. exiguum* without bacteria (○,●), with live bacteria (□,■), and by bacteria alone (△,▲). Upper bars show standard error of means (n = 2).
survived for only 2.5 d in the presence of antibiotics with autoclaved bacteria. In our study, we observed no differences in E. exiguum growth or starch digestion when cultured with antibiotics, either with or without autoclaved bacteria. It would appear that the dependence of E. exiguum on live bacteria is not simply nutritive.

Protozoa grew better when starch was autoclaved. We presume this is the result of increased substrate accessibility in the first 12 h of incubation (Figure 2 compared to Figure 3). Differences in digestion of cereal grains are primarily related to thickness of the protein matrix that surrounds starch granules (McAllister and Cheng, 1996). When the starch is autoclaved the matrix is disrupted, and the starch becomes more accessible to the microbial enzymes. However, untreated cornstarch is almost fully digestible because the thin protein covering that may remain after extraction of starch grains retards rather than limits digestion. Starch digestion among PA, PLB, and PAB was similar for both autoclaved and non-autoclaved cornstarch.

Differences in the rate of digestion of corn and rice starch may be attributed to the type or thickness of the protein coat, differences in the amylase:amylopectin ratio, or to a higher surface area available for microbial and enzymatic attack in the case of rice, because of smaller starch grains (3 to 8 μm vs 9 to 19 μm). Protozoal growth in the present study was fairly similar with both starches, at least up to 24 h, either with or without live bacteria. Within this range of particle sizes, 3 to 19 μm, the source of starch did not seem to effect growth or amylolysis in E. exiguum. Although total extent of digestion with protozoa was similar between the two sources, bacteria digest rice starch faster and to a greater extent than cornstarch (Figure 5), suggesting a higher importance of attachment in their starch digestion process.

Mendoza et al. (1995), using cows given a 75% cereal grain diet as donors, observed that specific amylase activity of mixed rumen protozoa was twofold that of bacteria, but Martin et al. (1999) reported the opposite with a 60% barley diet: a three- to sixfold higher specific amylase activity in solid-associated bacteria than in liquid-associated protozoa in the first 3 h after feeding. Similar discrepancies can be observed in the present data (Figure 5). The rate of bacterial amylolysis was much faster than that of protozoa for rice, whereas digestion of cornstarch was much slower and fairly similar between the bacteria and protozoa. These differences can possibly be explained by the different mechanisms involved in starch digestion; whereas Entodinium spp. engulf starch granules rapidly and digest them intracellularly (Coleman, 1989), enzymatic digestion in rumen bacteria occurs extracellularly, and end products are transported inside the cell (Stewart and Bryant, 1988).

An interesting result from our work is that the sum of bacterial and E. exiguum starch digestion in Trials 1 and 2, when cultured separately, does not equal the extent of digestion in cocultures at 6, 9, or 12 h in Figure 2; at 9, 12, or 24 h in Figure 3; or at 12 or 24 h for either rice or corn in Figure 5. Microbial concentrations in the coculture were similar to those observed in the individual bacterial and protozoal fermentations. No explanation for this synergism was obvious from our observations; however, the rapid engulfment of starch granules by Entodinium spp. reported by Coleman (1992) and verified in our studies by microscopic observation after 3-h incubation might allow for a synchronous availability of energy and nitrogen, increasing protozoal efficiency. Live bacteria may also supply as-yet unknown growth factors for the protozoa and stimulate their metabolic and intracellular digestion processes. Although there are no data available to support an increased metabolic activity for E. exiguum in the presence of bacteria, Coleman (1986) observed an increase in amylase activity of rumen contents of defaunated sheep 6.5 h after feeding when refaunated with E. simplex or E. caudatum populations. He did not measure protozoal activity without bacteria, but the presence of protozoa increased the specific (per unit of microbial protein) bacterial amylase activity. The increase was greater in the presence of E. simplex (2.7 times) than with E. caudatum (1.7 times).

### Implications

E. exiguum can grow in vitro in the absence of live bacteria, at least up to 48 h; however, both rate of growth and extent of starch digestion are lower. The type of starch affects both rate and extent of microbial digestion activity. The presence of bacteria and proto-

### Table 3. Estimated generation time of Entodinium exiguum when grown on autoclaved or non-autoclaved corn starch, without bacteria (PA), or with live bacteria (PLB)

<table>
<thead>
<tr>
<th>Time, h</th>
<th>PA</th>
<th>PLB</th>
<th>PA</th>
<th>PLB</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>37.3</td>
<td>23.6</td>
<td>42.8</td>
<td>37.7</td>
<td>10.37</td>
</tr>
<tr>
<td>24</td>
<td>22.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.38</td>
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<tr>
<td>36</td>
<td>20.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.91</td>
</tr>
<tr>
<td>48</td>
<td>20.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.91</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each value is the mean of two fermentation tubes.
<sup>b</sup>Means in the same row followed by different superscripts differ (P < 0.05).
zoa in coculture increases the digestion rate of starch. However, more studies are required to verify whether the effect is caused by an enhanced protozoal activity, if the presence of *E. exiguum* stimulates bacterial amylolytic activity, or whether this is simply an additive effect.

**Literature Cited**


