



HAL
open science

In vitro Antagonistic activities of *Lactobacillus* spp. against *Brachyspira hyodysenteriae* and *B. pilosicoli*

Marion Bernardeau, Micheline Gueguen, David G.E. Smith, Enrique Corona-Barrera, Jean Paul Vernoux

► To cite this version:

Marion Bernardeau, Micheline Gueguen, David G.E. Smith, Enrique Corona-Barrera, Jean Paul Vernoux. In vitro Antagonistic activities of *Lactobacillus* spp. against *Brachyspira hyodysenteriae* and *B. pilosicoli*. *Veterinary Microbiology*, 2009, 138 (1-2), pp.184. 10.1016/j.vetmic.2009.03.020 . hal-00490554

HAL Id: hal-00490554

<https://hal.science/hal-00490554>

Submitted on 9 Jun 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: In vitro Antagonistic activities of *Lactobacillus* spp. against *Brachyspira hyodysenteriae* and *B. pilosicoli*

Authors: Marion Bernardeau, Micheline Gueguen, David G.E. Smith, Enrique Corona-Barrera, Jean Paul Vernoux



PII: S0378-1135(09)00149-7
DOI: doi:10.1016/j.vetmic.2009.03.020
Reference: VETMIC 4390

To appear in: *VETMIC*

Received date: 15-8-2008
Revised date: 4-3-2009
Accepted date: 6-3-2009

Please cite this article as: Bernardeau, M., Gueguen, M., Smith, D.G.E., Corona-Barrera, E., Vernoux, J.P., In vitro Antagonistic activities of *Lactobacillus* spp. against *Brachyspira hyodysenteriae* and *B. pilosicoli*, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.03.020

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 *IN VITRO* ANTAGONISTIC ACTIVITIES OF *LACTOBACILLUS SPP.* AGAINST
2 *BRACHYSPIRA HYODYSENTERIAE* AND *B. PILOSICOLI*

3
4 **Running title**

5 **Antagonism between *Lactobacillus* and *Brachyspira***

6
7 Marion Bernardeau^{1,2}, Micheline Gueguen¹, David G E Smith^{3,5}, Enrique Corona-Barrera⁴,
8 Jean Paul Vernoux^{1*}

9
10 ¹Unité de recherche des Microorganismes d'Intérêt Laitier et Alimentaire (MILA), IFR 146
11 ICORE, Université de Caen Basse-Normandie, Esplanade de la Paix, Caen, France

12 ²Sorbial SAS, Route de Spay, 72700 Allonnes, France

13 ³Bacterial Functional Genomics, Moredun Research Institute, Pentlands Science Park,
14 Edinburgh, Scotland, UK

15 ⁴Centro de Investigaciones Regionales “Dr. Hideyo Noguchi” Universidad Autónoma de
16 Yucatán Mérida, Yucatán 97000, México

17 ⁵Division of Infection and Immunity, Faculty of Veterinary Medicine, University of Glasgow,
18 Glasgow, Scotland, UK

19
20
21 **Corresponding author :**

22 Jean-Paul Vernoux

23 Tel: +33 231 565 621 Fax: +33 231 566 179

24 E-mail address: jean-paul.vernoux@unicaen.fr (J.P. Vernoux)

25

26 **Abstract**

27

28 The sensitivity of *Brachyspira hyodysenteriae* and *B. pilosicoli*, respectively the causative
29 agents of Swine Dysentery and Porcine Intestinal Spirochaetosis to two probiotic
30 *Lactobacillus* strains, *Lb. rhamnosus* CNCM-I-3698 and *Lb. farciminis* CNCM-I-3699 was
31 studied through viability, motility and coaggregation assays. The cell-free supernatant of
32 these lactobacilli contains lactic acid, that is stressful for *Brachyspira* (leading to the
33 formation of spherical bodies), and lethal. It was demonstrated for the first time the *in vitro*
34 coaggregation properties of two probiotic *Lactobacillus* strains (active or heat-treated) with
35 two pathogenic strains of *Brachyspira*, leading to (1) trapping of spirochaetal cells in a
36 physical network as demonstrated by SEM; (2) inhibition of the motility of *Brachyspira*. Such
37 *in vitro* studies should encourage *in vivo* studies in animal model to evaluate the potential of
38 the use of probiotic lactobacilli through a feeding strategy for the prevention of *B.*
39 *hyodysenteriae* and *B. Pilosicoli*.

40

41 Keywords: *Lactobacillus*, *Brachyspira*, probiotic, *in vitro* antagonism, coaggregation

42

43 1. Introduction

44

45 *Brachyspira hyodysenteriae* and *B. pilosicoli* are important pathogenic intestinal spirochaetes
46 that cause costly diarrhoeal diseases for the pig industry (Smith, 2005), respectively Swine
47 Dysentery (SD) and Porcine Intestinal Spirochaetosis (PIS). Economical losses (poor gain
48 weight performance, high medication and death) have been recognized worldwide for
49 decades. Recently, *B. pilosicoli* was considered as a zoonotic agent (Hampson *et al.*, 2006).

50 Antimicrobial agents, mainly tylosin and tiamulin are widely used for extended periods to
51 control SD and PIS in affected herds. However, in the light of increasing multiple drug
52 resistance (Karlsson *et al.*, 2003), more consideration has been given to reduce the risk of
53 infection by applying preventive measures, and the use of antibiotic treatment should be
54 combined with hygienic, technical and nutritional management practices. A nutritional
55 strategy is the use of probiotics - live microorganisms which when administered in adequate
56 amounts confer a health benefit on the host (FAO/WHO, 2001). Supplementing animal feed
57 with probiotic lactobacilli is well documented (reviewed by Bernardeau *et al.*, 2006). Some
58 probiotics contribute to improve herd performance (Torres-Rodriguez *et al.*, 2007;
59 Vasconcelos *et al.*, 2008) and health management (Sargeant *et al.*, 2007). Multiple
60 mechanisms of action for the beneficial effect of probiotics have been postulated, including
61 preventing growth of pathogenic bacteria (Tsai *et al.*, 2008; Wagner *et al.*, 2008), production
62 of antimicrobial agents (Gillor *et al.*, 2008), coaggregation with pathogens (Golowczyc *et al.*,
63 2007) and stimulation of mucosal barrier function (Isolauri *et al.*, 2008).

64 To our knowledge, neither *in vitro* nor *in vivo* interactions between *Brachyspira* and probiotic
65 strains have been studied to date. Thus, the aim of this study was to investigate the *in vitro*
66 antagonistic activities of the cells of two *Lactobacillus* strains, *Lactobacillus rhamnosus*

67 CNCM-I-3698 and *Lb. farciminis* CNCM-I-3699, known as probiotics (Bernardeau, 2006)
68 and their cell-free supernatants against *B. pilosicoli* and *B. hyodysenteriae*.

69

70 **2. Materials and methods**

71

72 *2.1. Bacterial strains and microbial cell suspensions*

73 The two strains of lactobacilli, stored at - 80°C, were cultured for 24 hours in 9.0 ml of MRS
74 broth (MRS, De Man Rogosa Sharpe, AES, Combourg, France) or milk (Lait G, Standa
75 Industrie, France) in anaerobic conditions (GENbox anaer, Biomérieux, Marcy l'Etoile,
76 France) at 37°C prior to testing. The final *Lactobacillus* population was around 1×10^9 c.f.u.
77 ml^{-1} . *Lactobacillus* cells were separated from cell-free supernatants by centrifugation at
78 10,000 x g at 4 °C for 10 min, the pellet was washed three times with phosphate-buffer saline
79 (PBS: 0.8% NaCl, 0.02% KH_2PO_4 , 0.115% Na_2HPO_4 w/v, pH 7.4) and re-suspended with
80 PBS in the initial volume. Cell-free supernatant was filtrated (Cellulose acetate membrane 0.2
81 μm , Sartorius, VWR, France).

82 *Brachyspira pilosicoli* strain ATCC-51139 (pig isolate originally known as P43/6/78) was
83 obtained from the American Tissue and Cell Collection and kept at the Scottish Agricultural
84 College, Veterinary Sciences Division, Edinburgh, which was then donated to us by Dr. Jill
85 Thomson. Another strain of *Brachyspira* was obtained from LDA22 collection (Zoopôle
86 Ploufragan, France). It was isolated from a clinical case of post-weaning piglet (15 kg)
87 suffering from diarrhoea. It was identified by the LDA22 laboratory as *B. hyodysenteriae* and
88 numbered 9828978.

89 To conduct experiments, spirochaetes were propagated on blood agar (BA) made of Columbia
90 agar (AES, Combourg, France), supplemented with 5.0% sheep blood (AES, Combourg,
91 France) and incubated in an anaerobic jar with GENbox anaer (Biomérieux, Marcy l'Etoile,

92 France) at 37 °C for *B. pilosicoli* and 42 °C for *B. hyodysenteriae* for 3 to 5 days. Spirochaete
93 cells were harvested and suspended in PBS. Cells from several dishes were combined, washed
94 3 times and re-suspended in 1.0 ml of PBS to obtain a suspension containing 3×10^9 c.f.u. ml⁻¹
95 ($\sigma = 0.57$), which was the working bacterial suspension.

96

97 2.2. Coaggregation between *Lactobacillus* and *Brachyspira* cells

98

99 The coaggregation analysis was performed according to Collado *et al.*, (2007). Absorbances
100 (A_{600}) were monitored during the first 4 hours and after 18, 22 and 24 hours of incubation.
101 Coaggregation rate expressed as percentage was determined as $[(A_{\text{patho}} + A_{\text{Lb}})/2 -$
102 $(A_{\text{mix}})/(A_{\text{patho}} + A_{\text{Lb}})/2] \times 100$, where A_{patho} and A_{Lb} represent the absorbance at 600 nm in
103 tubes containing respectively only the pathogen or each *Lactobacillus* strains and A_{mix}
104 represents the absorbance of the mixed culture after the same period of incubation.
105 Coaggregation was qualitatively evaluated by scanning and transmission electron microscopy.
106 The impact of coaggregation on motility was evaluated using an agar motility test that we
107 developed for this work. Briefly, each strain of *Lactobacillus* (living cells or heat-inactivated
108 at 120°C for 15min) and *B. pilosicoli* ATCC 51139 cell suspensions were pre-incubated
109 (50/50 v/v) for 4 hours or 24 hours in an Eppendorf tube at 37°C. This test was performed in
110 two ways: a “square test” and a “spot test”, by spreading 100 μ l of the mixed suspension with
111 a loop on the surface of a blood agar plate respectively, in a 9 cm² defined square or by
112 spotting a 5 μ l drop. The plates were incubated for 8 days in anaerobic conditions as
113 described above. The extent of motility was assessed every day, visually examining the
114 hemolytic area and the spread of growth on the surface, compared to the natural spread of a
115 *Brachyspira* cell suspension (control) not mixed with viable or heat-treated lactobacilli cells.

116

117 2.3. *Electron microscopy*

118

119 Materials were washed with PBS and fixed overnight at 4 °C with a cacodylate buffer
120 (cacodylate 0.1M and saccharose 0.1M) containing 2.5% (v/v) glutaraldehyde. After washing
121 with PBS, the cells were post-fixed for 30 min in the dark at room temperature with a mixture
122 of cacodylate buffer containing 1% of osmium tetroxide OsO₄. For scanning electron
123 microscopic (SEM - JEOL Ltd 6400F, Tokyo, Japan) observations, cells were then washed
124 three times with the same mixture and dehydrated in a gradually increased concentration
125 series (30.0%; 50.0% and 70.0%) of ethanol solution for 10 min. The cells were then washed
126 in 100 % ethanol for 10 min before being dried in a critical-point dryer (Balzers CPD 020)
127 and coated with gold. For Transmission electron microscopic (TEM - JEOL 1011/micrograph
128 MegaviewIII SIS) observations, the protocol was adapted from Wood *et al.*, (2006). Cells
129 were washed through a cacodylate series (400, 300 and 200 mM), before being dehydrated
130 through an ethanol gradually increased concentration series and infiltrated with resin. The
131 resin was polymerized at 60°C for 24 hours. Ultra-thin sections (80 nm) obtained with a Leica
132 Microtome (Leica Ultra Cut R) were mounted on formvar-coated copper grids. Poststaining
133 was performed by soaking sections on 2.0% uranyl acetate for 15 min and lead citrate for 15
134 min.

135

136 2.4. *Statistical analysis*

137

138 The results for the viability, aggregation and coaggregation were expressed as the average of
139 3 independent experiments. Statistical analysis was performed by StatGraphicPlus software.
140 Data were subjected to a 1-way analysis of variance.

141

142 3. Results

143

144 3.1. Effect of cell-free *Lactobacillus* supernatant on viability and morphology of *Brachyspira* 145 *cells*

146

147 *Lactobacillus* supernatant obtained from MRS was an acidic solution (pH 3.5), and was shown to
148 contain 14.4 g of lactic acid/l (especially the L-form: 13.7 g/l as determined by D-Lactic acid/L-
149 Lactic acid Enzymatic kit, Boehringer Mannheim). A highly significant ($P < 0.001$) inhibitory
150 effect against *B. hyodysenteriae* 9828978 and *B. pilosicoli* ATCC-51139 was observed after 1
151 hour of contact time with *Lactobacillus* supernatant obtained from MRS since the initial
152 bacterial count of 1×10^9 c.f.u. ml⁻¹ diminished completely by this time point. Similar
153 inhibitory effects were observed with acidic solutions 14.4 g/L of lactic acid at pH 3.5.

154 The results of the adverse effect of the acidic *Lactobacillus* cell-free supernatant obtained
155 from milk (pH 4.5), on the cell morphology of the spirochaetes are shown on Fig. 1. In Fig. 1
156 (A), all spirochaetal cells from the PBS control exhibit a typical thin, smooth helical form
157 with pointed ends. The exposure of spirochaetal cells to a stressful acidic (pH 4.5)
158 environment for 1 hour showed besides scattered cells poles (data not shown), cells with
159 morphological variations presumably due to a progressive degree of injury ranging from
160 stress to lethality. For example, cells from Fig. 1 (B) exhibits progressive stressed stages
161 which could be summarised as: (1) cell retracts and folds in two, (2) exopolymers are
162 probably synthesised between the two parallel strands of the cell, (3) cell curls up, and (4)
163 spherical body is formed. The observations were similar for both *Brachyspira* species.

164

165 3.2. Coaggregation potential of *Lactobacillus* cells on *Brachyspira*

166

167 Each *Lactobacillus* strain -either viable or heat-inactivated- showed coaggregation abilities
168 towards *B. pilosicoli* ATCC-51139 (Table 1). Nevertheless, the rate of the formation of
169 coaggregates was delayed with heat-inactivated *Lactobacillus* strains compared to viable
170 cells. Similar coaggregation results were obtained with *B. hyodysenteriae* 9828978 (data not
171 shown). SEM was used to examine the morphological features of the interaction between the
172 *Lactobacillus* and *Brachyspira* cells (Fig. 2). Large masses of coaggregated bacteria were
173 evident in the scanning electron microscope preparations (Fig. 2-A) and there was typically a
174 mesh-like between the cells found internally in these coaggregates (Fig. 2-B). Areas of close
175 cell-to-cell contact between the two microorganisms are evident both on SEM (Fig. 2-C) and
176 on TEM (Fig. 2-D). No preferential location of cell-to-cell interaction (e.g. tip versus middle)
177 was found. The surface of *Brachyspira* had what appears to be thin filaments of exopolymers
178 emerging from the outer membrane (Fig. 2-E). These fibrils or exopolymers are associated in
179 many cases with other *Brachyspira* cells.

180

181 3.3. Inhibition of the spread of a motile *Brachyspira* strain

182

183 This was performed with the type strain *B. pilosicoli* ATCC 51139 which is very motile. The
184 motility of spirochaetal cells on the surface of BA plates was entirely inhibited by viable *Lb.*
185 *rhamnosus* 3698 after a 4 hour or 24h contact time contrary to viable *Lb. farciminis* 3699
186 which is only efficient after a 24h contact time (Table 2).

187 To by-pass the possible effect of competition for nutrients between the two bacterial species
188 and that of the synthesis of antimicrobial substances, similar experiments were conducted
189 with heat-inactivated lactobacilli cells. After 4 hours contact-time with heat-inactivated *Lb.*
190 *rhamnosus* 3698, *B. pilosicoli* ATCC-51139 remained viable as a spot of haemolysis was still
191 observed on the drop. However, the spirochaetal cells were not able to spread on the surface

192 of the agar media (Fig. 3-B). The same experiment conducted with heat-inactivated cells of
193 *Lb. farciminis* 3699 did not limit the spreading or haemolysis of the spirochaetes (Fig. 3-C).
194 After 24 hours contact-time, no haemolysis and no spreading were observed with either
195 strains of *Lactobacillus*, heat-inactivated (Fig. 3-E & F). So results were similar to those
196 obtained with viable lactobacilli cells (Table 2) and competition for nutrients between the two
197 bacterial genera or effect of antimicrobial substances produced by active *Lactobacillus*, are
198 not responsible for inhibiting the spirochaetes motility. The prevention of the motility of the
199 spirochaetes was interpreted as a consequence of the coaggregative properties of *Lb.*
200 *rhamnosus* 3698 and *Lb. farciminis* 3699.

201

202 4. Discussion

203

204 Here we demonstrated and illustrated the sensitivity of one strain of *B. hyodysenteriae* and of
205 one strain of *B. pilosicoli* (two pathogenic agents of economical importance in the pig
206 industry) to two probiotic *Lactobacillus* strains. Contact with cell-free *Lactobacillus*
207 supernatant had a lethal effect on *Brachyspira* cells, which was characterized by broken cells
208 walls. Sensitivity of *Brachyspira* to acidic compounds and disinfectants has already been
209 shown by one of us (Corona-Barrera, 2002 and 2004). Morphological changes due to stress
210 have been observed here and lead to “spherical body” formation. De Ciccio (1997) has
211 demonstrated that an accumulation of toxic products, a change in environmental conditions
212 caused by lactic acid have conducted to the formation of spherical body in *Treponema*
213 *denticola* (a member of the same phylum as spirochetes). Spherical bodies have also been
214 observed with *Brachyspira* strains and they can revert to normal helical form if conditions
215 improve (Wolf & Wecke, 1994). Wood *et al.*, 2006 described this kind of spherical body with

216 *B. hyodysenteriae* after a nutritional depletion and postulated that it is an adaptive survival
217 response to adverse environmental conditions.

218 Coaggregation -process by which genetically distinct bacteria become attached to one another
219 via specific molecules- is of considerable importance in several ecological niches (Jankovic *et*
220 *al.*, 2003). It could protect from specific diseases, as has been demonstrated for the oral cavity
221 (Willcox *et al.*, 1993), urogenital tract (Mastromarino *et al.*, 2002) and intestinal tract of
222 humans and pigs (Drago *et al.*, 1997; Kmet *et al.*, 1995). We demonstrated for the first time
223 the *in vitro* coaggregation properties of two probiotic *Lactobacillus* strains (active or heat-
224 treated) with two pathogenic strains of *Brachyspira*, leading to (1) trapping of spirochaetal
225 cells in a physical network as demonstrated by SEM; (2) inhibition of the motility of
226 *Brachyspira*. The effect on motility is very important result as motility (along with
227 chemotaxis) has emerged as a major virulence factor of intestinal spirochaetes, allowing them
228 to escape the physical effect of mucus (Witters and Duhamel, 1999). Mucus eliminates
229 unattached micro-organisms efficiently from the gastro-intestinal tract and, unlike other
230 enteric pathogens, *B. hyodysenteriae* and *B. pilosicoli* are highly motile in the presence of
231 mucus (Nakamura *et al.*, 2006).

232 The coaggregation properties of some lactobacilli cells have already been observed by
233 Schachtsiek *et al.* (2004) for *Lb. coryniformis* strains DSM 20001^T and LTH 1750, *Lb.*
234 *gasseri* 4B2, on cells of pathogens involved in enteric disorders: *Escherichia coli* K88,
235 *Campylobacter coli* and *Campylobacter jejuni*. The nature of the coaggregation of some
236 *Lactobacillus* strains with *Escherichia coli* has also been determined and linked to high level
237 of exopolysaccharides production (Aslim *et al.*, 2007). The coaggregative property of
238 *Lactobacillus* is neither genus- nor species-specific. *Lactobacillus* strains have also
239 demonstrated coaggregative properties with yeasts of urogenital importance such as *Candida*
240 *albicans* (Boris *et al.*, 1998; Ocana and Nader-Macias, 2002; Hronek *et al.*, 2005).

241 *In vitro* observations of antagonistic properties of two probiotic strains of *Lactobacillus*
242 against two strains of *Brachyspira* suggest a great potential as a preharvest strategy against
243 *Brachyspira* carriage. It agrees with our preliminary field observations of a reduce carriage of
244 *Brachyspira* following a feeding probiotic therapy with these two lactobacilli (Bernardeau
245 and Guilmoto, 2008). Further investigations are needed to explain the nature of coaggregative
246 properties and to determine whether lactobacilli and *Brachyspira* species compete for similar
247 specific receptors on the intestinal epithelium. Such *in vitro* studies should encourage *in vivo*
248 studies in animal model to evaluate the potential of the use of probiotic lactobacilli through a
249 feeding strategy for the prevention of *B. hyodysenteriae* and *B. Pilosicoli*.

250 **Acknowledgments**

251
252 This study was supported by a grant from the *Association Nationale de la Recherche*
253 *Technique* (France) and Sorbial, a French company. Authors wish to thank Atmane Asselate
254 and Raouf Tareb for their technical support. Authors gratefully acknowledge Dr. Didier Goux
255 from the Microscopy centre of the University of Caen (France) for his scientific and technical
256 assistance getting the electron micrographs.

258 **References**

- 259 Aslim, B., Onal, D., Beyatli, Y., 2007. Factors influencing autoaggregation and aggregation
260 of *Lactobacillus delbrueckii subsp. bulgaricus* isolated from handmade yogurt. *J Food*
261 *Prot.* 70, 223-227.
- 262 Bernardeau, M., 2006. Safety assessment and probiotic properties of two lactobacilli for use
263 in pig farming and prevention of *Brachyspira*'s disease. Thesis from the University of
264 Caen, France.

- 265 Bernardeau, M., Guéguen, M., Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed:
266 long-term use, biodiversity and proposals for specific and realistic safety assessments.
267 FEMS Microbiol Rev. 30, 487-513.
- 268 Bernardeau, M., Guilmoto, H., 2008. Diet supplementation with fermentative heat-inactivated
269 lactobacilli based product can help to prevent swine dysentery in pigs. 20th
270 International Pig Veterinary Society Congress 22-26 June 2008, Durban, South Africa.
271 Poster Proceedings, p.517.
- 272 Boris, S., Suarez, J.E, Vazquez, F., Barbes, C., 1998. Adherence of human vaginal lactobacilli
273 to vaginal epithelial cells and interaction with uropathogens. *Infect Immun.* 66, 1985-
274 1989.
- 275 Collado, M.C., Surono, I., Meriluoto, J., Salminen, S., 2007. Indigenous dadih lactic acid
276 bacteria: cell-surface properties and interactions with pathogens. *J Food Sci.* 72, M89-
277 93.
- 278 Corona-Barrera, E., 2002. Epidemiology, detection molecular typing and control of porcine
279 colonic spirochaetosis (*Brachyspira pilosicoli*). Thesis, University of Edinburgh, UK.
- 280 Corona-Barrera, E., Smith, D.G.E., Murray, B., Thomson J.R., 2004. The efficacy of seven
281 disinfectant-sanitisers on field isolates of *Brachyspira pilosicoli*. *Veterinary Record*
282 154, 473-474.
- 283 De Ciccio, A., 1997. Oral spirochetes : contribution to oral malodor and formation of sperical
284 bodies. Thesis from the Faculty of graduate Studies and research, McGill University,
285 Montreal, Quebec, Canada.
- 286 Drago, L., Gismondo, M.R., Lombardi, A., de Haën, C., Gozzini, L., 1997. Inhibition of *in*
287 *vitro* growth of enteropathogens by new *Lactobacillus* isolates of human intestinal
288 origin. *FEMS Microbiol. Lett.* 153, 455-463.

- 289 FAO/WHO, 2001. Health and Nutritional Properties of Probiotics in Food including Powder
290 Milk with Live Lactic Acid Bacteria. Report of a Joint FAO/WHO Expert
291 Consultation.
- 292 Gillor, O., Etzion, A., Riley, M.A., 2008. The dual role of bacteriocins as anti- and probiotics.
293 Appl Microbiol Biotechnol. 81, 591-606.
- 294 Golowczyc, M.A., Mobili, P., Garrote, G.L., Abraham, A.G., De Antoni, G.L., 2007.
295 Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella*
296 *enterica* serovar Enteritidis. Int J Food Microbiol. 118, 264-273.
- 297 Hampson, D.J., Oxberry, S.L., La, T., 2006. Potential for zoonotic transmission of
298 *Brachyspira pilosicoli*. Emerg Infect Dis. 12, 869-870.
- 299 Hronek, M., Vachtlova, D., Kudlackova, Z., Jilek, P., 2005. Antifungal effect in selected
300 natural compounds and probiotics and their possible use in prophylaxis of
301 vulvovaginitis. Ceska Gynekol. 70, 395-399.
- 302 Isolauri, E., Kalliomäki, M., Laitinen, K., Salminen, S., 2008. Modulation of the maturing gut
303 barrier and microbiota: a novel target in allergic disease. Curr Pharm Des. 14, 1368-
304 1375.
- 305 Jankovic, I., Ventura, M., Meylan, V., Rouvet, M., Elli, M., Zink, R., 2003. Contribution of
306 aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri*
307 4B2. J Bacteriol. 185, 3288-3296.
- 308 Karlsson, M., Fellström, C., Gunnarsson, A., Landén, A., Franklin, A., 2003. Antimicrobial
309 susceptibility testing of porcine *Brachyspira (Serpulina)* species isolates. J Clin
310 Microbiol. 41, 2596-2604.
- 311 Kmet, V., Callegari, M.L., Bottazzi, V., Morelli, L., 1995. Aggregation promoting factor in
312 pig intestinal *Lactobacillus* strains. Lett. Appl. Microbiol. 21, 351-353.

- 313 Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., Conte, U.,
314 Matteuzzi, D., 2002. Characterization and selection of vaginal *Lactobacillus* strains
315 for the preparation of vaginal tablets. *J Appl Microbiol.* 93, 884-893.
- 316 Nakamura, S., Adachi, Y., Goto, T., Magariyama, Y., 2006. Improvement in motion
317 efficiency of the spirochete *Brachyspira pilosicoli* in viscous environments. *Biophys J.*
318 90, 3019-3026.
- 319 Ocana, V.S., Nader-Macias, M.E., 2002. Vaginal lactobacilli: self- and co-aggregating ability.
320 *Br J Biomed Sci.* 59, 183-190.
- 321 Sargeant, J.M., Amezcua, M.R., Rajic, A., Waddell, L., 2007. Pre-harvest interventions to
322 reduce the shedding of *E. coli* O157 in the faeces of weaned domestic ruminants: a
323 systematic review. *Zoonoses Public Health.* 54, 260-277.
- 324 Schachtsiek, M., Hammes, W.P., Hertel, C., 2004. Characterization of *Lactobacillus*
325 *coryniformis* DSM 20001T surface protein Cpf mediating co-aggregation with and
326 aggregation among pathogens. *Appl Environ Microbiol.* 70, 7078-7085.
- 327 Smith, J.L., 2005. Colonic spirochetosis in animals and humans. *J Food Prot.* 68, 1525-1534.
- 328 Torres-Rodriguez, A., Donoghue, A.M., Donoghue, D.J., Barton, J.T., Tellez, G., Hargis,
329 B.M., 2007. Performance and condemnation rate analysis of commercial turkey flocks
330 treated with a *Lactobacillus* spp.-based probiotic. *Poult Sci.* 86, 444-446.
- 331 Tsai, C.C., Lin, P.P., Hsieh, Y.M., 2008. Three *Lactobacillus* strains from healthy infant stool
332 inhibit enterotoxigenic *Escherichia coli* grown *in vitro*. *Anaerobe.* 4, 61-7.
- 333 Vasconcelos, J.T., Elam, N.A., Brashears, M.M., Galyean, M.L., 2008. Effects of increasing
334 dose of live cultures of *Lactobacillus acidophilus* (Strain NP 51) combined with a
335 single dose of *Propionibacterium freudenreichii* (Strain NP 24) on performance and
336 carcass characteristics of finishing beef steers. *J Anim Sci.* 86, 756-762.

- 337 Wagner, R.D., Johnson, S.J., Kurniasih Rubin, D., 2008. Probiotic bacteria are antagonistic to
338 *Salmonella enterica* and *Campylobacter jejuni* and influence host lymphocyte
339 responses in human microbiota-associated immunodeficient and immunocompetent
340 mice. *Mol Nutr Food Res.* 2008 Dec 8. [Epub ahead of print]
- 341 Willcox, M.D., Patrikakis, M., Harty, D.W., Loo, C.Y., Knox, K.W., 1993. Coaggregation of
342 oral lactobacilli with streptococci from the oral cavity. *Oral Microbiol Immunol.* 8,
343 319-321.
- 344 Witters, N.A., Duhamel, G.E., 1999. Motility-regulated mucin association of *Serpulina*
345 *pilosicoli*, the agent of colonic spirochetosis of humans and animals. *Adv Exp Med*
346 *Biol.* 473, 199-205.
- 347 Wolf, V., Wecke, J., 1994. Formation of multiple treponemes. *Zentralbl Bakteriol.* 280, 297-
348 303.
- 349 Wood, E.J., Seviour R.J., Siddique, A.B., Glaisher, R.W., Webb, R.I., Trott, D.J., 2006.
350 Spherical body formation in the spirochaete *Brachyspira hyodysenteriae*. *FEMS*
351 *Microbiol Lett.* 259, 14-19.
- 352
- 353

354 TABLES

355

356 **Table 1:** Co-aggregation of viable or heat-inactivated cells of *Lb. rhamnosus* 3698 or *Lb.*
357 *farciiminis* 3699 and *B. pilosicoli* ATCC-51139 as determined by the spectrophotometry
358 assay; results expressed as average (n=3) \pm S.D.

359

360

361 **Table 2:** Effect of a 4 or 24 hours contact-time of viable or heat-inactivated (120°C/15min)
362 lactobacilli cells on haemolysis, motility (spot motility test) and growth of a *B. pilosicoli*
363 ATCC 51139 suspension.

364

365

366

367 **Figure caption**

368

369 **Fig. 1.** Scanning electron micrographs of *Brachyspira* strains subjected to cell-free
370 *Lactobacillus* culture supernatant obtained from milk (pH 4.5). *B. hyodysenteriae* 9828978
371 incubated 1 hour with PBS (control) (A) and with the supernatant (B). *B. hyodysenteriae*
372 9828978 incubated 1 hour with supernatant with various stages (1 to 4) of stress injury (C).

373

374 **Fig. 2.** Electron micrographs of co-aggregates of heat-inactivated *Lb. rhamnosus* 3698 and *B.*
375 *hyodysenteriae* 9828978. Scanning electron micrographs (A, B, C, E) demonstrating the
376 woven pattern of cells seen in co-aggregates (A, B), the close cell-to-cell interactions (C) and
377 the thin filaments of exopolymers (black arrows) produced by the spirochaetes from the outer
378 membrane (E). Transmission electron micrograph (D) through co-aggregate, demonstrating a
379 pattern of heat-inactivated *Lb. rhamnosus* 3698 (thick white arrow) surrounded by *B.*
380 *hyodysenteriae* 9828978 cells (thick black arrows); areas of cell contact (thin white arrows)
381 were evident.

382

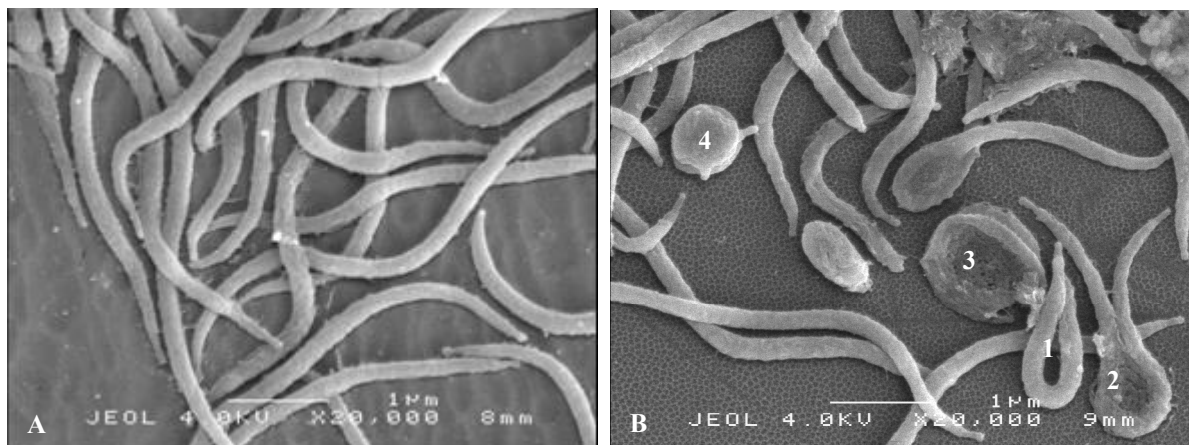
383 **Fig. 3.** Study of the motility (spot motility test) of *B. pilosicoli* ATCC 51139 pre-incubated
384 for 4 hours (A, B, C) and 24 hours (D, E, F) with : PBS (A, D) or with heat-inactivated cells
385 of *Lb. rhamnosus* 3698 (B, E) or *Lb. farciminis* 3699 (C, F) and inoculated via a drop on the
386 surface of agar medium (Columbia Agar supplemented with 5.0% sheep blood, plates
387 incubated for 8 days at 37°C in anaerobiosis).

388

389

1 **Figure 1**

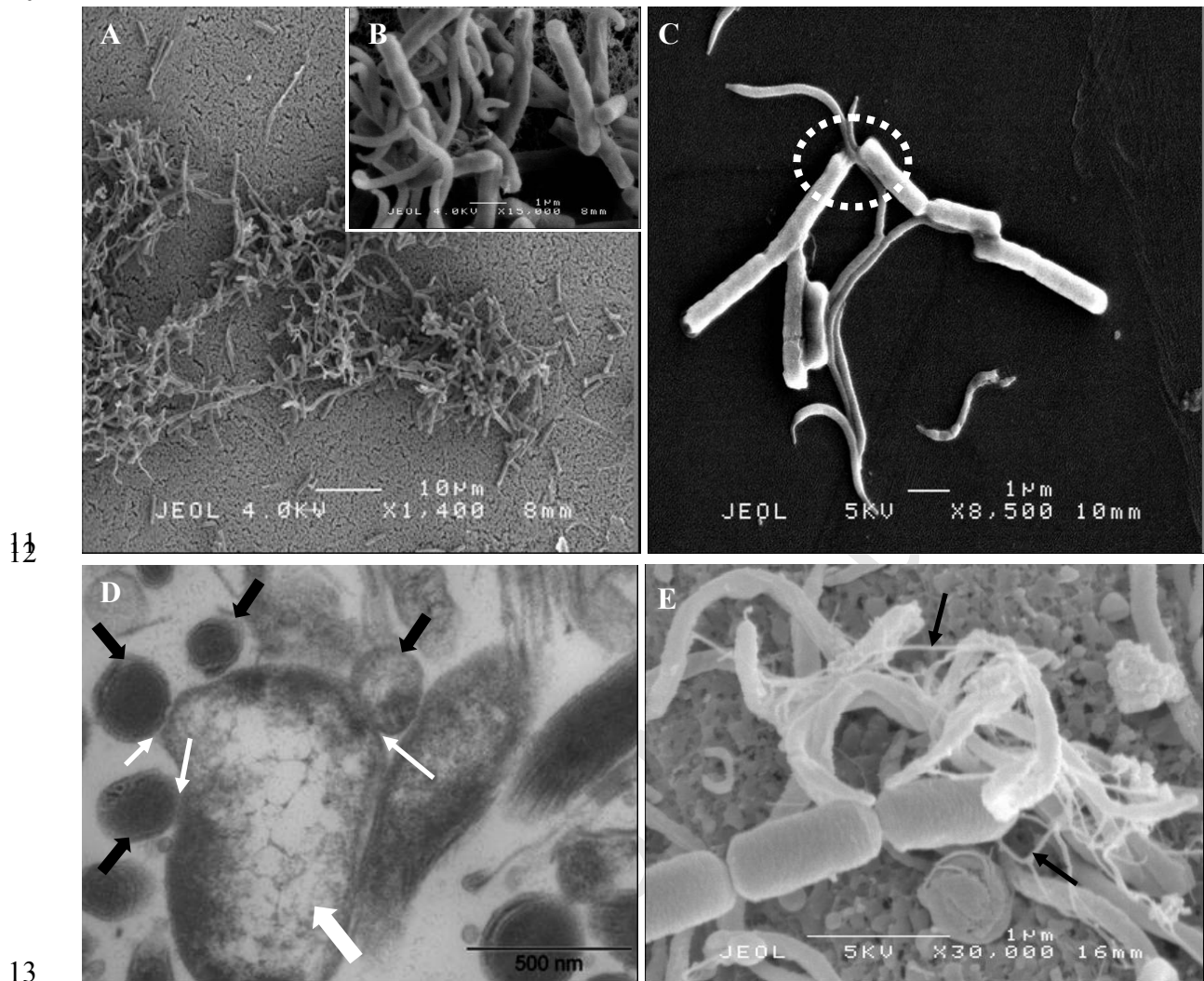
2
3



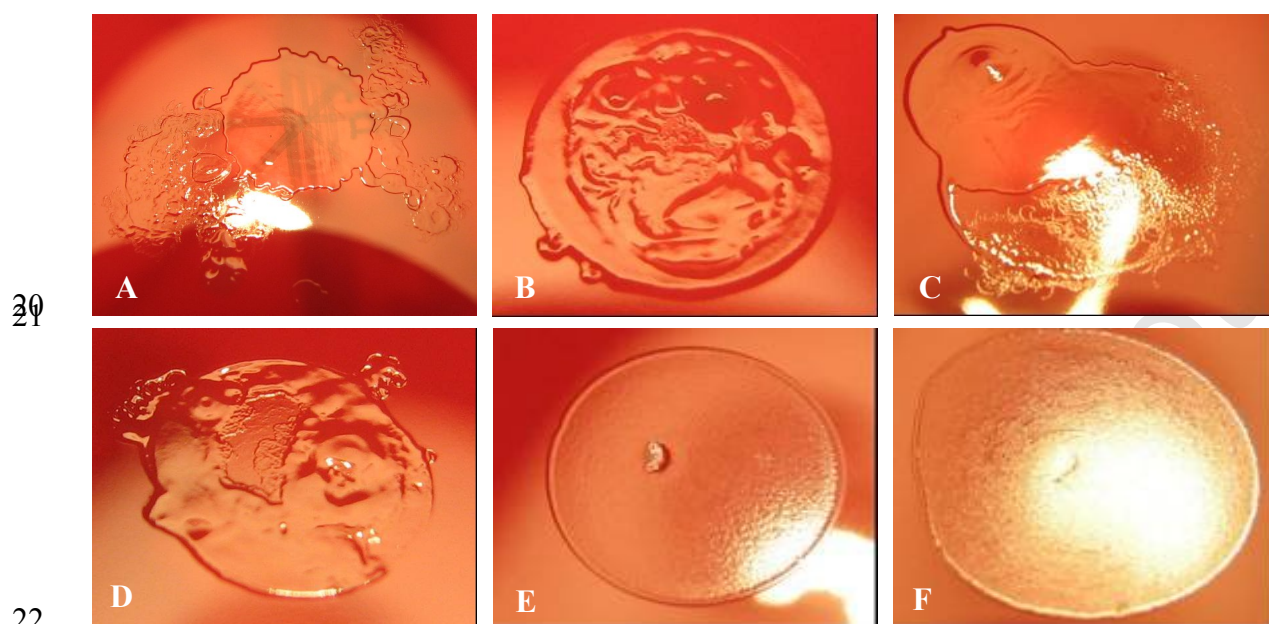
4
5
6
7
8

Accepted Manuscript

9 Figure 2
10



19 Figure 3



Accepted Manuscript

Table 1

Incubation (h)	% of coaggregation of <i>B. pilosicoli</i> ATCC 51139 with			
	<i>Lb. rhamnosus</i> 3698		<i>Lb. farciminis</i> 3699	
	Viable cells	HI-cells	Viable cells	HI-cells
1	3.50 ± 1.44	0.62 ± 0.47*	4.20 ± 1.77	0*
2	6.40 ± 2.13	3.17 ± 1.23	8.29 ± 2.03	3.22 ± 0.45
3	9.91 ± 2.6	5.87 ± 2.11	10.98 ± 0.98	6.28 ± 2.63
4	17.14 ± 6.93	8.57 ± 2.99	14.53 ± 5.31	8.69 ± 4.16
18	26.79 ± 3.24	25.27 ± 6.85	25.41 ± 6.44	20.02 ± 5.34
22	31.68 ± 3.86	32.22 ± 12.81	34.35 ± 7.54	25.07 ± 12.40
24	35.19 ± 3.75	36.51 ± 12.05	37.38 ± 7.12	31.11 ± 15.20

HI: Heat-Inactivated

*No significantly different ($P > 0.05$) from the control taken as 0% (no coaggregation)

Table 2

		Contact time (hrs)	<i>Brachyspira pilosicoli</i> ATCC 51139	
			Haemolysis	Motility
With PBS (Control)		4	+	++
		24	+	+
With viable Lb.	<i>Lb. rhamnosus</i> 3698	4	-	-
		24	-	-
	<i>Lb. farciminis</i> 3699	4	-	+
		24	-	-
With HI- Lb.	<i>Lb. rhamnosus</i> 3698	4	+	-
		24	-	-
	<i>Lb. farciminis</i> 3699	4	+	++
		24	-	-

HI: Heat-Inactivated; B: *Brachyspira pilosicoli* ATCC 51139; Lb: *Lactobacillus* sp.