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

Running title

Antagonism between *Lactobacillus* and *Brachyspira*

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Abstract

The sensitivity of *Brachyspira hyodysenteriae* and *B. pilosicoli,* respectively the causative agents of Swine Dysentery and Porcine Intestinal Spirochaetosis to two probiotic *Lactobacillus* strains, *Lb. rhamnosus* CNCM-I-3698 and *Lb. farciminis* CNCM-I-3699 was 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 formation of spherical bodies), and lethal. It was demonstrated for the first time the *in vitro* 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 physical network as demonstrated by SEM; (2) inhibition of the motility of *Brachyspira*. Such *in vitro* studies should encourage in vivo 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.*

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

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1. Introduction

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).

Antimicrobial agents, mainly tylosin and tiamulin are widely used for extended periods to 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 infection by applying preventive measures, and the use of antibiotic treatment should be combined with hygienic, technical and nutritional management practices. A nutritional 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 with probiotic lactobacilli is well documented (reviewed by Bernardeau *et al.*, 2006). Some probiotics contribute to improve herd performance (Torres-Rodriguez *et al.*, 2007; 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 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.*, 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*

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

2. Materials and methods

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 broth (MRS, De Man Rogosa Sharpe, AES, Combourg, France) or milk (Lait G, Standa Industrie, France) in anaerobic conditions (GENbox anaer, Biomérieux, Marcy l'Etoile, France) at 37°C prior to testing. The final *Lactobacillus* population was around $1x10^9$ c.f.u. ml^{-1}. *Lactobacillus* cells were separated from cell-free supernatants by centrifugation at 10,000 x g at 4 °C for 10 min, the pellet was washed three times with phosphate-buffer saline (PBS: 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4 w/v, pH 7.4) and re-suspended with PBS in the initial volume. Cell-free supernatant was filtrated (Cellulose acetate membrane 0.2 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,

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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 $3x10^9$ c.f.u. ml^{-1} ($\sigma = 0.57$), which was the working bacterial suspension.

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

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

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2.3. Electron microscopy

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 with PBS, the cells were post-fixed for 30 min in the dark at room temperature with a mixture of cacodylate buffer containing 1% of osmium tetroxide OsO_4. For scanning electron microscopic (SEM - JEOL Ltd 6400F, Tokyo, Japan) observations, cells were then washed three times with the same mixture and dehydrated in a gradually increased concentration series (30.0%; 50.0% and 70.0%) of ethanol solution for 10 min. The cells were then washed in 100 % ethanol for 10 min before being dried in a critical-point dryer (Balzers CPD 020) and coated with gold. For Transmission electron microscopic (TEM - JEOL 1011/micrograph MegaviewIII SIS) observations, the protocol was adapted from Wood *et al.*, (2006). Cells were washed through a cacodylate series (400, 300 and 200 mM), before being dehydrated through an ethanol gradually increased concentration series and infiltrated with resin. The resin was polymerized at 60°C for 24 hours. Ultra-thin sections (80 nm) obtained with a Leïca Microtome (Leïca Ultra Cut R) were mounted on formvar-coated copper grids. Poststaining was performed by soaking sections on 2.0% uranyl acetate for 15 min and lead citrate for 15 min.

2.4. Statistical analysis

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.

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3. Results

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

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.

The results of the adverse effect of the acidic *Lactobacillus* cell-free supernatant obtained from milk (pH 4.5), on the cell morphology of the spirochaetes are shown on Fig. 1. In Fig. 1 (A), all spirochaetal cells from the PBS control exhibit a typical thin, smooth helical form with pointed ends. The exposure of spirochaetal cells to a stressful acidic (pH 4.5) environment for 1 hour showed besides scattered cells poles (data not shown), cells with morphological variations presumably due to a progressive degree of injury ranging from 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 probably synthesised between the two parallel strands of the cell, (3) cell curls up, and (4) spherical body is formed. The observations were similar for both *Brachyspira* species.

3.2. Coaggregation potential of Lactobacillus *cells on* Brachyspira

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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 coaggregates was delayed with heat-inactivated *Lactobacillus* strains compared to viable cells. Similar coaggregation results were obtained with *B. hyodysenteriae* 9828978 (data not shown). SEM was used to examine the morphological features of the interaction between the *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 mesh-like between the cells found internally in these coaggregates (Fig. 2-B). Areas of close cell-to-cell contact between the two microorganisms are evident both on SEM (Fig. 2-C) and on TEM (Fig. 2-D). No preferential location of cell-to-cell interaction (e.g. tip versus middle) was found. The surface of *Brachyspira* had what appears to be thin filaments of exopolymers emerging from the outer membrane (Fig. 2-E). These fibrils or exopolymers are associated in many cases with other *Brachyspira* cells.

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

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

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

4. Discussion

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

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

Coaggregation -process by which genetically distinct bacteria become attached to one another via specific molecules- is of considerable importance in several ecological niches (Jankovic *et 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 humans and pigs (Drago *et al.*, 1997; Kmet *et al.*, 1995). We demonstrated for the first time the *in vitro* 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 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 chemotaxis) has emerged as a major virulence factor of intestinal spirochaetes, allowing them to escape the physical effect of mucus (Witters and Duhamel, 1999). Mucus eliminates unattached micro-organisms efficiently from the gastro-intestinal tract and, unlike other enteric pathogens, *B. hyodysenteriae* and *B. pilosicoli* are highly motile in the presence of mucus (Nakamura *et al.,* 2006).

The coaggregation properties of some lactobacilli cells have already been observed by Schachtsiek *et al.* (2004) for *Lb. coryniformis* strains DSM 20001^{T} and LTH 1750, *Lb. 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 *Lactobacillus* strains with *Escherichia coli* has also been determined and linked to high level of exopolysaccharides production (Aslim *et al.*, 2007). The coaggregative property of *Lactobacillus* is neither genus- nor species-specific. *Lactobacillus* strains have also demonstrated coaggregative properties with yeasts of urogenital importance such as *Candida albicans* (Boris *et al.*, 1998; Ocana and Nader-Macias, 2002; Hronek *et al.*, 2005).

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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 *Brachyspira* carriage. It agrees with our preliminary field observations of a reduce carriage of *Brachyspira* following a feeding probiotic therapy with these two lactobacilli (Bernardeau and Guilmoto, 2008). Further investigations are needed to explain the nature of coaggregative properties and to determine whether lactobacilli and *Brachyspira* species compete for similar specific receptors on the intestinal epithelium. Such *in vitro* studies should encourage in vivo 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.*

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TABLES

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) ± S.D.

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

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Figure caption

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).

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

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

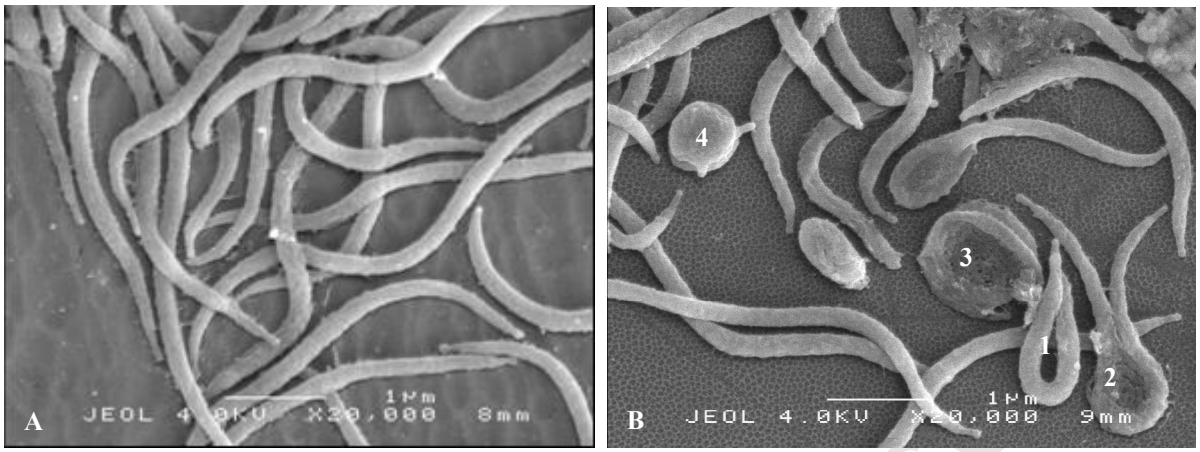

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Figure 2

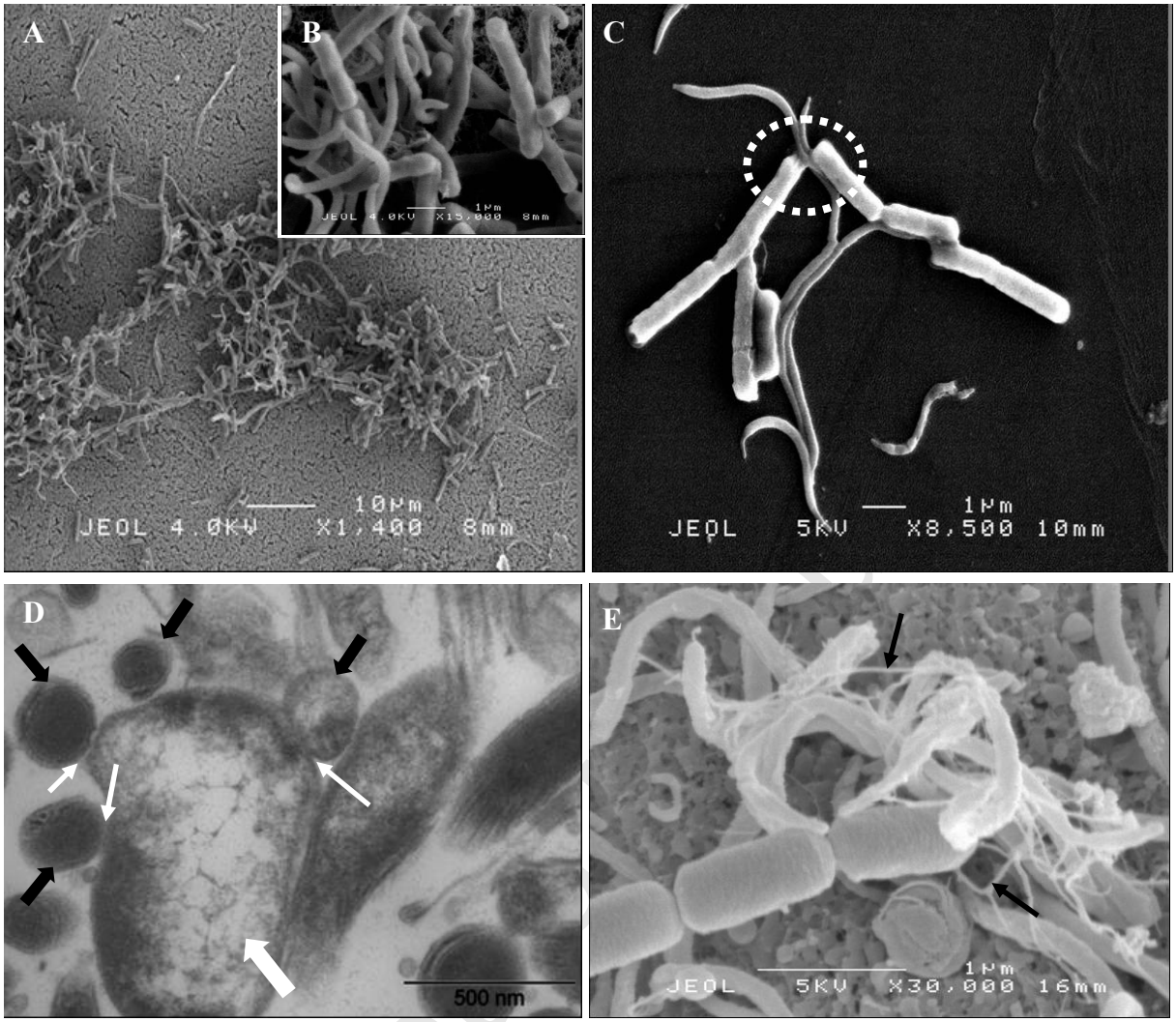

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Figure 3

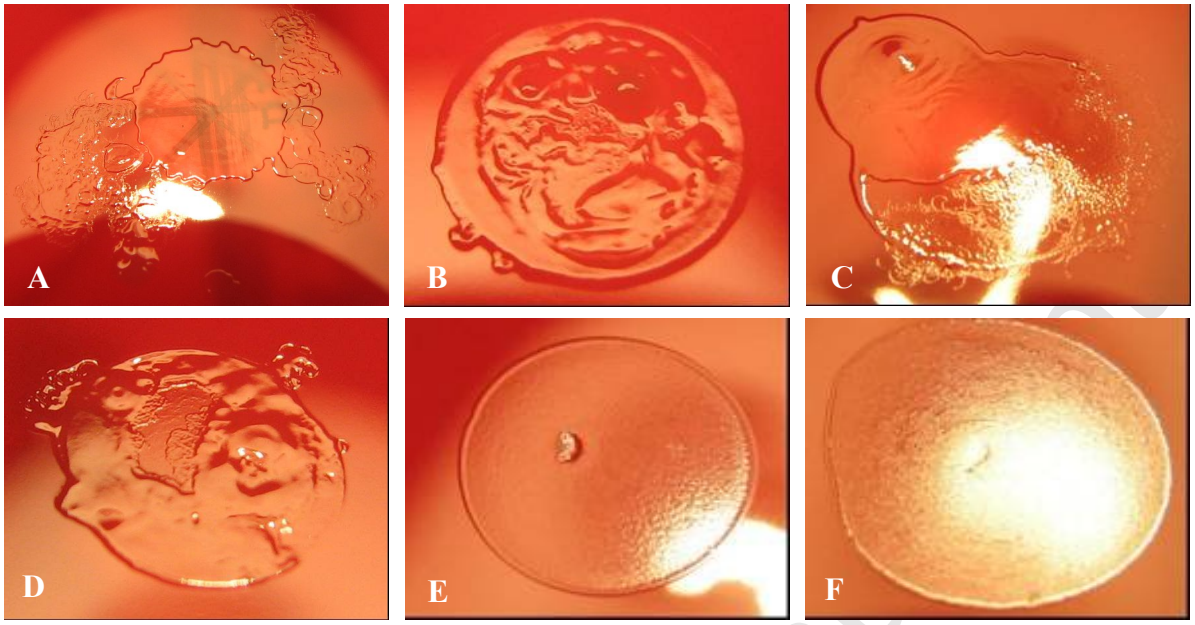

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Table 1

Incubation (h)	% of coaggregation of *B. pilosicoli* ATCC 51139 with			
	Lb. rhamnosus 3698		*Lb. farciminis* 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)

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Table 2

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

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

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