

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

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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	
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26 Abstract

27

28 The sensitivity of Brachyspira hyodysenteriae and B. pilosicoli, respectively the causative agents of Swine Dysentery and Porcine Intestinal Spirochaetosis to two probiotic 29 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 these lactobacilli contains lactic acid, that is stressful for Brachyspira (leading to the 32 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 two pathogenic strains of *Brachyspira*, leading to (1) trapping of spirochaetal cells in a 35 physical network as demonstrated by SEM; (2) inhibition of the motility of *Brachyspira*. Such 36 in vitro studies should encourage in vivo studies in animal model to evaluate the potential of 37 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

43 **1. Introduction**

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Brachyspira hyodysenteriae and *B. pilosicoli* are important pathogenic intestinal spirochaetes that cause costly diarrhoeal diseases for the pig industry (Smith, 2005), respectively Swine Dysentery (SD) and Porcine Intestinal Spirochaetosis (PIS). Economical losses (poor gain weight performance, high medication and death) have been recognized worldwide for 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 resistance (Karlsson et al., 2003), more consideration has been given to reduce the risk of 52 infection by applying preventive measures, and the use of antibiotic treatment should be 53 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 amounts confer a health benefit on the host (FAO/WHO, 2001). Supplementing animal feed 56 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 mechanisms of action for the beneficial effect of probiotics have been postulated, including 60 61 preventing growth of pathogenic bacteria (Tsai et al., 2008; Wagner et al., 2008), production of antimicrobial agents (Gillor et al., 2008), coaggregation with pathogens (Golowczyc et al., 62 63 2007) and stimulation of mucosal barrier function (Isolauri et al., 2008).

To our knowledge, neither *in vitro* nor *in vivo* interactions between *Brachyspira* and probiotic strains have been studied to date. Thus, the aim of this study was to investigate the *in vitro* 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

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72 2.1. Bacterial strains and microbial cell suspensions

The two strains of lactobacilli, stored at - 80°C, were cultured for 24 hours in 9.0 ml of MRS 73 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, France) at 37°C prior to testing. The final *Lactobacillus* population was around 1×10^9 c.f.u. 76 ml⁻¹. Lactobacillus cells were separated from cell-free supernatants by centrifugation at 77 10,000 x g at 4 °C for 10 min, the pellet was washed three times with phosphate-buffer saline 78 79 (PBS: 0.8% NaCl, 0.02% KH₂PO₄, 0.115% Na₂HPO₄ w/v, pH 7.4) and re-suspended with PBS in the initial volume. Cell-free supernatant was filtrated (Cellulose acetate membrane 0.2 80 81 μm, Sartorius, VWR, France).

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

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

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

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97 2.2. Coaggregation between Lactobacillus and Brachyspira cells

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99 The coaggregation analysis was performed according to Collado et al., (2007). Absorbances 100 (A₆₀₀) were monitored during the first 4 hours and after 18, 22 and 24 hours of incubation. Coaggregation rate expressed as percentage was determined as $[(A_{patho} + A_{Lb})/2 -$ 101 $(A_{mix})/(A_{patho} + A_{Lb})/2] \times 100$, where A_{patho} and A_{Lb} represent the absorbance at 600 nm in 102 tubes containing respectively only the pathogen or each Lactobacillus strains and Amix 103 104 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 µl of the mixed suspension with a loop on the surface of a blood agar plate respectively, in a 9 cm² defined square or by 111 spotting a 5 µl drop. The plates were incubated for 8 days in anaerobic conditions as 112 described above. The extent of motility was assessed every day, visually examining the 113 hemolytic area and the spread of growth on the surface, compared to the natural spread of a 114 115 Brachyspira cell suspension (control) not mixed with viable or heat-treated lactobacilli cells.

117 2.3. Electron microscopy

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119 Materials were washed with PBS and fixed overnight at 4 °C with a cacodylate buffer (cacodylate 0.1M and saccharose 0.1M) containing 2.5% (v/v) glutaraldehyde. After washing 120 121 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 Leïca 132 Microtome (Leïca 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

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

142 **3. Results**

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

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Lactobacillus supernatant obtained from MRS was an acidic solution (pH 3.5), and was shown to contain 14.4 g of lactic acid/l (especially the L-form: 13.7 g/l as determined by D-Lactic acid/L-Lactic acid Enzymatic kit, Boehringer Mannheim). A highly significant (P<0.001) inhibitory effect against *B. hyodysenteriae* 9828978 and *B. pilosicoli* ATCC-51139 was observed after 1 hour of contact time with *Lactobacillus* supernatant obtained from MRS since the initial bacterial count of $1x10^9$ c.f.u. ml⁻¹ diminished completely by this time point. Similar 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 which could be summarised as: (1) cell retracts and folds in two, (2) exopolymers are 161 162 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

167 Each Lactobacillus strain -either viable or heat-inactivated- showed coaggregation abilities towards B. pilosicoli ATCC-51139 (Table 1). Nevertheless, the rate of the formation of 168 169 coaggregates was delayed with heat-inactivated Lactobacillus strains compared to viable 170 cells. Similar coaggregation results were obtained with B. hvodvsenteriae 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 evident in the scanning electron microscope preparations (Fig. 2-A) and there was typically a 173 174 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.

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

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

To by-pass the possible effect of competition for nutrients between the two bacterial species and that of the synthesis of antimicrobial substances, similar experiments were conducted with heat-inactivated lactobacilli cells. After 4 hours contact-time with heat-inactivated *Lb. rhamnosus* 3698, *B. pilosicoli* ATCC-51139 remained viable as a spot of haemolysis was still 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 Lb. farciminis 3699 did not limit the spreading or haemolysis of the spirochaetes (Fig. 3-C). 193 194 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**

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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

B. hyodysenteriae after a nutritional depletion and postulated that it is an adaptive survival
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 (Willcox et al., 1993), urogenital tract (Mastromarino et al., 2002) and intestinal tract of 221 222 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 Brachyspira. The effect on motility is very important result as motility (along with 226 227 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 Schachtsiek et al. (2004) for Lb. corvniformis strains DSM 20001^T and LTH 1750, Lb. 233 234 gasseri 4B2, on cells of pathogens involved in enteric disorders: Escherichia coli K88, Campylobacter coli and Campylobacter jejuni. The nature of the coaggregation of some 235 236 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 Lactobacillus is neither genus- nor species-specific. Lactobacillus strains have also 238 239 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 against two strains of *Brachyspira* suggest a great potential as a preharvest strategy against 242 243 Brachyspira carriage. It agrees with our preliminary field observations of a reduce carriage of Brachvspira following a feeding probiotic therapy with these two lactobacilli (Bernardeau 244 245 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 feeding strategy for the prevention of *B. hyodysenteriae* and *B. Pilosicoli*. 249

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251

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- 352
- 353

- 354 TABLES
- 355

Table 1: Co-aggregation of viable or heat-inactivated cells of *Lb. rhamnosus* 3698 or *Lb. farciminis* 3699 and *B. pilosicoli* ATCC-51139 as determined by the spectrophotometry 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.

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367 Figure caption

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

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Fig. 2. Electron micrographs of co-aggregates of heat-inactivated *Lb. rhamnosus* 3698 and *B.* 374 hyodysenteriae 9828978. Scanning electron micrographs (A, B, C, E) demonstrating the 375 376 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.

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

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Figure 1



Figure 2





19 Figure 3



Table 1

	% of coaggregation of <i>B. pilosicoli</i> ATCC 51139 with				
Incubation (h)	Lb. rhamnosus 3698		Lb. farciminis 3699		
_	Viable cells	HI-cells	Viable cells	HI-cells	
1	3.50 ± 1.44	$0.62 \pm 0.47*$	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)

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Table 2

		Contact times (here)	Brachyspira pilosicoli ATCC 51139		Brachyspira pilosicoli ATC	coli ATCC 51139
		Contact time (nrs)	Haemolysis	Motility		
With PBS (Control)		4	+	++		
		24	+	+		
	Lb. rhamnosus 3698	4	-	-		
With vishle I h		24	-	-		
with viable Lb.	Lb. farciminis 3699	4	-	+		
		24	-	-		
	Lb. rhamnosus 3698	4	+			
With III Ib		24	-			
with HI- LD.	Lb. farciminis 3699	4	+	++		
		24	-	-		

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