Effect of fermented broth from lactic acid bacteria on pathogenic bacteria proliferation

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ABSTRACT

In this study, the effect that 5 fermented broths of lactic acid bacteria (LAB) strains have on the viability or proliferation and adhesion of 7 potentially pathogenic microorganisms was tested. The fermented broth from *Lactococcus lactis* C660 had a growth inhibitory effect on *Escherichia coli* K92 that reached of 31%, 19% to *Pseudomonas fluorescens*, and 76% to *Staphylococcus epidermidis*. The growth of *Staph. epidermidis* was negatively affected to 90% by *Lc. lactis* 11454 broth, whereas the growth of *P. fluorescens* (25%) and both species of *Staphylococcus* (35% to *Staphylococcus aureus* and 76% to *Staph. epidermidis*) were inhibited when they were incubated in the presence of *Lactobacillus casei* 393 broth. Finally, the fermented broth of *Lactobacillus rhamnosus* showed an inhibitory effect on growth of *E. coli* K92, *Listeria innocua*, and *Staph. epidermidis* reached values of 12, 28, and 76%, respectively. *Staphylococcus epidermidis* was the most affected strain because the effect was detected from the early stages of growth and it was completely abolished. The results of bacterial adhesion revealed that broths from *Lc. lactis* strains, *Lactobacillus paracasei*, and *Lb. rhamnosus* caused a loss of *E. coli* K92 adhesion. *Bacillus cereus* showed a decreased of adhesion in the presence of the broths of *Lc. lactis* strains and *Lb. paracasei*. *Listeria innocua* adhesion inhibition was observed in the presence of *Lb. paracasei* broth, and the greatest inhibitory effect was registered when this pathogenic bacterium was incubated in presence of *Lc. lactis* 11454 broth. With respect to the 2 *Pseudomonas*, we observed a slight adhesion inhibition showed by *Lactobacillus rhamnosus* broth against *Pseudomonas putida*. These results confirm that the effect caused by the different LAB assayed is also broth- and species-specific and reveal that the broth from LAB tested can be used as functional bioactive compounds to regulate the adhesion and biofilm synthesis and ultimately lead to

preventing food and clinical contamination and colonization of *E. coli* K92, *B. cereus*, and *Ls. innocua*.

Key words: lactic acid bacteria, *Lactobacillus*, *Lactococcus*, adhesion, probiotics

INTRODUCTION

Pathogenic bacteria or toxins produced by bacteria often enter the human body through food or drink, causing symptoms or illness with several mechanisms. *Staphylococcus aureus* and *Escherichia coli* belong to the normal flora of about 50% of people, although virulent strains, resistant to commonly used antibiotics, may cause sepsis and severe infections. Food-borne intoxication is caused by a heat-stable enterotoxin produced by *Staph. aureus* in consumed food, which produces nausea and vomiting (Nester, 2001). *Bacillus cereus* contaminates various types of final food products, such as cooked chilled meals, vegetables, meat, fish, milk, liquid egg, pastries, flavorings, oils, and fats (Wijnands et al., 2006; Baron et al., 2007). On the other hand, *Listeria monocytogenes* is a common bacterium in the environment and in animals, and it may be transferred to food and the human gastrointestinal tract via raw milk and contaminated dairy products. This organism may cause meningitis, sepsis, or abortion, but in practice only pregnant women and people with immune defects are in danger of catching the infection (Nester, 2001). *Staphylococcus epidermidis*, commensal coagulase-negative, may be transferred to the human gastrointestinal tract via breast milk because this microorganism belongs to skin microbiota (Iwase et al., 2010; Park et al., 2011).

Probiotics are defined as living microorganisms which act to keep the balance of intestinal microbiota after ingestion in sufficient numbers (Fuller, 1989). Lactic acid bacteria (LAB) are probiotic microorganisms known to produce antibacterial peptides and small proteins called bacteriocins, which enable them to compete against other bacteria in the environment. Lactic acid bacteria, such as *Lactobacillus* species, have protective effects against a variety of pathogenic infections in the gastrointestinal systems of humans and animals (Ahrné et al., 1998; Gill et al., 2001). These strains produce

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a wide range of antimicrobial agents and have been studied extensively with regard to their possible use in the field of probiotics. This bacteria may also be a useful alternative for the treatment and prevention of a variety of infectious diseases caused by oral, enteric, and urogenital pathogens (Shornikova et al., 1997; Caglar et al., 2006; Abad and Safdar, 2009).

Recently, probiotics have been studied principally for their potential as an alternative to antibiotics in the field of livestock production (Reid and Friendship, 2002; Reid, 2006). Commonly used probiotics such as *Bacillus*, *Lactobacillus*, *Bifidobacterium* species, and *Clostridium butyricum* have the potential to improve swine growth performance, to increase utilization of nutrients such as dietary starch, and to resist colonization by pathogens in the gastrointestinal tract of animals (Lee et al., 2001; Guo et al., 2006). Many studies suggest that LAB could protect sites against pathogenic bacterial invasion and colonization by preventing the attachment of these pathogens to sites. Lactic acid bacteria produce substances that inhibit the multiplication of pathogens by competing with other microorganisms for nutritional requirements. This might inhibit the multiplication of these agents by excreting substances, mainly H₂O₂, lactic acid, and bacteriocin-like substances (Andreu et al., 1995; Cadieux et al., 2002). Furthermore, the effect on growth is important when studying the relations established between different bacterial species. It is now known that only a very small fraction of bacteria are in the form of free-floating plankton. It is postulated that 99% of all bacterial cells exist as biofilms and only 1% live in a planktonic state (Ramadan et al., 2005; Sancelment et al., 2005). The development of biofilm communities is one of the main strategies for bacteria survival in a certain ecological niche. This state allows bacteria integrated into the biofilm to be protected from environmental fluctuation factors such as humidity, temperature, and pH and, in the case of infections antibacterial preparations applied to the host organism, lengthen the infection and also provide concentrated nutrients and waste disposal. In recent years interest has increased in eating healthy foods, mainly due to possible beneficial effects on the body.

In our laboratory, we studied different strains that may have probiotic potential (Monteagudo-Mera et al., 2011; Monteagudo-Mera et al., 2012). We then decided to focus on studying the competition that can exist between potentially probiotic bacterial strains and those that may be considered potentially pathogenic, both of which are common in the intestinal microbiota of the mammals. The former have been selected because of their antimicrobial activity and to achieve the displacement of potentially pathogenic strains from dairy

products that could attached to the gastric epithelium. The latter were chosen because of their presence and their possible capacity to adhere to the gastrointestinal epithelium. Our aim was to establish the characteristics that inhibit or reduce the growth of potentially pathogenic strains and enhance the proliferation of probiotic to increase the added value of functional foods. The objective we propose in this paper is, therefore, to determine the effect that different fermented broths of LAB strains have on the viability or proliferation and adhesion of potentially pathogenic microorganisms.

MATERIALS AND METHODS

Strains and Culture Conditions

To carry out this work, we studied 5 strains of LAB, obtained from the bacterial culture collection of the Department of Hygiene and Food Technology, at the University of León (these were isolated from human feces or milk products) and from the American Type Culture Collection (ATCC, Manassas, VA), which were selected according to their probiotic and antimicrobial properties (Monteagudo-Mera et al., 2011, 2012). Meanwhile, 7 potentially pathogenic strains were used; these came from the ATCC, Spanish Collection of Type Culture (Paterna, Spain), and the Culture Collection of the Hannah Research Institute (Ayr, Scotland), which were selected based on their potential as food contaminants (Tables 1 and 2).

All LAB strains were grown in trypticase soy broth (TSB) containing 0.6% (wt/vol) yeast extract at 30°C for 24 h, with the exception of *Lactobacillus casei* ATCC 393, which was incubated for 36 h at the same temperature. Potentially pathogenic strains were cultured in the same medium, but at 37°C for 12 h, except *Pseudomonas fluorescens* and *Pseudomonas putida*, which were incubated at 30°C. When it was necessary, the strains were grown on trypticase soy agar (TSA) petri plates or TSA slant tubes under the same growth conditions as TSB with yeast extract medium. The strains were maintained in a mixture of TSB-glycerol (1:1) at –80°C until further use.

Determining the Effect of LAB on Pathogenic Bacterial Growth

To determine the potential inhibitory effect of LAB over pathogenic bacteria, both strain types were grown in TSA slant tubes at the appropriate temperature (see above) and time (Monteagudo-Mera et al., 2011), and when the optimal growth was reached, the cells were resuspended in 4 mL of TSB. The bacterial grown was evaluated by measuring optical density (OD) at 620 nm

Table 1. Lactic acid bacteria (LAB) strains used in this study

Strain	Code	Source	Other features	References
<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	C660 ¹	Raw milk from sheep	Antibacterial activity against certain pathogens; potential probiotic microorganism	Monteagudo-Mera et al., 2012
<i>Lactococcus lactis</i> ssp. <i>lactis</i>	ATCC 11454	NE ²	Used to produce Swiss cheese to prevent the formation of gas by <i>Clostridia</i> ; produces nisin; potential probiotic microorganism	Monteagudo-Mera et al., 2012
<i>Lactobacillus casei</i>	ATCC 393	Milk products (cheese)	Biosynthesis of α -acetolactate; potential probiotic microorganism	Barnes and Keenan, 1972; Monteagudo-Mera et al., 2012
<i>Lactobacillus rhamnosus</i> (<i>Lactobacillus</i> GG)	ATCC 53103	Human feces	Probiotic microorganism; inhibits colon disorders and produces antibacterial agents	Isolauri et al., 1991
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i>	ATCC 27092	Human feces	Potential probiotic microorganism	Monteagudo-Mera et al., 2012

¹Internal codes assigned in the Department of Hygiene and Food Technology Collection, University of Leon.

²NE = nonspecific.

(LAB) and 540 nm (pathogenic strains). In all cases, 300 μ L of the above resuspension, adjusted to OD 1.0, were used to inoculate a flask containing 10 mL of TSB. Liquid cultures of LAB strains were incubated without shaking for 24 h and the pathogen strain cultures were incubated by shaking (250 rpm) for 12 h. To obtain the cell-free fermented broth, the cultures were centrifuged

at 10,000 $\times g$ for 5 min at room temperature, and these were combined with fresh medium to prevent the depletion of nutrients and used to check the growth capacity of the different pathogenic strains. The evaluation of the growth inhibition effect by the LAB fermented broth was carried out by incubation of 300 μ L (OD 1.0) of pathogenic strains in 2 mL of fresh medium (TSB)

Table 2. Potentially pathogenic strains used in this work

Strain	Code	Source	Other features	References
<i>Escherichia coli</i> K92	ATCC 35860	NE ¹	Opportunistic pathogen producer of capsular polymers and potential food contaminant.	Navasa et al., 2009
<i>Bacillus cereus</i> ²	PI-1	Milk	Opportunistic foodborne spore-forming pathogen isolated from a variety of both raw and processed foods.	Anderson Borge et al., 2001
<i>Listeria innocua</i> ³	125	—	A gram-positive, not pathogenic microorganism surrogate for <i>Listeria monocytogenes</i> , a pathogen able to grow in various food products as milk, dairy products, fruits, and vegetable juices.	McLauchlin, 1987; Mosqueda-Melgar et al., 2008; Guo et al., 2013
<i>Pseudomonas fluorescens</i> ²	83	Milk from sheep	Microorganisms that produce thermoresistant proteases that cause spoilage of milk products. Contaminant of fresh, low-acid cheese and other milk derivatives.	De Jonghe et al., 2011; Martin et al., 2011; Baglinière et al., 2012; Chiang et al., 2012
<i>Pseudomonas putida</i> U	CECT 4848	Urban sludges	Microorganisms that produce thermoresistant proteases that cause spoilage of milk products. Can cause an off-odor of taste in dairy products.	Whitfield et al., 2000; De Jonghe et al., 2011
<i>Staphylococcus aureus</i>	CECT 59	NE	Pathogenic microorganism associated with food poisoning including milk, dairy products, and egg derivatives by the presence of an enterotoxin that is highly heat stable. <i>Staphylococcus aureus</i> can be recognized as an opportunistic pathogen related to nosocomial infections on indwelling medical devices due to biofilm formation.	Jay et al., 2005
<i>Staphylococcus epidermidis</i>	CECT 231	NE	Commensal and not invasive microorganism that colonizes the human skin and competes with the invasive.	Otto, 2012; Scopel et al., 2014

¹NE = nonspecific.

²From the Culture Collection Hannah Research Institute (CCM, Ayr, Scotland).

³From the Department of Hygiene and Food Technology Collection, University of León.

with 1 mL of cell-free fermented LAB. Two controls of growth were carried out, one of them using 2 mL of fresh TSB and 1 mL of cell-free broth of the potentially pathogenic strains, and the other was performed using 3 mL of fresh medium (TSB).

The growth inhibition effect also was tested by bioassay using the methodology described by McGroarty et al. (1992) with some modifications. The cell-free fermented broths were obtained by centrifugation after different times of LAB incubation (from 0 to 24 h, at intervals of 2 or 3 h) and then were tested with the pathogenic strains. To prevent the broths from potentially losing a particular property, the assays were synchronized and the bacteria were cultured at different times so that no more than 5 h elapsed from collection to use in the bioassay; during this waiting time, the broths were stored in the refrigerator at 4°C. We proceeded to cover plates; for this purpose 100 µL (OD 1.0) of potentially pathogenic strains was added to 3 mL of TSA tempered in a bath at 42°C and then poured onto TSA plates annealed in an oven at 37°C and allowed to cool for 5 min in the refrigerator at 4°C. Finally, 60 µL of LAB broth or TSB (used as a control) was added to the wells performed on each plate. The plates were put into the refrigerator for 60 min to allow the diffusion of the well content and then were incubated at an adequate temperature. After 12 h, we checked the plates to see whether the LAB-fermented broths had any effect on the growth of the potentially pathogenic strains and, thus, corroborated the findings in the previous section.

The short-chain fatty acids (SCFA) released into the LAB broths were evaluated along with the bacterial growth time according to the procedure described by Zhao et al. (2006). The SCFA (ethanol, acetoin, acetic acid, propionic acid, 2,3-butanediol, butyric acid, and diacetyl) were identified on chromatograms by their specific retention times using a Perkin Elmer Autosystem XL GC equipped with a flame ionization detector (Perkin Elmer, Waltham, MA). A fused silica capillary column (TRB-FFAP, Teknokroma SL, Barcelona, Spain) coated with 1-µm thick film was used. The column was 30 cm long and had an inner diameter of 0.53 mm. Helium was supplied as the carrier gas at a pressure rate of 9.51×10^4 Pa. The initial oven temperature was 130°C and was maintained for 3 min. The temperature was then raised to 170°C at a rate of 40°C/min and held for 2.0 min. The temperature of the flame ionization detector and injection port was 250°C. The flow rates of hydrogen and air (used as makeup gases) were 50 and 500 mL/min, respectively. The injected sample volume for the GC analysis was 2 mL. Volatile fatty acid standards were obtained from Sigma Aldrich (St. Louis, MO). To quantify the peak area in terms of

concentration, the relative response factor was used, following the methodology described by Ranilla et al. (2001).

To check the effect of these SCFA on pathogenic bacteria growth, we added to the growth medium each fatty acid (ethanol, acetoin, acetic acid, propionic acid, 2,3-butanediol, butyric acid, and diacetyl) at (1) the maximal concentration and (2) a concentration 5-fold higher, in both cases using the levels of production registered in LAB as reference values. Moreover, to test if the inhibitory effect could be caused by the liberation of H₂O₂ during the growth of LAB, catalase (1 mg/mL) was added to the broths to prevent this effect in accordance with the methodology described by Yang et al. (2012). In addition, pH measurements were taken for all broths obtained and in all cases and times assayed were near to neutrality.

Determining the Effect of LAB on Pathogenic Bacterial Adhesion

Adhesion assays were performed essentially according to the method of Nagant et al. (2010). Briefly, LAB and potentially pathogenic strains were grown in TSA slant tubes at the appropriate temperature for 24 and 12 h, respectively (Monteagudo-Mera et al., 2011). Flasks of TSB were then inoculated with 50 µL at OD 1.0 and cells were obtained from the exponential phase of growth. The broths were recovered after pelleting of cells by centrifugation. The 96-well microtiter plates (Deltalab, Barcelona, Spain) were filled with 120 µL of cell-free broth of LAB strains and 60 µL of TSB, and others with 120 µL of cell-free broth of potentially pathogenic strains and 60 µL of TSB. All wells were inoculated with 20 µL at OD 2.0 with cells of each potentially pathogenic strain. The plates were incubated for 12 h at 37°C, except the *Pseudomonas* strains which were incubated at 30°C. To establish the positive controls, wells were filled with 180 µL of TSB and 20 µL at OD 2.0 of potentially pathogenic strains; to establish the blanks, some wells were filled with 200 µL of sterile TSB. Controls of these assays were carried out in the presence of cell-free broths boiled for 5 min at 100°C to test the possible inhibition caused by thermosensitive agents. To evaluate the adhesion we used the method of Kubota et al. (2008); after removal of the broths from the wells, these were washed 3 times with 200 µL of PBS. Adherent cells were then stained with 125 µL of crystal violet solution (0.2% wt/vol). After 30 min, the excess of crystal violet was removed and the wells were washed twice with 200 µL of PBS. The dye was dissolved with absolute ethanol and the plate was read on the Anthos 2020 (Biochrom, Cambridge, UK) at 540 nm. The results were expressed as an increasing of the

absorbance measured at 540 nm. Each experimental condition was evaluated in quadruplicate. To check the inhibitory effect to the SCFA on adhesion of potentially pathogenic strains we added these compounds to the adhesion assay at a concentration that was calculated as the average of all the fatty acid LAB production values and also at a concentration 5-fold higher.

Statistical Analysis

The results are presented as means ± standard error of the mean. Significant differences between means were calculated with Student’s *t*-test; *P*-values of 0.05 or less were considered statistically significant.

RESULTS AND DISCUSSION

In this work we studied the competition between 5 potentially probiotic LAB strains previously characterized in our laboratory and 7 potentially pathogenic and food-contaminating strains. *Lactococcus lactis* C660 LAB was selected from a previous work carried out in our laboratory at the University of León (Monteagudo-Mera et al., 2011) based on its antimicrobial activity, whereas the rest were obtained from the ATCC and were selected based on their origin (human or milk), potential as a probiotic, or antimicrobial properties (Monteagudo-Mera et al., 2011). Indeed, one of the bacteria obtained from the ATCC corresponds to the strain *Lactobacillus rhamnosus* GG (ATCC 53103), one of the most researched in the world because of its potential probiotic properties (Gorbach and Goldin, 1989).

Effect of LAB Culture Broths on the Growth Pathogenic Strains

The microbial world has a wide variety of competitors, and a large range of mechanisms may be responsible for the appearance of dominant microbial populations. Because food resources are key in microbial competition, once the collection time of the bacterial

culture broths was established we studied if the LAB fermented broths had any effect over the proliferation of the pathogenic strains. Preliminary results from our laboratory showed that the broth from *Lc. lactis* ATCC 11454 caused a clear inhibition of *B. cereus* growth, but no clear effect was seen for most of the other strains tested (Monteagudo-Mera et al., 2011). We then evaluated the effect of LAB fermented broths on the growth of pathogenic bacteria and by bioassay (see Materials and Methods) and we observed that *E. coli* K92, *Ls. innocua*, *P. fluorescens*, *Staph. aureus*, and *Staph. epidermidis* (but not *P. putida*) were also affected (Table 3 and Figure 1). The inhibitory effect study revealed that, as Figure 1A shows, the antibacterial broth effect of *Lc. lactis* C660 increased at the end of the *E. coli* K92 growth. However, in the case of *P. fluorescens* using *Lc. lactis* broth (Figure 1B), *P. fluorescens* and *Staph. aureus* using *L. casei* 393 broth (Figure 1D and E), and *E. coli* K92 and *Ls. innocua* using *Lb. rhamnosus* broth (Figure 1G and H), the inhibitory effect occurred during the exponential phase of growth. It is worth remarking on the inhibitory effect caused on *Staph. epidermidis* by all the lactic broth from *Lc. lactis* C660 (Figure 1C), *Lc. lactis* 11454 (Table 3), *Lb. casei* 393 (Figure 1F), and *Lb. rhamnosus* (Figure 1I). In all these cases we observed that the growth was totally inhibited from the early stages of growth (Figures 1C, F, and I).

In line with our results, different authors have observed the effect caused on the growth of different pathogenic bacteria of culture medium in which LAB have grown (Drago et al., 1997; Olivares et al., 2006; López-Brea et al., 2008; Jara et al., 2011), although in some cases the effect could be observed only if these broths were previously concentrated (López-Brea et al., 2008) or at specific pH (Yang et al., 2012). Thus, Yang et al. (2012) described that *Lc. lactis* is only able to inhibit *E. coli* growth when the pH of the broth was not adjusted to pH 7 and the effect disappeared after adjusting the pH. Nevertheless, in our work *Lc. lactis* C660 is also able to inhibit *E. coli* K92 growth (Figure 1A) even when the culture broth is a pH near to neutral-

Table 3. Results of the effect of lactic acid bacteria (LAB) culture broths on the growth of potentially pathogenic strains¹

Strain (%)	<i>Escherichia coli</i> K92	<i>Bacillus cereus</i>	<i>Listeria innocua</i>	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas putida</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>
<i>Lactococcus lactis</i> C660	31	— ²	—	19	—	—	76
<i>L. lactis</i> ATCC 11454	—	—	—	—	—	—	90
<i>Lactobacillus casei</i>	—	—	—	25	—	35	76
<i>Lactobacillus paracasei</i>	—	—	—	—	—	—	—
<i>Lactobacillus rhamnosus</i>	12	—	28	—	—	—	76

¹Percent inhibition is the difference between the optical density at the point of maximal inhibition by the LAB broth and control thereof cultured in trypticase soy broth.
²Nondetectable inhibition effect of growth.

Figure 1. Growth curves and bioassays (maximal inhibition time) of *Escherichia coli* K92 (A and G), *Pseudomonas fluorescens* (B and D), *Staphylococcus epidermidis* (C, F, and I), *Staphylococcus aureus* (E), and *Listeria innocua* (H) in the presence of (●) trypticase soy broth, (▼) their own culture broth, and (○) 24-h fermented broth from *Lactococcus lactis* C660 (A–C); *Lactobacillus casei* 393 (D–F), and *Lactobacillus rhamnosus* (G–I). OD_{540nm} = optical density at 540 nm. The error bars indicate the standard deviation. Color version available online.

ity (see Materials and Methods). Furthermore, (Enan et al., 2013) described that broths of different strains of *Lc. lactis* are able to inhibit the growth of *Staph. aureus*. However, in our assay conditions, we observed that neither *Lc. lactis* C660 nor *Lc. lactis* ATCC 11454 produced any effect on this pathogenic strain (Table 3).

With regard to the genus *Lactobacillus*, similar to results published by Yang et al. (2012), *Lb. rhamnosus* broth was able to inhibit the growth of *Ls. innocua* mainly between 5 and 12 h (Figure 1H). Previous results (Silva et al., 1987) also revealed that this broth bacterium was able to inhibit the growth of *E. coli* B-44, but not *E. coli* O157:H7. In our case, *E. coli* K92 growth was inhibited by *Lb. rhamnosus* broth (Figure 1G). These findings corroborate those related by other authors (Johnson-Henry et al., 2008) with respect to the different behaviors that probiotics, such as *Lb. rhamnosus*, have on different strains of the same bacterial species. This variability is also observed in the

Lactobacillus genus; Abriouel et al., (2012) describes how the broth of *Lactobacillus pentosus* is able to inhibit the growth of *B. cereus*, *Staph. aureus*, and *P. fluorescens*, whereas our results reveal that broth from *Lb. casei* is able to cause an inhibitory effect on the growth of *Staph. aureus* and *P. fluorescens* (Figure 1D and E) but not on *B. cereus* (Table 3). All of these cases indicate that 2 bacteria do not have the same behavior, and, even within the same species, different strains may have quite different and sometimes contrasting effects. Therefore, to establish the antimicrobial behavior of LAB it is necessary to study each directly against specific pathogen strains.

Lactic acid bacteria exert their antibacterial activity through the production of different factors, such as hydrogen peroxide, lactic acid, media acidification, SCFA, and specific antibacterial compounds such as antibiotics or bacteriocins, that have been identified in the culture media of several LAB (Drago et al.,

Figure 2. Maximal production of short-chain fatty acids by lactic acid bacteria. Propionic acid and 2,3-butanediol showed the same retention time using this methodology.

1997; Monteagudo-Mera et al., 2011, 2012). Although the precise mechanisms by which the broths from our strains inhibit growth of pathogens are not fully understood at present, the fact that all the broths tested in our experiments exhibited a pH near neutrality (data not shown) indicate that the production of lactic acid is not enough to cause the pathogen viability inhibition observed. Hydrogen peroxide also does not appear to be involved in the bacterial activity, as the addition of catalase (see Materials and Methods) did not modify the bactericidal effect (data not shown). The SCFA have been associated as determinant factors in the inhibition of growth caused by different LAB (Piard and Desmazeaud, 1991; Salminen et al., 2004; Olvera-García et al., 2015). The SCFA produced by the LAB analyzed in the present work were identified and quantified using GC (see Materials and Methods). As shown in Figure 2, except in the case of acetic acid (between 87 mg/L to *Lb. rhamnosus* and 150 mg/L to *Lc. lactis* C660), the production of the other compounds was very low (not exceeding 32 mg/L in the case of ethanol to *Lb. rhamnosus*; 33 mg/L of acetoin to *Lb. casei* 393; 2.4 mg/L of propionic acid/2,3-butanediol to *Lc. lactis* C660; 1.27 mg/L of butyric acid to *Lc. lactis* C660; and 6 mg/L of diacetyl to *Lb. rhamnosus*). These maximal concentrations (Figure 2) and those 5-fold

higher of each fatty acid were tested to evaluate their effect on the growth of the pathogenic bacteria tested. No inhibitory bacterial growth was detected even when a fatty acid concentration 5-fold higher was used (data not shown). These results indicate that the inhibitory growth effect obtained by the addition of different LAB broths (Figure 1) is not a consequence of the presence in the media of these SCFA.

Effect of LAB Culture Broths on the Adhesion of Potentially Pathogenic Strains

The modification in adhesion and biofilm formation caused by binding and displacement of pathogenic microorganisms by LAB led to the conclusion that it is most likely due to steric hindrance or related to mechanisms other than those involving competition for the specific binding sites (Tuomola et al., 1999; Larsen et al., 2007). Furthermore, the participation of any antibacterial lipophilic factor (Liévin-Le Moal et al., 2002), proteinaceous factor (Teneberg et al., 2004), and antimicrobial compounds of uncertain composition that prevents adherence (Coconnier et al., 2000) has been reported.

Our previous results had reported that the LAB are capable of adhering and forming biofilm in both abiotic

and biotic surfaces, such as Caco-2 cells, and, in the case of *Lc. lactis* C660, with a high capacity (Monteagudo-Mera et al., 2012). Therefore, through competition for the same binding sites, these LAB can have the ability to impede the adhesion and biofilm formation of pathogenic bacteria. In the previous section we observed that cell-free fermented broths are able to inhibit the growth of pathogenic bacteria, and it is possible that they may also affect the ability of adhesion and, consequently, the biofilm formation of pathogenic bacteria. As observed in Figure 3, our results reveal that the presence of fermented broth from 4 LAB (*Lc. lactis* C660, *Lc. lactis* 11454, *Lb. paracasei*, and *Lb. rhamnosus*) reduced the *E. coli* K92 adhesion capacity, although to different degrees (from 15% in the case of *Lb. rhamnosus* to 40% for *Lc. lactis* C660). Only the addition of broth from *Lc. casei* showed a significant increase in adhesion formation (up to 185%). Several investigators have reported results on the effect of diverse LAB on the adhesion of different *E. coli* strains; thus, it has been described that some *Lactobacillus* do not modify the adhesion of *E. coli* (Gagnon et al., 2004; Collado et al., 2005), whereas probiotic strains are capable of inhibiting bacterial attachment, as in our case (Collado et al., 2005; Johnson-Henry et al., 2007; Larsen et al., 2007; Candela et al., 2008). Furthermore, our data on increased adhesion caused by *Lb. casei* agree with the results obtained by Parassol et al., (2005) with *E. coli* using the enteropathogenic strain E2348/69. The cellular growth of *B. cereus* was inhibited by *Lc. lactis* ATCC 11454 broth (Monteagudo-Mera et al., 2011); we showed a decrease of adhesion by this bacterium in the presence of both the *Lc. lactis* strains tested and *Lb. paracasei* broths (Figure 3). This antiadhesion effect was similar to that reported by (Ksontini et al., 2013). However, broth from *Lb. rhamnosus* and *Lb. casei* 393 caused a *B. cereus* attachment increase (10 and 70%, respectively). *Listeria innocua* adhesion inhibition was observed in the presence of *Lb. paracasei* broth, but the greatest inhibitory effect on adhesion (up to 80%) of all the cases tested was registered when this bacterium was incubated in the presence of *Lc. lactis* 11454 broth (Figure 3). However, broths from the other LAB slightly increased the binding of this microorganism. With regard to the 2 *Pseudomonas* species selected in our study, we observed that, except for the slight adhesion inhibition showed by *Lb. rhamnosus* broth against *P. putida*, no antiadhesion effect was achieved (Figure 3). In contrast, bacterial attachment increase was registered in the presence *Lc. lactis* C660 and *Lb. casei* 393 broths for the 2 *Pseudomonas* tested, *Lc. lactis* 11454, and *Lb. rhamnosus* broths for *P. fluorescens*. All LAB broths used in our work caused an adhesion stimulation in the 2 *Staphylococcus* species tested (*Staph. aureus*

and *Staph. epidermidis*). As Figure 3 shows, bacterial attachment increased between 20 and 300% for *Staph. aureus* (with *Lc. casei* broth) and for *Staph. epidermidis* (with *Lb. rhamnosus* broth), respectively.

The results summarized in Figure 3 confirm that the effect caused by the different LAB assayed is also broth- and species-specific and reveal that the broth from LAB tested in this work can be used as functional bioactive compounds to regulate the adhesion and biofilm synthesis and ultimately lead to preventing the food and clinical contamination and colonization of *E. coli* K92, *B. cereus*, and *Ls. innocua*. Nevertheless, it should be noted that the broth from LAB stimulate the biofilm formation of the potentially pathogenic *P. putida*, *P. fluorescens*, *Staph. aureus*, and *Staph. epidermidis*. In spite of this, the fact that the LAB broths stimulated *Staph. epidermidis* adhesion can be useful for promoting the colonization of this bacterium when it behaves as a mutualistic microorganism, such as in balancing skin microbiota (Otto, 2009; Christensen and Brüggemann, 2014; Morán et al., 2014). All these results allow us to conclude that the adhesion changes of potentially pathogenic bacteria (positive or negative) registered in the presence of cell-free probiotic fermented broths are caused by a product released into the medium during the growth of LAB, and this product is heat-resistant because no differences have been achieved (data not shown) when the LAB broths were boiled (see Materials and Methods). In this regard, considerable evidence supports the action of H₂O₂ produced by probiotics on the changes in the structure and viability of the biofilms (Saunders et al., 2007). Moreover, the broth acidification or production of organic acids can affect biofilm genesis (Coconnier et al., 2000). However, our LAB-fermented broths did not show significant pH changes after bacterial growth, and the adhesion was not modified when we prevented the H₂O₂ presence by the addition of catalase (data not shown).

Evaluation of the effect of SFCA produced by LAB was carried out only with those pathogenic bacteria that showed a significant adhesion inhibition (*E. coli* K92, *B. cereus*, and *Ls. innocua*) when different LAB broths were added (Figure 3). The results obtained are summarized in Figure 4. Although the tested fatty acids may have individual effects on pathogenic bacteria, our aim was to correlate the bacterial adhesion inhibitory effect shown by the LAB broths (Figure 3) with the production of SFCA by these bacteria (Figure 2). The addition of acetic acid to the adhesion assay (see Materials and Methods) did not modify the bacterial attachment (positive or negative) on the 3 pathogenic strains tested (Figure 4). This result indicates that acetic acid is not responsible of the inhibitory effect on pathogenic bacteria adhesion (see Figure 3). Butyric

Figure 3. Effect of the broths obtained from the lactic acid bacteria (LAB) on the bacterial adhesion developed by the different pathogen strains. Values are expressed as the percentage biofilm of bacteria in presence of LAB broth treatment, compared with the control (black line), which does not contain broth of LAB, expressed as means $\pm$ SEM of the 3 assays in triplicate. An asterisk (*) indicates no significant differences ($P < 0.05$) with respect to control. OD_{540nm} = optical density at 540 nm.

acid and acetoin, which are produced by *Lc. lactis* C660, *Lc. lactis* 11454, and *Lb. rhamnosus*, but not by *Lb. paracasei* (Figure 2), modified the adhesion of *E. coli* K92 and *Ls. innocua* (Figure 4). However, these fatty acids would not be responsible of the inhibition of adhesion of *E. coli* K92 and *Ls. innocua* (see Figure 3), which is also supported by the fact that *Lb. paracasei*, lacking production of these fatty acids, also shows a significant inhibitory adhesion effect. Ethanol and propionic acid or 2,3-butanediol produced by all LAB, except *Lc. casei* 393, did not show inhibitory adhesion effect of *B. cereus* (Figure 4) when were tested at 30 and 2 mg/L, respectively (concentrations produced by the LAB, see Figure 2). Although we observed an inhibitory adhesion effect (15%) when the concentration was 5-fold higher (Figure 4), these results indicate that inhibition of *B. cereus* adhesion observed in Figure 3 caused by the different broths is not a direct consequence of the presence of these fatty acids in the LAB broths. Ethanol, propionic acid, and 2,3-butanediol also showed inhibitory effects of *E. coli* K92 and *Ls. innocua* adhesion (Figure 4); the presence of ethanol caused a decrease of the *E. coli* K92 (25%) and *Ls. innocua* (70%) adhesion. With propionic acid and 2,3-butanediol, the adhesion inhibition reached values of 10% to *E. coli* K92 and between 22 and 40% to *Ls. innocua* at the SFCA concentrations

produced by LAB (Figure 2). Analyzing these results and those obtained in the study of the effect of the addition of different culture broths together (Figure 3), it can be concluded that *E. coli* K92 inhibition was due to the presence of these compounds. Whereas the lack of inhibition of *Ls. innocua* by *Lc. lactis* C660, *Lb. casei* 393, and *Lb. rhamnosus* broths led us to conclude that the adhesion inhibition of this bacteria caused by *Lc. lactis* 11454 and *Lb. paracasei* broths is not caused by these fatty acids.

Other bioactive substances such as bacteriocins, biosurfactants, and other related molecules have been associated with adhesion and biofilm genesis modification (Jeon et al., 2011). Indeed, in some cases they have received considerable attention and the target of their action has begun to be known. This is true of *Lb. rhamnosus*, a prototypical probiotic whose production of bacteriocins produces inhibitory activity on the biosynthesis of exopolysaccharide of the pathogenic bacteria (Jeon et al., 2011). It is also known that the biosynthesis of glucans plays a crucial role in biofilm formation (Koo et al., 2013), and several LAB are able to regulate the expression of genes encoding glucosyltransferases through still unknown mechanisms. Tahmourespour et al. (2011) described how the expression of *gtfB/C* genes related to glucan from *Streptococcus mutants*

Figure 4. Effect of the short-chain fatty acids (SCFA) on the bacterial adhesion (*Escherichia coli* K92, *Bacillus cereus*, *Listeria innocua*). Values are expressed as the percentage of bacteria adhesion in the presence of SCFA, compared with the control (black line), which does not contain any fatty acid. Values are expressed as means $\pm$ SEM of the 3 assays in triplicate. An asterisk (*) indicates no significant differences ($P < 0.05$) with respect to control. OD_{540nm} = optical density at 540 nm.

are modified for the product released by *Lactobacillus fermentum*. In other cases it has been reported that the exopolysaccharides released from LAB are able to decrease pathogenic biofilm formation by affecting the genes related to curli production (*crl*, *csgA*, and *csgB* genes) and chemotaxis (*cheY* gene; Kim et al., 2009). It is clear that LAB produce specific molecules that, when released into the medium, modify the viability, adhesion, and biofilm synthesis capacity of other microorganisms. As previously (Viswanathan et al., 2009) noted, we have a new alternative for the treatment and prevention of the effects caused by potentially pathogenic bacteria capable of developing antibiotic resistance. The use of LAB can be an adequate or complementary antibiotic strategy to prevent the growth and proliferation of undesired bacteria and represent a growing area of current biotechnological research (food and clinical areas). Further analysis of the factors directly involved in these effects would allow the direct use of these molecules in preventing the harmful effects these bacteria cause to health to be studied. More in this regard is currently in progress.

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