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Anti-bacterial and anti-adherence activities of a probiotic strain of *Lactobacillus paracasei* against *Listeria monocytogenes*

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ABSTRACT

***Lactobacilli* are very ubiquitous, frequently associated to human microbial flora and foodstuffs; where they have being observed to accomplish various protective roles against adverse microorganisms. A human strain of *Lactobacillus paracasei* subsp. *paracasei* originally isolated from newborn faeces was investigated on its anti-listerial activities. Using intestinal Caco-2 cell line in an *in vitro* model and abiotic surfaces, stainless steel and Teflon (polytetrafluoroethylene) which are the most largely used materials in food industry, it was observed that this strain exhibited adherence and anti-adherence properties. The inhibitory effects of this strain on the adherence of *Listeria monocytogenes* were determined. A decrease in the number of adhering pathogen cells was observed, using either pre-incubation or co-incubation of the pathogen with *Lb. paracasei*. Moreover, the anti-listerial and anti-adherence activities of its cell-free supernatant were examined. An antibacterial activity related to the production of a bacteriocin-like substance and a displacement of the pathogen from all the surfaces (Caco-2 cell line, stainless steel and Teflon) were registered. Together, these findings suggest that this strain could be used to prevent colonization of the gastrointestinal tract and the food-contact surfaces by *Listeria monocytogenes*.**

INTRODUCTION

The term biofilm was created to describe the sessile form of microbial life, characterized by adhesion of micro-organisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances (Nikolaev and Plakunov, 2007). *Listeria monocytogenes* is a Gram-positive, facultatively intracellular, food-borne pathogen that has the capacity to cause severe infections, such as gastroenteritis, septicemia, abortion and meningitis in humans and animals (Farber and Peterkin, 1991; Lecuit, 2007). The virulence of *L. monocytogenes* stems from its capacity to adhere, invade, and multiply within professional and non-professional phagocytes (Vazquez-Boland et al., 2001; Seveau et al., 2007). Since the invasion of the intestinal barrier is the first step in the infection process, maintenance of the intestinal ecological flora is important in preventing disease by controlling overgrowth of potentially pathogenic bacteria. Nowadays, there is growing consumers and scientific interests in probiotic bacteria, especially lactic acid bacteria. It is increasingly accepted that these bacteria might represent effective

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tools for controlling overgrowth of pathogens and thereby control or prevent infections. Indeed, numerous *in vitro* and *in vivo* studies performed with different genera of probiotics bacteria have shown the capacities of these bacteria to interfere with both growth and virulence properties of various pathogens (Coconnier et al., 1997; Koga et al., 1998; Tejero-Sarinera et al., 2012). Since, inhibition of pathogen adhesion to the intestinal epithelium may prevent colonization and limit opportunity for systemic infection **(**Humphrey, 2004; Burkholder and Bhunia, 2009), this study investigated in the first part, the *in vitro* ability of a strain of *Lactobacillus paracasei* sub-species *paracasei* to impair adherence of *L. monocytogenes* to Caco-2 cells. In addition, since researchers have reported the occurrence of *L. monocytogenes* on the surface of equipments and utensils in meat and dairy processing industries (Chambel et al., 2007; Cruz et al., 2008), and that surface-adhered microbial cells contaminate food products during processing, the authors decided to test in the second part of this study, the anti-adherence properties of *Lb. paracasei* strain against this pathogen, on stainless steel and Teflon (PTFE®, polytetrafluoroethylene) materials widely used in food industry.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Lb. paracasei sub-species *paracasei* was isolated from 1-year infant faeces. It was identified phenotypically by classical tests (temperature, pH, carbohydrate pattern) and genotypically by sequencing the 16S rDNA. The strain was previously demonstrated as active against *Listeriae* (*L. monocytogenes* EGDe and *Listeria innocua* CLIP 74915), enteropathogenic *Escherichia coli, Salmonella* Typhimurium and *Staphylococcus aureus* (Bendali et al., 2008) and to fulfill probiotics criteria (Bendali et al., 2011). Culture of *Lb. paracasei* was carried out in de Man Rogosa and Sharp (MRS) broth (Fluka, Sweetzerland) and agar (Carl Roth, Sweetzerland) at 37°C under aerobic conditions. Brain Heart Infusion (BHI) broth (Difco, France) was used for culture of *L. monocytogenes* EGDe at 37°C in aerobiosis. Palcam agar (Merck, France) was used for *Listeria* strain counts.

Anti-listerial activity

The well diffusion assay

An overnight anaerobic MRS or Tryptic Soy Broth supplemented with 0.6% Yeast Extract (TSB-YE Merck), culture of *Lb. paracasei* was centrifuged at 8000 × g for 20 min at 4°C (Jouan, thermoelectronic corporation) and the supernatant was filter sterilized through 0.22 µm pore-size Acrodisc® Syringe filters (Pall Gelman Laboratory, USA). Anti-listerial activity of the cell-free supernatant (CFS) was tested by the well diffusion assay as follows: 20 ml of MRS agar poured into a sterile Petri plate was overlaid with 5 ml of soft BHI agar (0.75%, w/v) seeded with *L. monocytogenes* (10^6 CFU ml^{-1}). After solidification, the CFS was placed in duplicate into wells made in the agar. The plates were left at 4°C for 2 h to allow diffusion of tested supernatant and then incubated aerobically for 18 h at 37°C. The absence and presence of inhibitory zones around the wells was recorded.

Characterization of the antibacterial substances in the CFS

Cell free supernatant was divided in three samples. Sample 1 was directly tested while sample 2 was adjusted to a pH of 6.5 with 1 N NaOH (Merck-eurolab, Briare Le Canal, France) to rule out acid inhibition. Inhibitory activity from the hydrogen peroxide was ruled out by the addition of catalase (300 U ml^{-1}) (C- 3515, Sigma-Aldrich Chemie, Steinheim, Germany) to sample 3. The antagonistic activities of the three samples were determined in duplicate by the well diffusion assay as described previously. The sensitivity to different proteolytic enzymes of antibacterial substances was tested as follows. The CFS was treated with several enzymes (all from Sigma-Aldrich Chemie, Steinheim, Germany): α-chymotrypsin, proteinase K, trypsin and papain at final concentration of 2 mg ml^{-1}. All samples were adjusted to a pH of 6.5 with 1.0 mol l^{-1} NaOH (Merck), Filtered-sterilized (0.22 µm pore-size Acrodisc® syringe filters, Pall Gelman Laboratory; USA) and were held for 1 h at 30°C with the proteolytic enzymes. The treated and control samples (supernatant not treated with the enzymes and enzymes preparations) were heated at 100°C for 5 min and then immediately cooled at 4°C in order to inactivate the enzymes. The residual activity of treated and control samples was determined by measuring the diameter of the inhibition zones in the well diffusion assay as described previously.

Adherence to Caco-2 cells

Caco-2 cell culture

The Caco-2 human colon adenocarcinoma cell line was obtained from the American Type Culture Collection and frozen in liquid nitrogen. The cells used in this study were from passage 19. All chemicals used in preparing the cell culture medium were obtained from the GIBCO/BRL

Division of Life Technologies, Invitrogen. Cells were routinely grown at 37°C in a 95% air-5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) containing 4,5 g l^{-1} (+) D-glucose, 110 mg l^{-1} sodium pyruvate, L-glutamate and red phenol. The medium was supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (100 ×), 1% Pen-Strep solution 100 × (100 U of penicillin G per ml and 100 µg of streptomycin sulfate per ml) before it was used.

Monolayers of Caco-2 cells, which were used in the adherence assays, were prepared by inoculating six-well tissue culture plates with 10^6 cells per well in 4.0 ml of culture medium. Cells were grown in 75 cm^2 flasks until they were confluent before they were cultured in the plates. The concentration of the cells was determined under optical microscopy using a Malassez chamber and the number of cells inoculated in each well was found within the range of 1×10^6 to 2×10^6/ well. The plates used were covered with collagen before use. The culture medium was replaced every other day and the monolayers were used in the adherence and invasion assays after 7 days of incubation. The cell culture medium was changed to fresh medium without antibiotics prior to treatment of the cells with bacteria (Johnson-Henry et al., 2008).

Adherence of *Lb. paracasei* to Caco-2 cells

The study of the probiotic adhesion potential was performed as described by Bendali et al. (2011). Briefly, *Lactobacillus* cells were harvested from MRS cultures (18 h) and washed twice with 5 ml Phosphate Buffered Saline (PBS) solution of pH of 7.2. Cells were re-suspended in 1 ml of PBS and then properly diluted in non-supplemented DMEM (GIBCO) to achieve a concentration of 10^8 CFU ml^{-1}. The growth medium in six-well tissue culture plates of Caco-2 monolayers (7 days old) was aspirated and the cells washed twice with PBS. Subsequently, 1 ml of bacterial DMEM suspension was transferred onto the Caco-2 monolayers.

The plates were incubated at 37°C in a 5% CO_2/95% air atmosphere for 1 and 2 h, and then the bacterial suspension was aspirated and the Caco-2 monolayers were washed twice with PBS before 1 ml of Tween 80 (0.04%, w/v; Sigma) was added to detach the adhered bacterial cells. The bacterial suspension was then enumerated as described previously. The adhesion of the strain of *Lb. paracasei* to Caco-2 cells was expressed as a percentage of the viable bacteria compared to their initial population in the DMEM suspension. Adhesion experiments were performed in triplicate. The enumeration of the adhered *Lactobacillus* cells was performed in duplicate.

Adherence of *L. monocytogenes* to Caco-2 cells

Exponentially-phase bacteria (≈10^7 CFU ml^{-1}) were added to tissue culture cells grown in six-well tissue culture plates at a multiplicity of infection (MOI) of 10, for 1 and 2 h at 37°C in antibiotic free tissue culture medium, containing 1 g l^{-1} glucose. After being washed three times with PBS to remove non-adherent bacteria, Caco-2 cells with adherent bacteria were washed with 1 ml 0.04% (w/v) Tween 80 in PBS for 5 min at room temperature to detach the adhered bacterial cells. Bacteria were then serially diluted in PBS and plated onto Palcam agar, and CFU counts were calculated after overnight growth at 37°C to determine the number of viable bacteria adherent to the tissue culture cells. As for *Lb. paracasei*, the adhesion of the strain of *L. monocytogenes* to Caco-2 cells was expressed as a percentage of the viable bacteria compared to their initial population in the DMEM suspension.

Cell invasion assay

To study invasion by *L. monocytogenes*, gentamicin protection assay was performed as described by Werbouck et al. (2006) with the following modifications: Caco-2 cells were infected with 1 ml of bacterial cell suspension containing ≈10^7 *L. monocytogenes* cells (MOI 10) grown or treated as described previously for 1, 2 and 3 h. After two washings with PBS, the Caco-2 cells were re-incubated for 90 min in fresh DMEM containing gentamicin (Sigma Chemical Co.) at a concentration of 5 mg l^{-1}. However, to determine total counts of bacteria associated with the cell (counts of adhered bacteria plus counts of intracellular bacteria), duplicate wells with Caco-2 cells were analyzed without gentamicin treatment. The cell monolayers with or without gentamicin treatments were then washed with PBS and lysed with 1% Triton X-100 (Sigma). Appropriate dilutions were plated on Palcam plates and the CFU were enumerated. The invasion efficiency (invasion index) was calculated according to Jaradat and Bhunia (2003) by dividing the number of CFU that invaded the cells (with gentamicin) by the total number of CFU obtained without gentamicin treatment (both the invasion and the adhesion counts).

***L. monocytogenes* adherence and invasion inhibition assays**

Three different procedures were used in order to differentiate exclusion, competition and displacement of *L. monocytogenes* by *Lb. paracasei* and its CFS. For exclusion tests, Caco-2 cell monolayers were cultured and washed as previously described and incubated with *Lactobacillus* (10^8 CFU ml^{-1}, MOI 100) for 1 and 2 h.

Afterwards, non-adhering lactobacilli were removed by two washes with PBS solution, *L. monocytogenes* (10^7 CFU ml^{-1}, MOI 10) was added and incubation was continued for further 1 and 2 h. Regarding competition tests, *Lb. paracasei* (10^8 CFU ml^{-1}, MOI 100) and the pathogen (10^7 CFU ml^{-1}, MOI 10) were mixed and added to the intestinal cells and then incubated for 2 h. For displacement assays, *L. monocytogenes* was first added to Caco-2 cells and then incubated for 2 h before the addition of *Lb. paracasei* CFS, and left for 30 min. The number of bacteria detached from the intestinal cells was determined by plating serial dilutions on Palcam agar plates. Each assay was conducted at least twice with two determinations per assay (Forestier et al., 2001). For invasion inhibition assay, the same experiments (pre- and co-incubation) were performed but the bacterial enumeration was realised after re-incubation of the Caco-2 cells in the presence of gentamicin (5 mg l^{-1}).

Microbial adherence to stainless steel and PTFE®

Stainless steel and PTFE® treatment

The two surfaces used for biofilm experiments were AISI 304 stainless steel and PTFE® (polytetrafluoroethylene, Teflon). Each surface was cut into rectangular coupons (3 by 1.5 cm) and washed using the procedure described by Bellon-Fontaine and Cerf (1990) with some modifications before adhesion assays. The coupons were washed by immersion for 10 min with agitation in 200 ml of an alkaline detergent of 2% (v/v) RBS 35 (Société des Traitements Chimiques de Surface, France) solution (initial temperature 50°C), and then were rinsed by immersion in 200 ml of tap water (initial temperature 50°C) with agitation for 25 min. Five further 1 min immersions with agitation in 200 ml of distilled water at ambient temperature were performed. The coupons were then autoclaved at 120°C for 20 min and were dried in a laminar air flow hood.

Adhesion experiments

Adhesion tests were conducted using the procedure of Chavant et al. (2002) with slight modifications. Bacterial cells (*Lb. paracasei* and *L. monocytogenes*) were harvested by centrifugation (8,000 × g at 4°C, 20 min), washed with sterile PBS (pH 7.2) solution and re-suspended in the same solution to give 10^8 CFU.ml^{-1}. Ten (10) ml of the bacterial suspension was poured into a Petri plate (55 mm diameter) containing a stainless steel or a PTFE® coupon and was incubated at 30°C for 3 h. Next, coupons were washed twice with PBS and the remaining cells detached from the inert surfaces by vortexing the coupons in 20 ml of sterile PBS in the presence of sterile glass beads for 2 min. Cells detachment was also done after a prolonged incubation of the adhered cells for 18 h as follows. The washed coupons were placed in another Petri plate containing 10 ml of sterile TSB-YE and were incubated for 18 h. Next, the coupons were washed twice again with PBS and the cells were detached as described previously. After ten-fold serial dilutions, viable adherent bacteria were counted after cultivation on Palcam agar (Merck). Each experiment was performed in triplicate using independently grown cultures.

Inhibition of *L. monocytogenes* adhesion to abiotic surfaces

Using Lb. paracasei cells

Lb. paracasei (10^8 CFU ml^{-1}) and *L. monocytogenes* (10^8 CFU ml^{-1}) strains were grown in TSB-YE and incubated at 30°C then a pre-incubation procedure was used as follows. 10 ml of *Lb. paracasei* culture at 10^8 CFU ml^{-1} were poured in a Petri plate containing the stainless steel or the PTFE® coupon and incubated for 3 h at 30°C. The culture broth was then aspirated and the coupon rinsed twice with PBS solution. Ten (10) ml of a listerial culture at 10^8 CFU ml^{-1} were used to inoculate the Petri plate and then incubated at 30°C for additional 3 and 18 h as described for adhesion assays. The same experiment was performed in a co-incubation procedure: 5 ml of each culture (10^8 CFU ml^{-1} for *Lb. paracasei* and for *L. monocytogenes*) were mixed together and poured into the Petri plate containing the stainless steel or the PTFE® coupon and incubated at 30°C for 3 and 18 h. At the end of the two procedures, the coupons were rinsed twice with PBS and the adherent *L. monocytogenes* cells were detached and enumerated as described previously. Each experiment was performed in triplicate using independently grown cultures. *Lb. paracasei* (10^8 CFU ml^{-1}) was used for the displacement of mature *L. monocytogenes* biofilms (7 days) produced on the same surfaces (stainless steel and PTFE®) using post-incubation for 1 h. The mature biofilms were obtained as described by Bellon-Fontaine et al. (1996). The number of detached cells from the two surfaces was determined by plating appropriate dilutions on Palcam agar plates.

Using the Lb. paracasei culture supernatant

In the pre-incubation procedure, TSB-YE culture supernatant of the *Lactobacillus* strain was recovered after 18 h culture (10^8 CFU ml^{-1}) by centrifugation at 8,000 × g for 20 min, at 4°C. Ten (10) ml of the CFS were poured in a Petri plate containing the stainless steel or the PTFE® coupon and was left for 1 h at 30°C, then the

Table 1. Anti-listerial activity of *Lb. paracasei* culture supernatant (mm).

Culture broth	pH values of the culture supernatant		
	4.0	**5.6**	**6.5**
MRS	18	12	12
TSB-YE	24	15	15

MRS: de Man, Rogosa and Sharp medium; TSB-YE: Tryptic Soy Broth-Yeast Extract.

supernatant was aspirated and the coupon rinsed twice with PBS solution. Ten (10) ml of a listerial culture in TSB-YE broth (10^8 CFU ml^{-1}) were used to inoculate the Petri plate and then incubated at 30°C for additional 3 and 18 h, as described previously. The coupons were rinsed twice with PBS and the adherent *L. monocytogenes* cells were detached and enumerated as aforementioned. Each experiment was performed in triplicate using indepen-dently grown cultures. The same experiment was performed in co-incubation procedure, using 5 ml *Lactobacillus* CFS and 5 ml of *L. monocytogenes* culture (10^8 CFU ml^{-1}) in TSB-YE as an adhesion medium. As for *Lb. paracasei* cells, *Lb. paracasei* (10^8 CFU ml^{-1}) CFS was used for the displacement of mature *L. monocytogenes* biofilms (7 days) produced on the same surfaces (stainless steel and PTFE®) using post-incubation for 1 h.

Statistical analyses

All the results were expressed as mean ± standard deviation. Statistical analyses was performed using ANOVA test and paired Student's test to compare the concentration of *L. monocytogenes* at different times and conditions of the trials. Probability values of $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Anti-listerial activity

Using MRS or TSB-YE broths as growth media, large differences were observed regarding the anti-listerial activity. The *Lb. paracasei* CFS recovered from MRS broth had a pH of 4.0 whereas that obtained from TSB-YE was just 5.6. This difference in pH value could be attributed to the low carbohydrate content of the TSB-YE compared to MRS (2% glucose). The CFS whether at pH 6.5, 5.6 or 4.0 inhibited the growth of the pathogenic strain tested at different degrees as revealed by the disparity of diameters of inhibition zones (Table 1). If pH neutralization reduced the anti-listerial activity of the MRS supernatant, no reducing effect was registered in the case of the TSB-YE supernatant (pH 5.6) which demonstrated the involvement of the acidic pH in the MRS supernatant anti-listerial activity but not in the TSB-YE one. Since inhibition was observed when the pathogen was grown in the presence of near-neutral supernatant (pH 6.5) or with the appropriate control medium (MRS and TSB-YE at pH 5.6 and 6.5), inhibition effects cannot be explained by only organic acids production (along with the low pH in MRS broth). They are most probably due to other substances (most probably due to a bacteriocin) since proteases treatment abolished completely the anti-listerial activity and no effect on the anti-listerial activity was noted after catalase treatment of either MRS or TSB-YE culture supernatant.

Adherence of *Lb. paracasei* and *L. monocytogenes* to Caco-2 cells

Since bacterial adhesion to intestinal cells is considered one of the most crucial selection criteria for probiotic strains (Dunne et al., 2001), we determined the adherence capacities of the *Lb. paracasei* strain on Caco-2 cells. As already reported by Bendali et al. (2011), the probiotic strain was able to adhere to the cell surface monolayer with an average of 6.0×10^6 CFU ml^{-1} after 1 and 2 h incubation at levels ranging from 1.33 to 1.71%, respectively. Adhesion of *Lb. paracasei* to intestinal epithelial cells would allow colonization of the intestinal mucosa and therefore could limit the overgrowth of pathogens. Previous studies indicated that *Lactobacillus* species are able to adhere to the surface of intestinal epithelial cells in tissue culture (Granato et al., 1990; Greene and Klaenhammer, 1994; Kaushik et al., 2009). Similarly, *L. monocytogenes* was able to adhere to Caco-2 cells within different time contact (1 and 2 h). 2.5% of the cells population adhered to Caco-cells within 1 h contact time; this level reached a value of 7.5% after further 1 h incubation (Figure 1).

Inhibiton of *L. monocytogenes* adherence to Caco-2 cells

Lb. paracasei inhibited *L. monocytogenes* adhesion. This

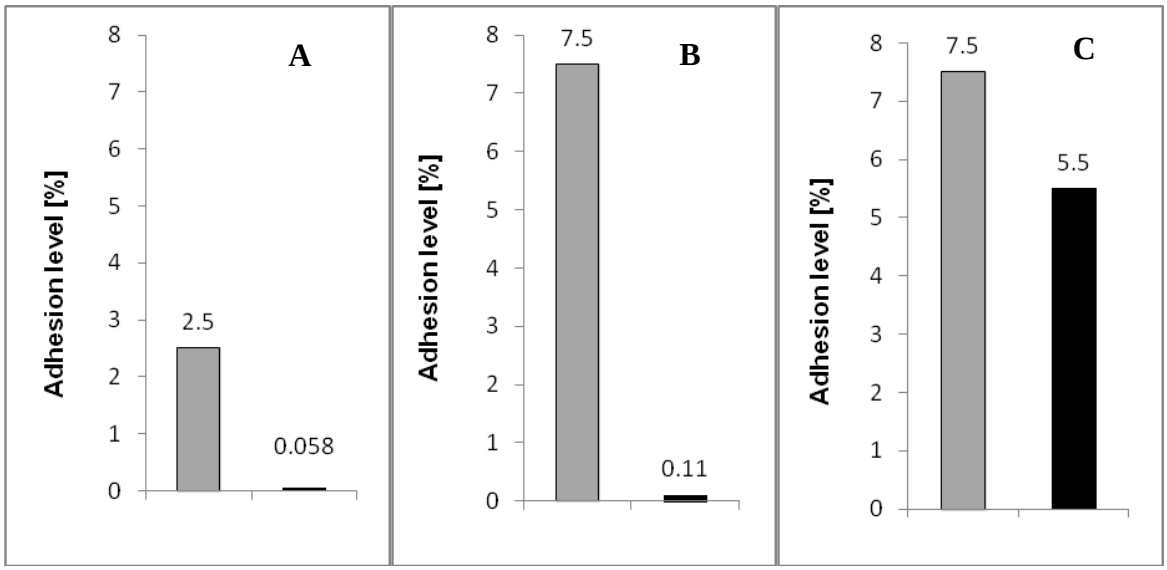

Figure 1. Adhesion level (%) of *L. monocytogenes* to Caco-2 cells in the absence (■) and in the presence of *Lb. paracasei* cells (■). In the case of post-incubation procedure, the remaining attached cells after *Lb. paracasei* culture supernatant treatment was indicated (■). A, Pre-incubation [contact 60 min, multiplicity of infection (MOI) 10]; B, co-incubation (contact 120 min, MOI 10); C, post-incubation (contact time 120 min, MOI 10).

inhibition was dependent on the time of contact, the inhibition agent (live *Lb. paracasei* cells or its cell-free culture supernatant) and on the mode of contact (pre-incubation, co-incubation or post-incubation). The inhibition level varied from 99.37% (2 h) to 99.77% (1 h) in the pre-incubation experiments and from 98.26% (2 h) to 99.47% (1 h) in the co-incubation experiments. The best inhibition was registered in the pre-incubation procedure than the co-incubation while the best inhibition rates were obtained within 1 h than after 2 h. Whereas in the post-incubation procedure with the use of the *Lb. paracasei* CFS, the detached cells level was 2% as determined by the enumeration of the remaining adhered cells after 30 min CFS treatment and no viable detached cell was detected. In the pre-incubation experiments, *Lb. paracasei* (≈ 10^8 CFU ml^{-1}) was maintained in contact to the Caco-2 cells for 2 h. After elimination of the non-adherent bacteria, *L. monocytogenes* cells (≈10^7 CFU ml^{-1}) were added and the plates were further incubated for 1 and 2 h, respectively. At the end of the incubation periods, very low adhesion levels (0.058 and 0.006 %) were recorded for *L. monocytogenes* after 1 and 2 h (Figure 1). In the co-incubation experiments, adhesion levels of 6.0×10^6 and 9.2×10^4 CFU ml^{-1} were obtained for *L. monocytogenes* (initial level 8×10^7 CFU ml^{-1}) in the absence (control) and in the presence of *Lb. paracasei* (7.2×10^8 CFU ml^{-1}) which represents an inhibition percentage of 98.47% compared to the control (Figure 1). This indicates that the probiotic strain adhered more easily and quickly to the Caco-2 cells than the pathogen. Whereas in the post-incubation procedure, the *Lb. paracasei* culture supernatant was also examined for its ability to impair the adherence of *L. monocytogenes* to Caco-2 cells. Using a MOI of 10, the level of adhesion of this pathogen (in the absence of the active supernatant) was 6.0×10^6 CFU ml^{-1} and in the presence of *Lb. paracasei* culture supernatant, the adhesion of the pathogen was reduced. As shown in Figure 1, incubation with the *Lb. paracasei* culture supernatant for 30 min resulted in displacement levels of 2.0% of the adhered *L. monocytogenes* population using post-incubation procedure. Previous reports (Jankowska et al., 2008; Banerjee et al., 2009) have showed that *Lactobacilli* inhibit binding and cytotoxic effect of pathogens with a Caco-2 cell model. The pre-installation of *Lb. paracasei* was shown to be the best procedure in impairing the pathogen adhesion. This can be the result of several factors. Indeed, it has been demonstrated that probiotics increased expression of mucins (Mack et al., 1999; Mack et al., 2003) and as expected for *Lactobacillus* Lcr35, *Lb. paracasei* could interact with the level of mucins produced by Caco-2 cells and thus impair the adhesion of *L. monocytogenes*. The presence of *Lb. paracasei* may impede the access of pathogen to cell surface by steric hinderance and that may explain the decrease of adhesion of the pathogen in the presence of *Lb. paracasei*. However, this hypothesis could not account for the whole inhibition process. It is also possible that *Lb. paracasei*-specific products inhibit the adhesion of *Listeriae* since it has been previously shown that

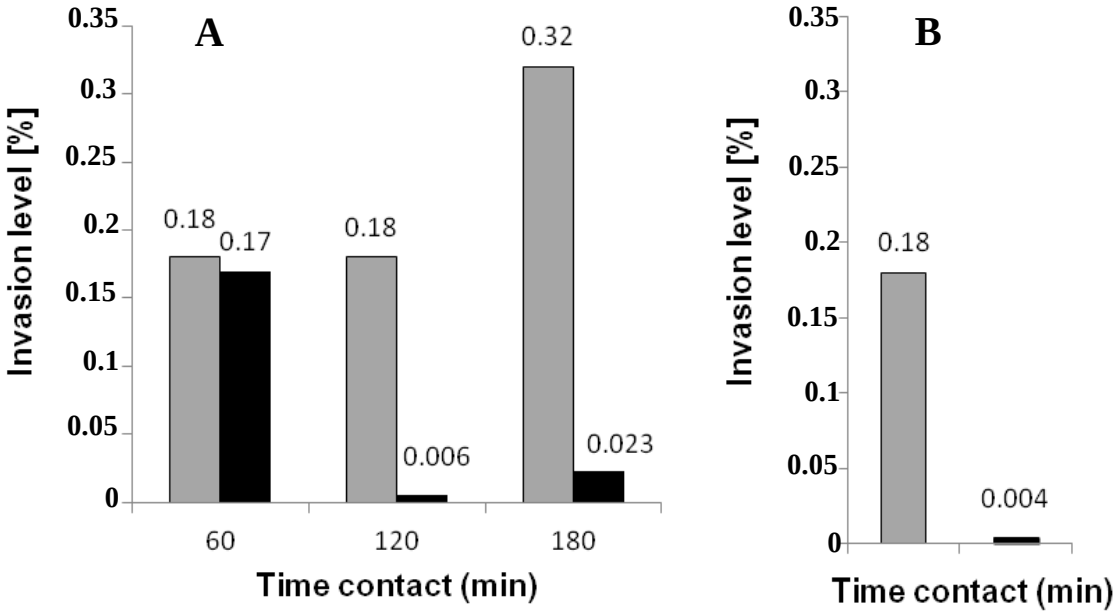

Figure 2. Invasion of Caco-2 cells by *L. monocytogenes* in the absence (■) and in the presence of *Lb. paracasei* (■). A, Pre-incubation [contact 60 min, 120 and 180 min, multiplicity of infection (MOI) 10]; B, co-incubation (contact 120 min, MOI 10).

production of biosurfactants by some strains of *Lactobacillus* can prevent adhesion of pathogens to intestinal cells (Vignolo et al., 1993; Gudina et al., 2010). Probiotics were also reported to exert their beneficial effects by producing bacteriostatic or bactericidal agents (Takahashi et al., 2004; Corr et al., 2007), competitively excluding pathogenic bacteria such as bacteriocins, proteinases, peroxides, and exopolysaccharides (Bernet-Camard et al., 1997; Lebeer et al., 2008).

L. monocytogenes invasion of human Caco-2 cells

To study the invasion potential of *L. monocytogenes* in specific Caco-2 cells, gentamicin protection assay was performed. The strain was able to invade with low capacity (≈10^4 CFU ml^{-1}) into Caco-2 cells after 1 and 2 h (1.8×10^4 CFU ml^{-1}), and 3 h (3.2×10^4 CFU ml^{-1}) of contact with an invasion index of 5.8×10^{-4}.

Inhibition of *L. monocytogenes* invasion of human Caco-2 cells

Using *Lb. paracasei*, Caco-2 cells invasion by *L. monocytogenes* was inhibited. This inhibition was dependent on the time of contact, the inhibition agent (live *Lb. paracasei* cells or its cell-free supernatant) and the mode of contact (pre-incubation, co-incubation or post-incubation procedure) (Figure 2). The inhibition levels of *L. monocytogenes* varied from 99.26 to 99.77% (with the *Lb. paracasei* bacterial cells) and from 92.90 to 99.33% (with the sole *Lb. paracasei* culture supernatant). However, no effect was observed using the post-incubation procedure (Figure 2). Very little is known about the mechanism of inhibition of adhesion/invasion of pathogens by *Lactobacilli*. In a study by Jaradat and Bhunia (2003), using the Caco-2 cell line, they found significant variation in efficiencies of invasion among *L. monocytogenes* strains, ranging from 1.8 to 31.4% of the initial *inoculum*. Similarly, Chaterjee et al. (2006) reported a large variability in invasiveness among *L. monocytogenes* serotypes. Roberts et al. (2009) found that *L. monocytogenes* outbreak strains showed variation in invasion efficiencies in Caco-2 human intestinal epithelial cells. The mean invasion efficiency ranged from 0.01 to 3.17%.

Bacterial adhesion to stainless steel and PTFE®

Bacterial cells adhesion to stainless steel and PTFE® surfaces is presented on Figure 3. *Lb. paracasei* showed maximum adherence levels of 2×10^3 CFU cm^{-2} on AISI 304 and 3.5×10^4 CFU cm^{-2} on PTFE® after 3 h incubation (Figure 3A). After prolonged incubation period (18 h), the maximum adherence levels of 6.2×10^5 and 6.6×10^7 CFU cm^{-2} were obtained on AISI 304 and PTFE® respectively

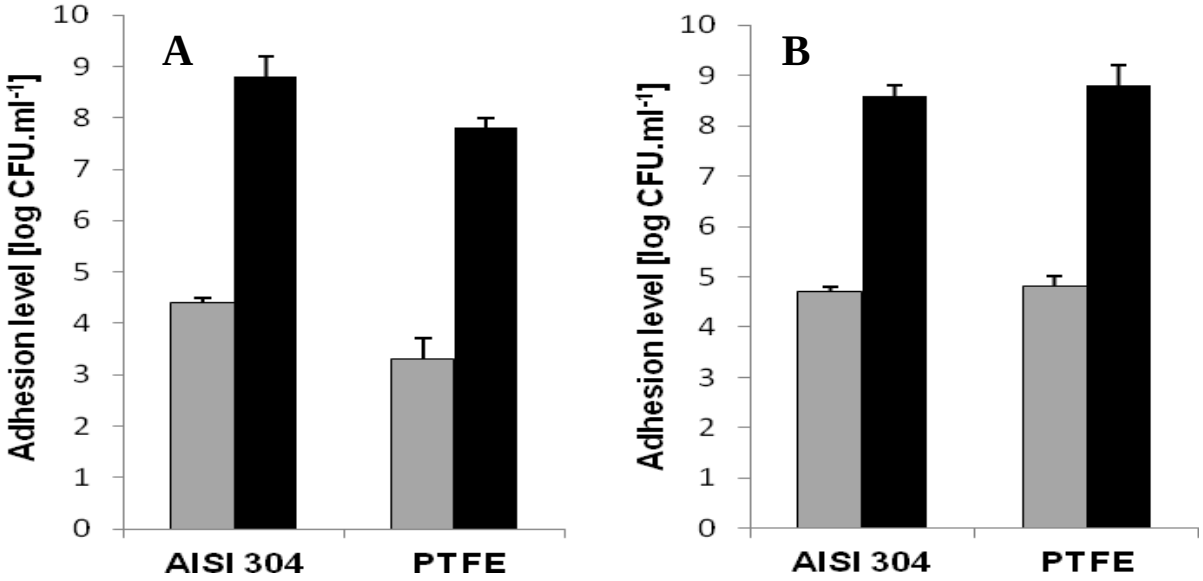

Figure 3. Adhesion levels *Lb. paracasei.* A, adhesion level of *Lb. paracasei*; B, *L. monocytogenes* on AISI 304 and PTFE® after 3 (■) and 18 h (■) contact times.

(Figure 3C). We observed that Lb. paracasei adheres strongly to PTFE® surfaces than to stainless steel 304. On the contrary, *L. monocytogenes* strain adhered strongly to both surfaces. The adhesion level was a function of the contact time (3 and 18 h). Stainless steel and PTFE® were selected because of their common use in food-processing plants and because they have different physic-chemical characteristics (Blackman and Frank, 1996; Arnold and Bailey, 2000). Several authors (Mafu et al., 1990; Blackman and Frank, 1996; Chavant et al., 2002) have reported the capacity of different strains of *L. monocytogenes* to colonize such surfaces. Results from analysis of variance showed that the nature of the surface and the contact time were the main factors which statistically affected adhesion and colonization ($P< 0.05$). Thus, better adhesion and colonization were observed on the stainless steel, confirming the hydrophilic character of *L. monocytogenes* EGDe and the importance of this property in these processes (Van Loosdrecht et al., 1987; Chavant et al., 2002). This results are close to that obtained by de Oliveira et al. (2010), who reported that *L. monocytogenes* adhered to the stainless steel surface and presented a count of 4.89 Log CFU.cm^{-2} after 3 h of contact. In their study, initial adhesion capacity measured during 3 h was 58.75±0.90% and this corresponded to an *inoculum* of 8.26±0.18 Log CFU ml^{-1} (OD_{600nm} = 0.873±0.04). Chavant et al. (2002) observed that the colonization of stainless steel coupons by *L. monocytogenes* LO28 was fast; and at least 80% of the coupon surface was covered by the biofilm after 2 h at 37 and 20°C. For both surfaces (AISI 304 and PTFE®), the colonization reached 100% after 24 h at 37°C, and under their experimental conditions, a minimum of 10^7 CFU cm^{-2} were observed after 2 h of contact with the substrata.

Inhibition of *L. monocytogenes* adhesion

Using the Lb. paracasei cells

Lb. paracasei showed an inhibition effect on adhesion and consequently the biofilm formation of *L. monocytogenes* on AISI 304 and on PTFE®. Significant differences ($P<0.05$) were recorded between the adhesion levels in the absence and in the presence of the strain following either the pre-incubation or the co-incubation procedure. The highest efficiency displayed by *Lb. paracasei* was in the pre-incubation procedure with a total inhibition of the adhesion of *L. monocytogenes* to AISI 304 and an inhibition of 98.6% on PTFE®. However, using the co-incubation procedure, the adhesion of the pathogen was inhibited only by a mean of 95% (Figure 4). Yet, the application of *Lb. paracasei* post-installation of *L. monocytogenes* onto the abiotic surfaces did not influence the adhesion level. The presence of *Lb. paracasei* may impede the access of pathogen to material surface by steric hindrance and that may explain the decrease of adhesion of the pathogen in the presence of *Lb. paracasei*. However when tested individually, the level of adhesion of the pathogen was at least 10 times higher than those of *Lb. paracasei* at the

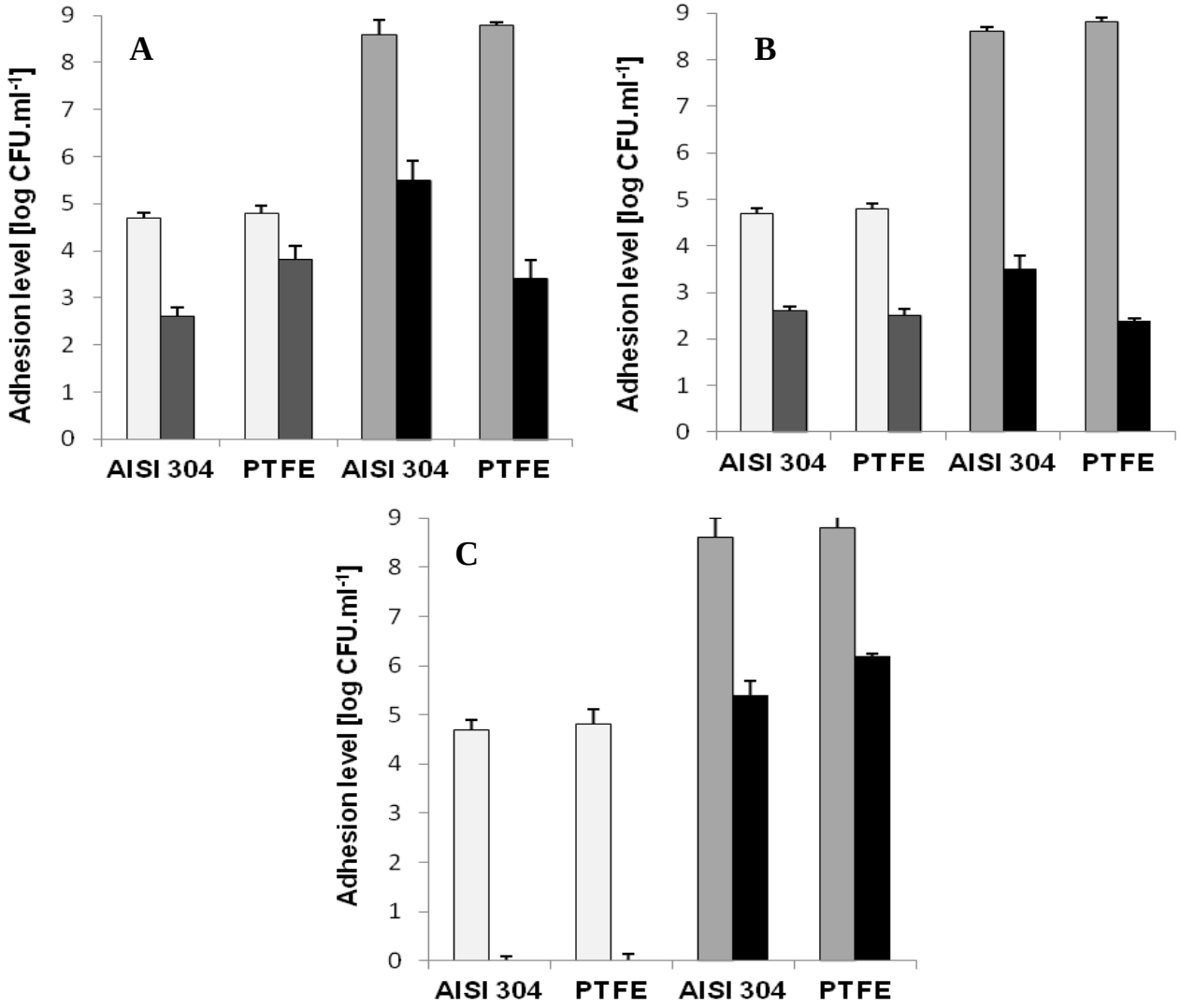

Figure 4. Adhesion level of *L. monocytogenes* on stainless steel AISI 304 and PTFE® in the absence (□, ■) and in the presence (■,■) of *Lb. paracasei* after 3 h contact (□,■) and prolonged period 18 h (■,■) contact time following the pre-incubation (A), co-incubation (B) and post-incubation (C) procedures. In the later case, the detachment level in the presence of the culture supernatant rather than the adhesion level was indicated.

same contact time. Therefore, this hypothesis could not account for the whole inhibition process.

Using the cell-free supernatant

Cell-free culture supernatant of *Lb. paracasei* showed an inhibition effect on adhesion and consequently on biofilm formation of *L. monocytogenes*. Similar to the use of the bacterial cells, significant differences ($P<0.05$) were recorded between the adhesion levels in the absence and in the presence of the culture supernatant. Similar efficiency was displayed by the *Lb. paracasei* cell-free supernatant in the co-incubation and in the pre-incubation procedures ($P<0.05$), indicating that adsorption of the supernatant constituents to the inert surfaces was substantial (Figure 5). Interestingly, significant detachment levels of the installed biofilms (7 days) were registered when treated with the cell-free supernatant compared to the non-treated ones. Adherence of bacteria to the material surface is an important prerequisite for colonization by microorganisms and biofilm formation. Inhibiting the adhesion of pathogenic bacteria to the material surfaces could decrease the surface colonization and in consequence block the process of biofilm formation. In this study, we showed that *Lb. paracasei* interfered with the adhesion process to stainless steel and PTFE® surfaces of *L. monocytogenes*. The

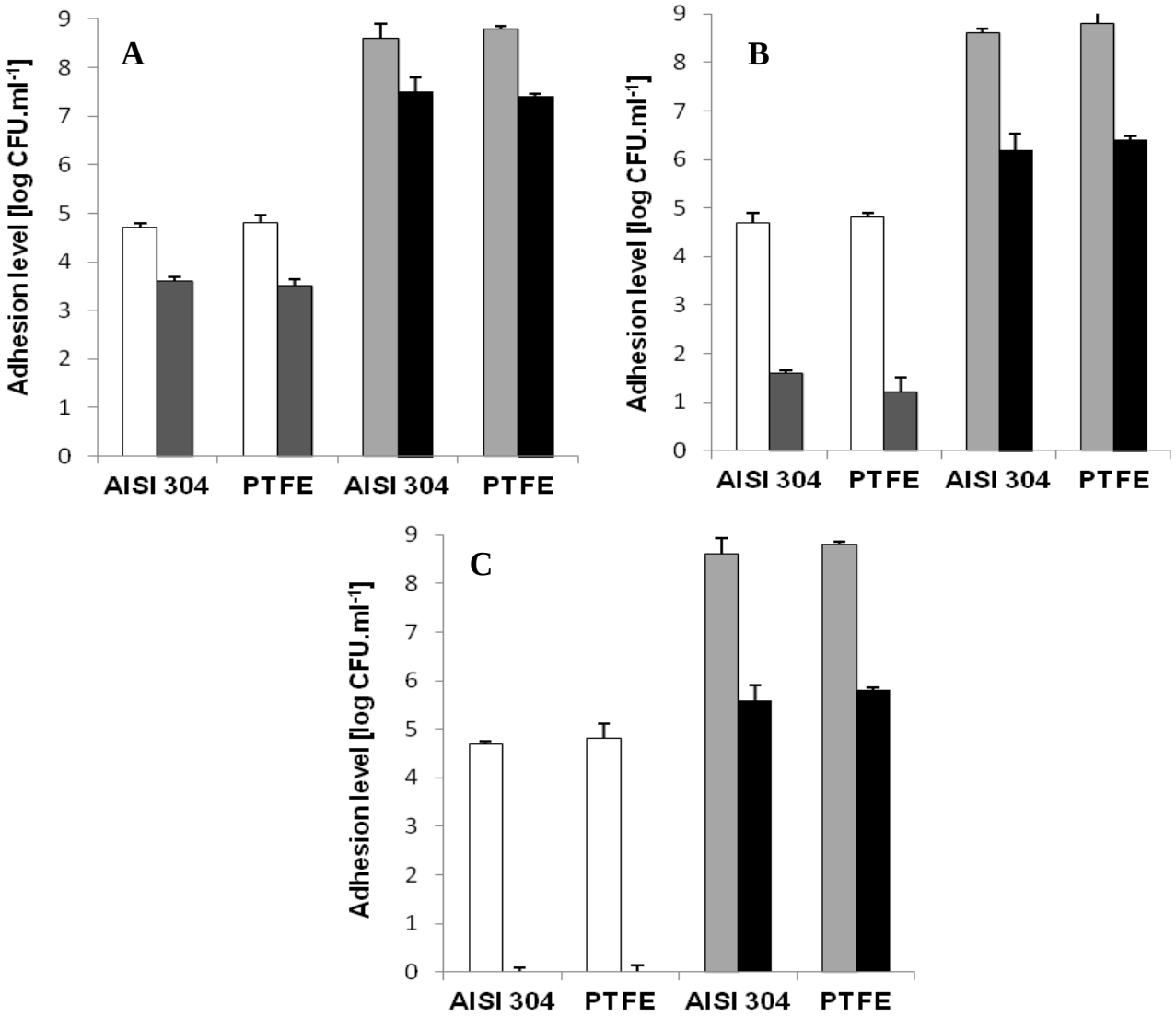

Figure 5. Adhesion level of *L. monocytogenes* on stainless steel AISI 304 and PTFE in the absence (□, ■) and in the presence (■,■) of *Lb. paracasei* culture supernatant after 3 contact (□,■) and 18-h (■,■) contact time following the pre-incubation (A), co-incubation (B) and post-incubation (C) procedures. In the later case, the detachment level in the presence of the culture supernatant rather than the adhesion level was indicated.

adherence of the pathogen was decreased by addition of *Lb. paracasei* or its cell-free supernatant regardless of whether the *Lb. paracasei* or its supernatant was added before or during the incubation with the pathogen. Zhao et al. (2004) have already reported the inhibition of *L. monocytogenes* settlement on stainless steel by competitive exclusion *Enterococcus* and *Lactococcus* strains.

Conclusion

Adherence of bacteria to biotic and abiotic surfaces is an important prerequisite for colonization by micro-organisms, biofilm formation and invasion (mucosa). Inhibiting the adhesion of pathogenic bacteria to the surfaces could decrease the surface colonization and in consequence block the process of biofilm formation and propagation. In this study, we showed that *Lb. paracasei* interfered with the adhesion process of *L. monocytogenes* to Caco-2 cells, stainless steel and PTFE® surfaces. The adherence of the pathogen was decreased by addition of *Lb. paracasei* or its cell-free supernatant regardless of whether the *Lb. paracasei* or its supernatant was added before or during the incubation with the pathogen. Therefore, the *Lactobacillus* strain

used in this study may be an excellent candidate for eventual use as prophylactic and therapeutic agent. The protective role of lactic acid bacteria against biotic and abiotic surfaces colonization by pathogenic micro-organisms has gained more credibility and may represent a new effective tool to control pathogen overgrowth inside the gastrointestinal tract of patients and onto food contact surfaces.

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