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Chemotactic response of the flavonoid daidzein and its effect on the composition of the rumen bacterial community

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Abstract

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Daidzein is one of common metabolites in plants and has chemotactic effect on soil bacteria that colonize the plants. There are several tests to assess bacterial chemotaxis, but none focused on rumen bacteria. Therefore, the aim of this study was to test the chemotactic response of the rumen microflora towards daidzein using a standardized bacterial chemotaxis assay. It consisted in a modifying capillary technique and employing technology for measuring in vitro gas production. Ruminal fluids and cellulose were used as controls. The response of bacteria to daidzein was greater than the response to cellulose, supporting the hypothesis that when fodder is chewed by the ruminant it releases daidzein which can attract rumen bacteria towards feed particles (chemotaxis) for attachment and subsequent degradation.

Keywords: Daidzein, cellulose, chemotaxis, ruminal bacteria, rumen

1. Introduction

The rumen is a highly dynamic environment, and none of the changes are permanent within the influence due to microbial species (bacteria, archaea, fungi and protozoa) which are found in the rumen, since each species has an affinity for a substrate and / or fermentation byproducts (Church, 1974; Janssen and Kirs, 2008). The potentially bioactive compounds found in plants which constitute ruminant diet have become an area of interest in animal nutrition (Hammes and Hertel, 2002). Recent studies have shown that extracts of plants containing secondary metabolites (i.e. saponins, tannins and essential oils) can modify rumen microbial population. However, it is not clear how these metabolites affect rumen bacteria populations and therefore ruminant production efficiency. Daidzein is one of the most common isoflavonoid in plants, and it has chemoattractant effects on soil bacteria which colonize plants (Gough et al., 1997; Peck et al., 2006). Chemotaxis is a mechanism

by which the bacteria respond quickly and efficiently to an attractant concentration gradient, either towards (positive chemotaxis) or away (negative chemotaxis) of such compound (Murialdo et al., 2009). Daidzein can stimulate the growth of some rumen bacteria and lactobacilli in the intestine of piglets (Yu et al., 2004), and may have stimulating effects on rumen microbial fermentation, suggesting an interaction between rumen microorganisms and this isoflavonoid (Mao et al., 2007). However, many aspects remain to be elucidated to understand how bacteria sense and respond in the rumen environment. Therefore the aim of this work was to elucidate the chemotactic effect of daidzein over the rumen microflora. To achieve that, we standardized a chemotaxis assay for rumen bacteria by modifying a capillary method (Adler, 1973) developed for aerobic bacteria by combining it with technology commonly used for measuring *in vitro* gas production (Theodorou et al., 1994). We included parallel assays for studying the isoflavone daidzein as a possible attractant and looked through the profile fingerprints of Denaturing Gradient Gel Electrophoresis (DGGE) the effect of different chemoattractants (sterile rumen fluid, cellulose and daidzein) on ruminal bacterial consortium.

2. Material and methods

2.1. Culture medium (artificial saliva)

The medium was prepared according to Menke and Steingass (1988). It consisted of various components: trace minerals solution, buffer solution, a solution of macrominerals, cysteine as reducing solution and resazurin as an indicator of anaerobiosis, prepared under CO_2 (100%).

2.2. Obtaining rumen fluid inoculums

64 The rumen fluid content (solid and liquid) was collected from 3 fistulated cows with 12
65 hours of fasting, placed in sealed plastic bags (to maintain anaerobiosis) and transported at
66 39 °C to the Nutrition Laboratory (100m away), Faculty of Veterinary Medicine,
67 Universidad Autónoma de Yucatán, México. Ruminal fluid of 3 cows was leaked (together)
68 using sterile cheese cloth and collected in a beaker under constant flow of CO_2.The
69 remaining solids were blended under CO_2 (100%) for 20 seconds with artificial saliva in a
70 volume equal to that extracted as rumen liquour (ratio 1:1) (Menke and Steingass 1988).
71 Rumen solids were filtered again and liquor added to the initial rumen liquid obtained.
72 Then, rumen liquor was placed in 45 ml Falcon tubes and centrifuged at 10,000 rpm
73 (16,770 x g) min at 4 °C. The pellet containing the bacteria was recovered and resuspended
74 in 80 ml of artificial saliva medium and incubated at 39 °C overnight. All procedures were
75 carried out under constant flow of 100% CO_2.

76 ***2.3. Chemotaxis assay***

77 The capillary method of Adler (1973) was adapted to material for measuring *in vitro* gas
78 production (Theodorou et al., 1994). There is evidence of chemotaxis in the rumen bacteria
79 towards cellulose (Miron et al., 2001, Morris, 1998) and compounds in the rumen fluid
80 (Orpin and Bountiff, 1978). Thus, these compounds were chosen as positive controls. Then,
81 50 mg/L daidzein (attractant tested), 50 mg/L of cellulose (positive control), artificial saliva
82 (negative control), and rumen fluid (positive control) were prepared. These solutions
83 (daidzein, cellulose, rumen fluid and artificial saliva) were sterilized by filtration (0.22 µm
84 pore size). Then, capillaries (75 mm lenght, 1.1-1.2 mm and 1.5-1.6 mm inside and outside
85 Ø respectively, Marienfeld, Germany) were filled with with 60 µl of the solutions and one

end placed inside a serum bottle (100 ml nominal capacity) and one end was kept outside. Capillaries were sealed with clay and inserted trough the septum until the open end was in contact with the culture media (80 ml) containing rumen bacteria (Figure 1). The septa were sealed with parafilm "M" and incubated at 39 °C for 1 h. Subsequently, bacteria that had been attracted by the flavonoid and entered the capillary tubes were transferred into individual sterile eppendorf tubes and transported in a cooler at 4 °C to the Laboratory of Biotechnology, Faculty of Chemical Engineering, University of Yucatan. Samples were centrifuged for 30 min at 13000 rpm (28,341 x g), and then the cell pellet was resuspended in 30 µl sterile distilled water. For each attractant (cellulose, daidzein, rumen liquour) Each treatment had 30 replicates, 15 were stored at -20 °C for subsequent DNA extraction and 15 repetitions were kept at 4 °C for subsequent cell count by direct counting using the Nuebauer camera.

2.4 Statistical analysis

The chemotactic effect of attractants: ruminal fluid and cellulose (controls), and daidzein (unknown response substance) on rumen bacteria, was performed by analysis of variance (ANOVA). Differences in means were determined by Fisher's Least Significant Difference (LSD) test, using a significance level of $\alpha = 0.05$ and the Minitab statistical program (2007).

2.5. PCR-denaturing gradient gel electrophoresis (DGGE) analysis

For each attractant replicate, the cells pellets were lysed by five consecutive cycles of freezing (-20 °C) and heating (65 °C) for 3 min. Two microliters of cell lysate were used for amplification. The primer set, 338f (5´ACT CCT ACG GGA GGCAGC AG-3´) and 518r (5´ATT ACC GCG GCT GCTGG-3´), spanned the V3 region of the 16S rDNA. The

338f GC primer has a GC clamp (5′CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGA GGG G3′) attached to the 5′end of primer 338f (Cocolin et al., 2001). The PCR (25 µL reaction mixtures with appropriate template) amplification program consisted of preheating at 94 °C for 5 min and 10 cycles of denaturing (94 °C, 1 min.), annealing (65 °C, 1 min. decresing 1°C per cycle) and extension (72 °C, 1 min), continued, with three steps 20 cycles of denaturing (94 °C, 1 min.), annealing (55 °C, 1 min. decresing 1°C per cycle) and extension (72 °C, 1 min), followed by final extension at 72 °C for 10 min. The DGGE analysis of PCR amplicons was performed (DCode Universal Detection System, Bio-Rad). The amplicons were separated in 8% polyacrylamide gel containing a 100 to 40% gradient of 8 M urea and formamide increasing in the direction of electrophoresis. The electrophoresis was conducted in 1× TAE buffer under 70 V at 60 °C for 18 h. The DNA bands in gels were visualized by SYBR Gold. The similarities of PCR-DGGE profiles were analyzed using the program Quantity One (BioRad Imaging Systems) analysis was performed on the image. The presence (1) or absence (0) of the band through patterns jolting generated a binary matrix to obtain a similarity dendrogram using the method Euclidean nearest neighbor and the degree of similarity was represented by a similarity coefficient was determined, using the program Paleontological Statistics Software Package for Education and Data Analysis (PAST) (Hammer et al., 2001).

3. Results

3.1 Chemotaxis assay

The technique proposed by Alder (1973) is based on a capillary tube containing a solution as an attractant at one end. It is placed in a chamber containing a suspension of motile bacteria in buffer without a carbon source. The positive response is observed as an

131 accumulation of bacteria near the attractant in the tip of the capillary. However, this
132 chemotaxis assay was not designed for the study of anaerobic bacterial consortia, thus,
133 some modification have been developed for different purposes (Bharat et al., 2004). In the
134 present report, the technique was integrated with material used for measuring *in vitro* gas
135 production (Theodorou et al., 1994). Four capillaries per bottle were used simultaneously
136 (although a higher number could be used) to test if daidzein, cellulose and rumen liquour
137 had a chemotactic effect (attractant or repellent). When each capillary content was observed
138 under a light microscope, using bright field and differential Gram stain, microscopy
139 revealed Gram negative rod-shaped bacteria, forming palisades groups, diplobacillus and
140 estreptobacillus, while in artificial saliva no bacteria was not found. The higher chemotactic
141 response (P =0.0001) was found with ruminal fluid followed by daidzein and finally
142 cellulose (Table 1).

143 ***3.2.PCR-DGGE analysis of bacterial profiles***

144 Partial sequences of 16s rDNA genes (DGGE) from the capillaries showed the V3 region
145 (Ampe et al., 1999). Profiles of starved bacteria and those found in capillaries with different
146 the chemoattractants tested showed similar patterns (Figure 2). We considered 4 different
147 phylotypes involved in chemotaxis. The phylotype 1 (presente solo en daidzeina) was
148 positioned at the same place in the denaturing gradient as DNA from *R. albus* 7 (not
149 showed information). No similarity was found with *F. succinogenes*, and *R. flavefaciens*.
150 This agrees with the results of Galicia Jiménez et al., (2011) where a search of sequences of
151 *F. succinogenesis*, *R. albus* and *R. flavefaciens* in the NCBI database and comparative
152 analysis of chemotactic genes encoding a protein involved in chemotaxis was found only in
153 *R. albus* 8 sequences.

4. Discussion

In the present studio, the higher response of ruminal fluid in the test of quimiotaxis was probably due to its rich and complex composition (Orpin and Bountiff, 1978). Several solutes (amino acids, peptides, ammonia, soluble sugars, starch and VFA's) can be found in the rumen liqour considering its heterougenous origin, feeds, saliva, microorganisms and digestion byproducts, etc. (Araujo, 2007), some of them might also have chemotactic effect and as a result the response observed with rumen fluid could be an additive effect. In analysis of bacterial profiles, to sorrow that the fluid ruminal presented major number of bacterial cells, only he presented two phylotype, whereas in cellulose and daidzeina 3 presented phylotype, only the phylotype 1 was present in the daizeina. Provided that the phenomenon quimiotáctico has been little studied in bacteria ruminales, this investigation and the detection of genes responsible for the quimiotaxis in these microorganisms, as the brought in *Ruminococcus albus* (Galicia Jiménez et al., 2011), they provide a tool in the molecular dissection of this phenomenon in ruminants.

5. Conclusion

In summary, the anaerobic chemotaxis assay developed allowed to: 1) work directly with rumen samples without the need to separate bacteria 2) study chemotaxis testing simultaneously several rumen bacteria sources or, 3) using multiple chemoattractant with the same bacterial consortium, 4) quantify microorganisms accumulated inside the capillary tube and 5) Provide quick and relative clean samples to characterize bacterial consortium chemotactically attracted (to compounds of choice) through the profile of DGGE-fingerprints. 6) Obtain evidence of the chemotactic effect of daidzein upon rumen bacteria consortia.

177 This work tries to contribute in knowledge of the beginning that govern the communication
178 of the microbial populations, his principal interactions and products of the microbial
179 metabolism might raise the manipulation of the fermentation ruminal, creating this way the
180 cultures probióticos for the cattle, acting charitably in the intestinal flora of the individual.

181 **Conflict of interest**

182 All the authors have no conflict interest.

183 **Acknowledgment**

184 *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, and *Ruminococcus albus*
185 7 DNA sequences provided by D.M. Stevenson and Paul J. Weimer (Research
186 Microbiologist, USDA-ARS-US Dairy Forage Research Center)

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*Conflict of Interest Statement

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Sincerely,
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Figure

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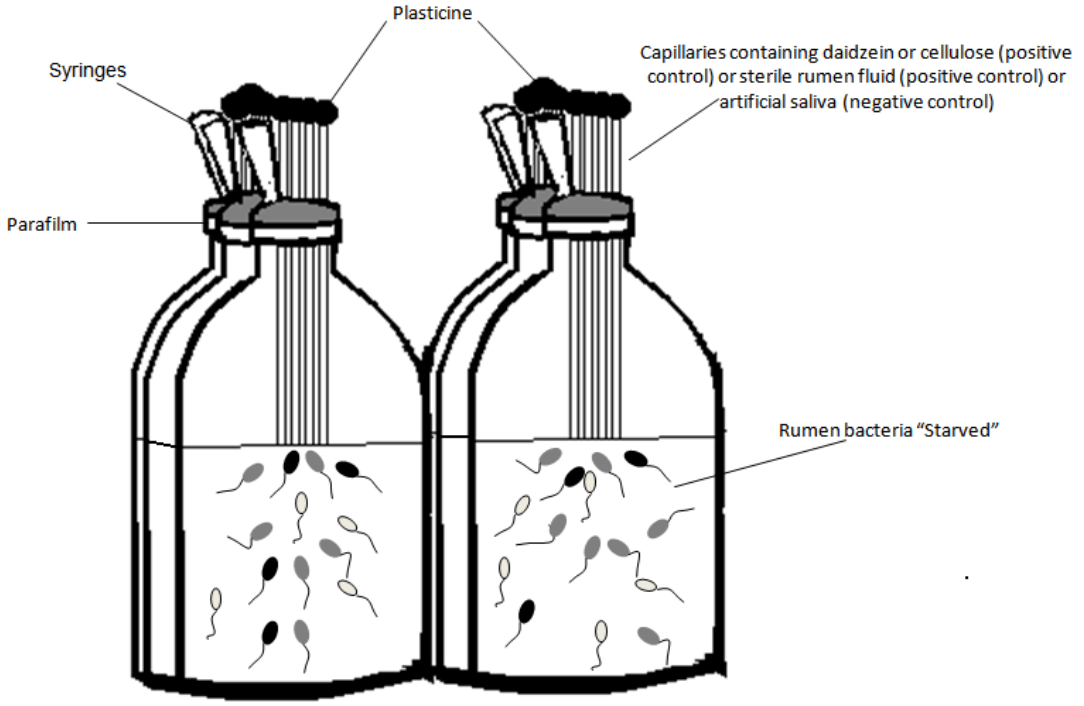

Figure 1. Material and setup of a modified capillary chemotaxis assay.

Figure

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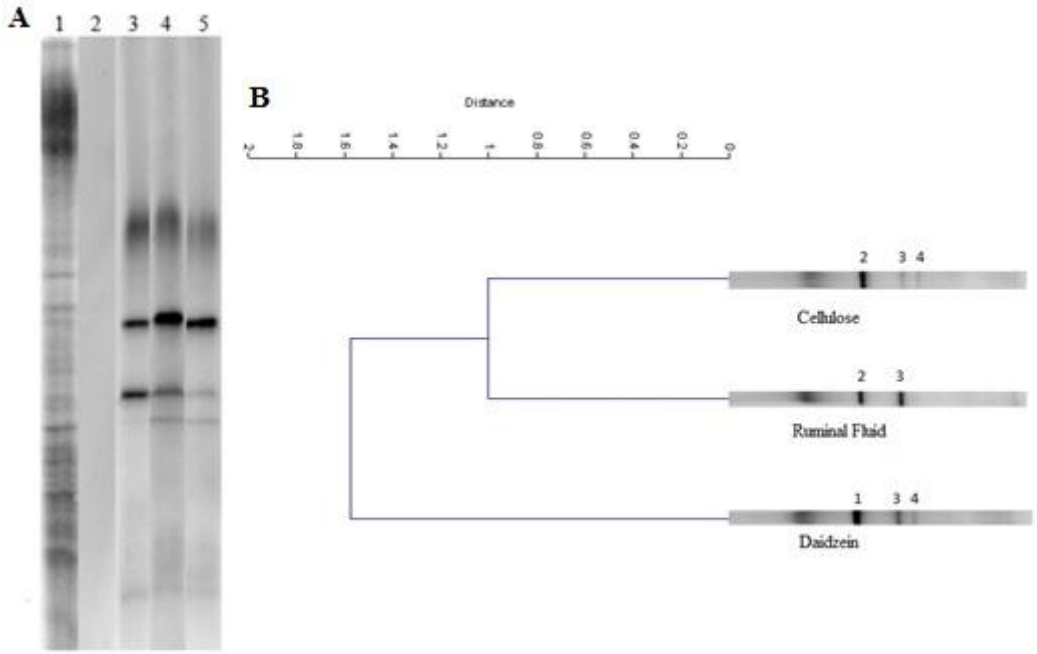

Figure 2 **A**. DGGE profile of 16S rDNA fragments amplified with primers 338f and 518r. (1) Starved rumen bacteria (2), artificial saliva (negative control), bacterial community accumulated in the capillaries with the attractant (3) sterile rumen fluid (positive control), (4) daidzein flavonoid (5) cellulose (positive control). Negative image of the gel stained with SYBR. **B.** Euclidean distance generated dendrogram with the nearest neighbor method using a binary presence or absence of bands in the banding profiles obtained rDNA DGGE bacterial chemotaxis assay. The numbers indicate the profiles identified phylotype (1 to 4).

Table

Table 1. Ruminal bacteria found in capillary chemotaxis assay (bacterias /microliter).

Substrate	Mean	S.D.	N
Ruminal Fluid	17.32251[a]	0.1134	15
Daidzein	5.6514[b]	0.1215	15
Celullose	5.2869[c]	0.1619	15
Control[1]	0.0284[d]	0.0393	15

Different literals (a, b, c, d) in the same column, differs statistically ($P < 0.001$)

[1]Artificial saliva (negative control). Menke and Steingass (1988).