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Corresponding Author: Dr. monica galicia, Dra

Corresponding Author's Institution: Universidad del Mar

First Author: monica galicia, Dra

Order of Authors: monica galicia, Dra; Rafael Rojas Herrera, Dr.; Carlos Sandoval Castro, Dr.; Silvia E. Murialdo, Dra.; Héctor Magaña Sevilla, Dr.

Abstract: 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.

Suggested Reviewers: Wen Yao Dr.
Research Professor , Laboratory of Gastrointestinal Microbiology,, Nanjing Agricultural University
yaowen67jp@yahoo.com
His line of investigation is related to the topic, it it endorses multiple articles related to the daidzeina and the ruminants

X Zhang Dr.
Research Professor , College of Animal Science and Technology, Nanjing Agricultural University
zhangxunnj@gmail.com
His line of investigation is related to the topic, it it endorses multiple articles related to the daidzeina and the ruminants

W.Y. Zhu Dr.
Research Professor , College of Animal Science and Technology, Nanjing Agricultural University

zhuweiyunnjau@hotmail.com
His line of investigation is related to the topic, it it endorses multiple articles related to the daidzeina and the ruminants

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

3 Mónica Marcela Galicia-Jiménez[1*], Rafael Rojas-Herrera[2], Carlos Sandoval-Castro[3], Silvia
4 E. Murialdo[4], and Héctor Magaña-Sevilla[5]
5 [1]Instituto de Genética. Universidad del Mar. Campus Puerto Escondido. Ciudad
6 Universitaria, Carretera Vía Sola de Vega, Puerto Escondido, San Pedro Mixtepec, Juquila,
7 Oax., México C.P. 71980. E-mail: monicagalicia@zicatela.umar.mx,
8 [2]Facultad de Ingeniería Química, Campus de Ingenierías y Ciencias Exactas, Universidad
9 Autónoma de Yucatán Periférico Norte Kilometro 33.5, Tablaje Catastral 13615, Col.
10 Chuburna de Hidalgo Inn, Mérida, Yucatán, México. C.P. 97203. r.rojas@uady.mx ,
11 [3]Facultad de Medicina Veterinaria y Zootecnìa. Universidad Autónoma de Yucatán.
12 Carretera a Xmatkuil Km. 15.5. Xmatkuil, Yucatán, México. CP 97315. ccastro@uady.mx ,
13 [4]Facultad de Ingeniería. Universidad Nacional de Mar del Plata. Diagonal J. B. Alberdi
14 2695 (7600) Mar del Plata, Argentina. silviaem@fi.mdp.edu.ar
15 [5]Instituto Tecnológico de Conkal. Km. 16.3 Antigua Carretera Mérida-Motul. Conkal,
16 Yucatán, México. C.P. 97345. hectorms68@hotmail.com

17

18 **Abstract**

[*] Corresponding author at: [1]Instituto de Genética. Universidad del Mar. Campus Puerto Escondido. Ciudad Universitaria, Carretera Vía Sola de Vega, Puerto Escondido, San Pedro Mixtepec, Juquila, Oax., México C.P. 71980. Tel.: +52 954 582 49 90 ext. 312; Fax: +52 954 582 49 92. E-mail addres: monicagalicia@zicatela.umar.mx

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

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

57 **2. Material and methods**

58 ***2.1. Culture medium (artificial saliva)***

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

63 ***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

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

98 ***2.4 Statistical analysis***

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

103 ***2.5. PCR-denaturing gradient gel electrophoresis (DGGE) analysis***

104 For each attractant replicate, the cells pellets were lysed by five consecutive cycles of
105 freezing (-20 °C) and heating (65 °C) for 3 min. Two microliters of cell lysate were used
106 for amplification. The primer set, 338f (5´ACT CCT ACG GGA GGCAGC AG-3´) and
107 518r (5´ATT ACC GCG GCT GCTGG-3´), spanned the V3 region of the 16S rDNA. The

108 338f GC primer has a GC clamp (5´CGC CCG CCG CGC GCG GCG GGC GGG GCG
109 GGG GCA CGA GGG G3´) attached to the 5´end of primer 338f (Cocolin et al., 2001).
110 The PCR (25 μL reaction mixtures with appropriate template) amplification program
111 consisted of preheating at 94 °C for 5 min and 10 cycles of denaturing (94 °C, 1 min.),
112 annealing (65 °C, 1 min. decresing 1°C per cycle) and extension (72 °C, 1 min), continued,
113 with three steps 20 cycles of denaturing (94 °C, 1 min.), annealing (55 °C, 1 min. decresing
114 1°C per cycle) and extension (72 °C, 1 min), followed by final extension at 72 °C for 10
115 min. The DGGE analysis of PCR amplicons was performed (DCode Universal Detection
116 System, Bio-Rad). The amplicons were separated in 8% polyacrylamide gel containing a
117 100 to 40% gradient of 8 M urea and formamide increasing in the direction of
118 electrophoresis. The electrophoresis was conducted in 1× TAE buffer under 70 V at 60 °C
119 for 18 h. The DNA bands in gels were visualized by SYBR Gold. The similarities of PCR-
120 DGGE profiles were analyzed using the program Quantity One (BioRad Imaging Systems)
121 analysis was performed on the image. The presence (1) or absence (0) of the band through
122 patterns jolting generated a binary matrix to obtain a similarity dendrogram using the
123 method Euclidean nearest neighbor and the degree of similarity was represented by a
124 similarity coefficient was determined, using the program Paleontological Statistics
125 Software Package for Education and Data Analysis (PAST) (Hammer et al., 2001).

126 **3. Results**

127 ***3.1 Chemotaxis assay***

128 The technique proposed by Alder (1973) is based on a capillary tube containing a solution
129 as an attractant at one end. It is placed in a chamber containing a suspension of motile
130 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)

187 **References**

188 Adler J. 1973. A Method for Measuring Chemotaxis and Use of the Method to Determine
189 Optimum Conditions for Chemotaxis by *Escherichia coli*. Microbiology 74:77-91.
190 Ampe, F., Miambi, E., 2000. Cluster analysis, richness and biodiversity indexes derived
191 from denaturing gradient gel electrophoresis fingerprints of bacterial communities
192 demonstrate that traditional maize fermentations are driven by the transformation
193 process. International Journal of Food Microbiology. 60: 91–97.
194 Araujo FO, Vergara-López J. 2007. Propiedades físicas y químicas del rumen. Archivos
195 Latinoamericanos de Producción Animal. 15:133-140.
196 Bharat Bhushan, Annamaria Halasz, Sonia Thiboutot, Guy Ampleman, and J. H. 2004.
197 Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and
198 CL-20 by Clostridium sp. EDB2. Biochem Biophys Res Commun, 316:816–821.

199 Cocolin, L., Manzano, M., Aggio, D., Cantoni, C., Comi, G., 2001. A novel polymerase
200 chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) for the
201 identification of Micrococcaceae strains involved in meat fermentation. Its application
202 to naturally fermented Italian sausages. Meat Science. 57, 59–64.
203 Church, D. C. 1974. Fisiología digestiva y nutrición de los rumiantes. Editorial Acribia.
204 Zaragoza, España.
205 Galicia Jiménez MM, Sandoval Castro C, Rojas Herrera R, Magaña Sevilla H. 2011.
206 Possible chemotaxis in *Ruminococcus albus*: comparative genomics. Journal of
207 Applied Animal Research 39:189-191
208 Gough C, Galera C, Vasse J, Webster G, Cocking EC, Dénarié J. 1997. Specific flavonoids
209 promote intercellular root colonization of Arabidopsis thaliana by Azorhizobium
210 caulinodans ORS571. Molecular Plant-Microbe Interactions 10:560-70.
211 Hammes WP, Hertel C. 2002. Research approaches for pre- and probiotics: challenges and
212 outlook. Food Research International 35:165-170.
213 Hammer Ø, Harper DAT, Ryan PD. 2001. Paleontological statistics software package for
214 education and data analysis. Palaeontologia Electronica 4: 9pp
215 Janssen H P, Kirs M. 2008. Structure of the Archaeal Community of the Rumen. Applied
216 and Environmental Microbiology 74: 3619–3625
217 Mao SY, Zhu WY, Wang QJ, Yao W. 2007. Effect of daidzein on in vitro fermentation by
218 microorganisms from the goat rumen. Animal Feed Science and Technology 136:154-
219 163.

220 Menke KH, Steingass H. 1988 Estimation of the energetic feed value obtained from
221 chemical analysis and *in vitro* gas production using rumen fluid. Animal Research 28:
222 7-55.

223 Miron J, Ben-Ghedalia D, Morrison M. 2001. Invited Review: Adhesion Mechanisms of
224 Rumen Cellulolytic Bacteria. Journal of Dairy Science 84:1294-1309.

225 Murialdo SE, Sendra GH, Passoni LI, Arizaga R, Gonzalez JF, Rabal H. 2009. Analysis of
226 bacterial chemotactic response using dynamic laser speckle. Journal of Biomedical
227 Optics 14:064015-064015-6.

228 Orpin CG, Bountiff L. 1978. Zoospore Chemotaxis in the Rumen Phycomycete
229 Neocallimastix frontalis. Microbiology 104:113-122.

230 Peck MC, Fisher RF, Long SR. 2006. Diverse flavonoids stimulate NodD1 binding to nod
231 gene promoters in Sinorhizobium meliloti. Journal of Bacteriology 188:5417-27.

232 Theodorou MK; Williams BA; Dhanoa MS; McAllen AB; France J. 1994. A simple gas
233 production method using a pressure transducer to determine the fermentation kinetics
234 of ruminant feeds. Animal Feed Science and Technology 48:185-197.

235 Yu Z, Yao W, Mao S, Zhu W. 2004. Effect of daidzein on the intestinal flora of piglets.
236 Acta Nutrimenta Sinica 2007-01.

***Conflict of Interest Statement**

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Figure
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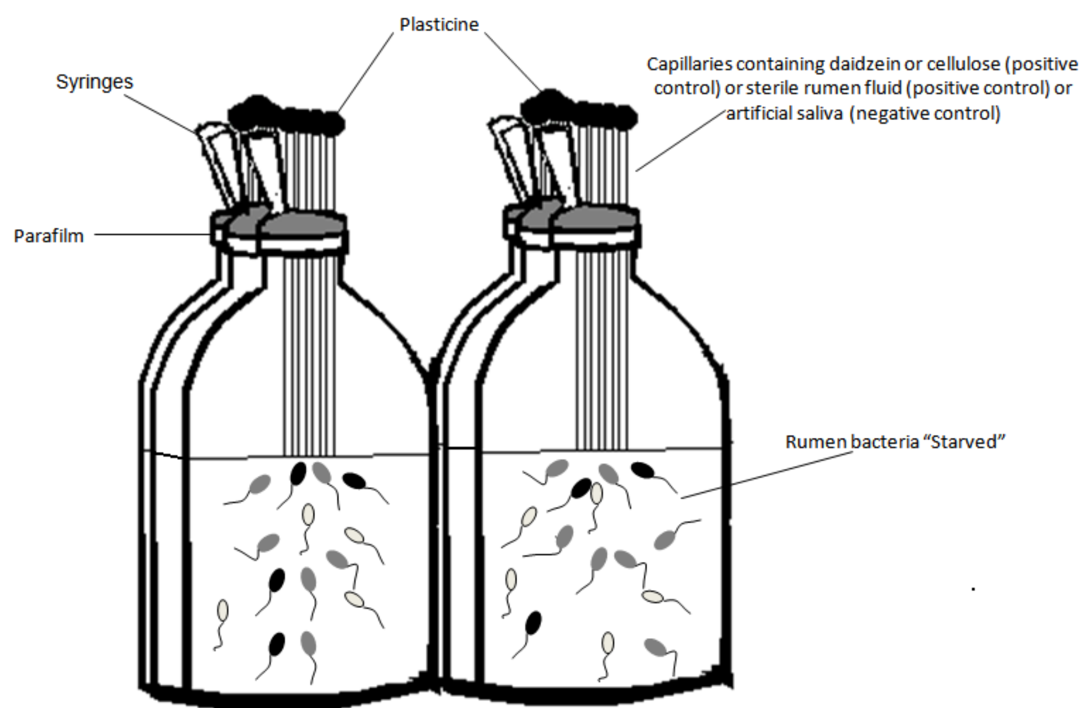

Figure 1. Material and setup of a modified capillary chemotaxis assay.

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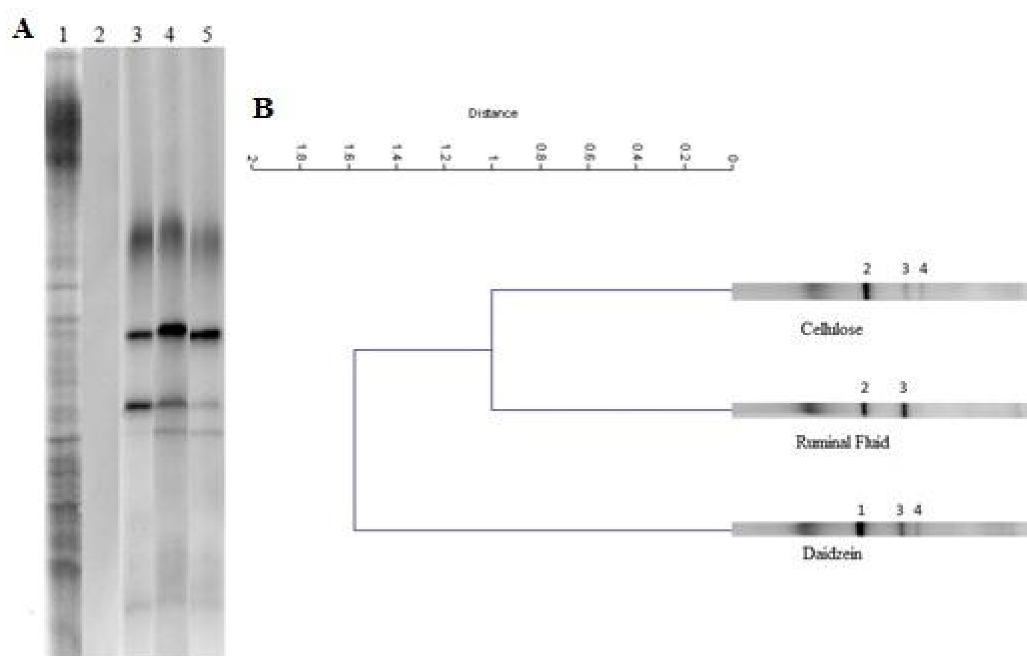

Figure 2 **A**. DGGE profile of 16S rDNA fragments amplified with primers 338f and 518r. (1) Starved rumen bacteria (2), artificial saliva (negative control), bacterial community accumulated in the capillaries with the attractant (3) sterile rumen fluid (positive control), (4) daidzein flavonoid (5) 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).