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**Topical application of probiotics in skin: adhesion, antimicrobial and
antibiofilm *in vitro* assays**

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Running head: Topical application of probiotics in skin

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Abstract

Aims: When skin dysbiosis occurs as a result of skin disorders, probiotics can act as modulators, restoring microbial balance. Several properties of selected probiotics were evaluated so that their topical application could be considered.

Methods and Results: Adhesion, antimicrobial, quorum sensing and antibiofilm assays were carried out with several probiotic strains and tested against selected skin pathogens. All tested strains displayed significant adhesion to keratin. All lactobacilli with the exception of *L. delbrueckii*, showed antimicrobial activity against skin pathogens, mainly due to organic acid production. Most of them also prevented biofilm formation, but only *P. innocua* was able to break down mature biofilms.

Conclusions: This study demonstrates that although all tested probiotics adhered to human keratin, they showed limited ability to prevent adhesion of some potential skin pathogens. Most of the tested probiotics successfully prevented biofilm formation, suggesting that they may be successfully used in the future as a complement to conventional therapies in the treatment of a range of skin disorders.

Significance and Impact of study: The topical use probiotics may be a natural, targeted treatment approach to several skin disorders and a complement to conventional therapies which present many undesirable side effects.

Keywords: Biofilms – quorum sensing – antimicrobials – Staphylococci – Probiotics

Introduction

The skin is a major organ that has a very typical microbiota, reflective of the harsh environment it offers. As the skin is in direct contact with the surrounding environment, it is inhabited by, and constantly exposed to microorganisms (Rosenthal *et al.* 2011).

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Metagenomic analysis using 16S rRNA sequencing has uncovered a rich and highly diverse bacterial community. The skin microbiome is also notable for having more interpersonal variation than is generally seen at other body sites. Despite these variations by age, site, individual and time, the skin microbiota is dominated by four phyla: Actinobacteria, Proteobacteria, Bacteroidetes and Firmicutes. From these, three genera account for 60% of the bacterial species present. These are *Staphylococcus*, *Corynebacterium* and *Propionibacterium* (Scharschmidt and Fischbach 2013). This resident skin microbiota interacts with other external invaders, such as pathogenic microorganisms, and act as a first line of defence. Resident bacteria produce small molecules that act as antimicrobials, promoting the immune arsenal of toll-like receptors, Langerhans cells and T cells that enhance the barrier function (Chen and Tsao 2013). The skin is a complex and dynamic ecosystem, determined by a number of physical and biochemical factors. Therefore, the bacterial equilibrium can be easily disturbed. Imbalances on skin microbiota can result from a change in the composition of skin bacteria, or an alteration of the host immune response, or both; in either case the result is excessive inflammation (Scharschmidt and Fischbach 2013). This dysbiosis contributes to the development of noninfectious pathologies such as, dermatitis, acne, psoriasis, eczema and skin rashes.

The use of probiotics is emerging as a complementary therapeutical tool in the treatment of several skin disorders, namely atopic dermatitis (AD) (Kalliomaeki *et al.* 2001). AD is a chronic inflammatory skin condition characterized by intense pruritus, exacerbations and eczematous changes and scaly lesions. The current treatment of patients with AD comprises basic skin care, including repair and protection of the skin barrier with proper hydration and topical therapy, which includes the use of moisturizers and anti-inflammatory medications. Besides that, topical or systemic antimicrobial therapy can be incorporated (Chase and

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Armstrong 2012). However, these traditional treatment options are often insufficient or are associated with undesirable side effects thus, is crucial the search for a targeted and non-pharmacological treatment approaches.

Probiotics are viable microorganisms that confer health benefits when administered in adequate amounts (Holzapfel *et al.* 1998). Scientific evidence on the role of probiotics in the stimulation of systemic, cell-mediated immunity with consequent reduction of allergy risk, better resistance to infections and limited development of several pathological conditions does exist (Matilla-Sandholm and Blum 1999). Recently, the use of probiotics to equilibrate the skin microbiota and promote alleviation from the symptoms associated with some skin conditions, due to modulation of the immune system, effect upon commensal or pathogenic microorganisms or by acting on microbial products, has raised interest among the scientific community (Oelschlaeger 2010).

The studies in this field refer mainly to the effects of ingestion of probiotics on AD (Rosenfeldt *et al.* 2003; Weston *et al.* 2005). The results obtained are not, however, consistent to recommend the use of probiotics as part of a standard therapy for the treatment of AD.

In mice, topical *L. Plantarum* inhibited *Pseudomonas aeruginosa* colonization, improved tissue repair, and enhanced phagocytosis in burn wounds in mice (Valdéz *et al.* 2005). Clinical studies on patients with second- and third-degree burns found that the application of *L. Plantarum* was as effective as silver sulfadiazine in decreasing bacterial load, promoting the appearance of granulation tissue, and wound healing (Peral *et al.* 2009). Nevertheless, the effect of the topical application of probiotics in the management of some skin disorders has been barely investigated and might be beneficial, since the adhesion at the site may promote faster action (Weston *et al.* 2005).

In this work, we explored the potential adherence capabilities of probiotic strains to human keratin, and their antimicrobial activity against selected pathogens was assessed. Furthermore, cell-free culture supernatants (CFCS) of probiotic strains were also evaluated in their ability to prevent the formation or destruction of an established biofilm by selected pathogens and the search for potential quorum sensing antagonists.

Materials and Methods

Microorganisms and culture conditions

Probiotics and pathogenic strains used in this study as well as their culture conditions are summarized in Table 1. All strains were preserved at - 80 °C in the appropriate media with 15% (v/v) of glycerol in sterile cryovials until use.

***In vitro* adhesion assays**

Adhesion of probiotic and pathogenic strains to human keratin

Adhesion of probiotic/pathogenic strains to human keratin was carried out following the methodology described by Ouwehand *et al.* (2003) slightly modified with the protocol described by Laparra *et al.* (2011). Keratin from human epidermis (Sigma-Aldrich, Germany,) was used. A fluorescence-based method was used for the detection of adhesive properties of probiotic strains as well as pathogenic strains. So, these suspensions were incubated with 75 $\mu\text{mol L}^{-1}$ carboxyfluorescein diacetate (CFDA; Sigma-Aldrich,) at 37 °C during 30 min. Then, labelled bacteria were added to the wells (100 μL) and incubated for 1 h at 37 °C. Non-bound bacteria were removed by washing twice with PBS. Bound bacteria were released and lysed by incubating 1 h at 37 °C with 200 μL of 1% (w/v) sodium dodecyl sulphate (SDS) in 0.1 mol L^{-1} NaOH. After this time, the mixtures were homogenized by pipetting and the supernatants were transferred to black 96-well plates (Thermo Fisher

Scientific Inc.). The fluorescence was measured in a multiscan fluorometer (Fluorstar optima, BMG Lab Tech, Germany) at λ_{ex} 485 nm and λ_{em} 520 nm. Negative controls of labelled bacteria were used to calculate the percentage of adhesion. This percentage was expressed as the percentage of fluorescence recovered after attachment to keratin relative to the initial fluorescence of the bacterial suspension added to the wells. This experiment was carried out three times in duplicate (n=6).

Inhibition of pathogen adhesion by probiotic strains

Adhesion assays of pathogens in the presence of the best probiotic strains adhering to keratin were carried out to determine the possible exclusion of pathogens. Thus, the protocol was the same described in the previous section, but using non-labelled probiotic strains. After the step referred to the removal of unbound non-labelled probiotic strains, 100 μ L fluorescence labelled pathogenic strains were added to the wells. The percentage of inhibition was assessed by comparing the adhesion of pathogenic strains with and without the presence of the probiotic strains. This experiment was carried out three times in duplicate (n=6).

Antimicrobial assays

Preparation of cell-free culture supernatants (CFCS) from probiotic strains

Each probiotic strain was incubated in 20 mL of adequate medium (Table 1), for 16 h. Then, an aliquot of the cultures from the stationary phase was used for quantification of bacterial growth ($\approx 10^9$ CFU mL⁻¹). The remaining spent medium was centrifuged at 13,000 g for 10 min at 4 °C. The pellet was discarded and the CFCS neutralized to pH 7 by addition of 4 mol L⁻¹ NaOH. Then, the CFCS were sterilized through 0.2 μ m cellulose filters (GVS - Filter Technology, Italy) into sterile tubes. Both neutralized and non-neutralized CFCS were used on the following assays. The CFCS were stored at -20 °C until use.

Antimicrobial activity of cell-free culture supernatants against pathogens by a well diffusion assay

The detection of the potential mechanisms involved in the inhibition of pathogenic bacterial growth by the CFCS were investigated by a slightly modified well diffusion assay described by Tejero-Sariñena *et al.* (2012). Un-neutralized and neutralized CFCS were used. The antimicrobial activity was recorded as growth-free inhibition zones around the wells. Inhibition zones were measured in mm from the edge of the wells. For the controls, 80 µL of Man, Rogosa and Sharpe (MRS) medium or Yeast Tryptic Soy Broth (YTSB) medium, were used. This experiment was carried out three times in duplicate (n=6).

Quorum sensing assays

The detection of potential QS antagonists contained in CFCS of the selected probiotic strains was performed using *C. violaceum* as a biosensor. The amount of violacein depletion, a violet pigment, produced by *C. violaceum* was recorded as a result of potential QS inhibition (McLean *et al.* 2004). In this assay, un-neutralized CFCS of the selected probiotic strains were inoculated into each well on the agar plate previously incorporated with *C. violaceum*. The anti-quorum sensing activity was recorded as growth-free inhibition zones around the wells. Inhibition zones were measured in mm from the edge of the wells to the border of the clear halo. For the controls, 80 µL MRS or YTSB were used. This experiment was carried out three times in triplicate (n=9).

Antibiofilm formation assays

Inhibition of cell attachment

The determination of antibiofilm activity of the selected probiotic strains against pathogens was performed as described by Sandasi *et al.* (2010). One hundred microliters of CFCS of the probiotic to be tested were added to the wells of a microtiter plate. Then, 100 µL

of the pathogenic strains in the stationary phase (10^7 – 10^8 CFU mL⁻¹) were pipetted into each well. The plates were incubated for 8 h at 37 °C to allow cell attachment and biofilm development. Following incubation, the modified crystal violet assay was performed to assess biofilm biomass, and the results expressed as percent inhibition. This experiment was carried out three times in duplicate (n=6).

Destruction of pre-formed biofilms

The determination of destruction of pre-formed pathogen biofilms by the selected probiotics was performed as described above. A biofilm was allowed to form for 4 h, prior to the addition of the CFCS. Biofilm formation was achieved by adding 100 µL of the pathogenic bacteria from 16-h-old cultures (10^7 – 10^8 CFU mL⁻¹) into a 96-well microtiter plate. Following the 4 h incubation period, 100 µL of each supernatant was added to yield a final volume of 200 µL. The plates were further incubated for 24 h, afterwards the modified crystal violet assay was performed and the results expressed as percent destruction. This experiment was carried out three times in duplicate (n=6).

Assessment of biofilm biomass

The crystal violet staining was used as an indicator of the total biomass of biofilm by adapting the procedure described by Djordjevic *et al.* (2002). After the incubation time required testing the inhibition of cell attachment and destruction of pre-formed biofilms, 8 h and 4 h, respectively, the supernatant cultures were removed. Then, the wells from the 96-wells plate were washed twice with sterile water. Afterwards, the attached pathogenic bacteria were fixed with 100 µL of 99% methanol (Merck, USA) per well and after 15 min the plates were emptied and left to dry. Then, the wells were stained with 100 µL of 1% crystal violet (Merck, USA) and incubated at room temperature for 15 min after which the

plates were washed three times with sterile distilled water to remove unabsorbed stain.

Afterwards, the plates were air-dried; the dye bound to the adherent cells was re-solubilized with 100 μ L of 33% (v/v) acetic acid (Merck, USA). Then, 100 μ L of this solution was transferred to a new plate, and the absorbance read at 620 nm using a microplate reader.

Determination of organic acids in the cell-free culture supernatants by liquid chromatography

CFCS from the selected probiotic bacteria were filtered through 0.2 μ m cellulose filters (GVS - Filter Technology, Italy) membranes and organic acids (acetic and lactic) analysed in an Agilent 1200 series HPLC instrument with a refractive index detector (Agilent, Waldbronn, Germany). Separation was carried out in an Aminex HPX-87H column (BioRad, Hercules, CA, USA) operated at 50 °C with 0.003 M H₂SO₄ as mobile phase and a flow rate of 0.6 mL min⁻¹. Solutions of pure standards of the organic acids (acetic, lactic, propionic and butyric acids) of known concentrations were used for quantitative analysis.

Statistical analysis

Statistical analysis was conducted using an analysis of variance (one-way ANOVA); the Tukey's HSD test was conducted for multiple comparisons between treatments and the Dunnett's test for comparisons with the control. Statistical significance was defined at the 5% level ($P < 0.05$). All statistical analysis was carried out using Graph Pad Version 6.0 (Graph Pad Software Inc; USA).

Results

Adhesion to human keratin

Probiotic adhesion to the skin is the first necessary step for colonization to occur, after which the beneficial effects can be exerted, so it was the first point evaluated in this study. Figure 1 depicts the adherence capabilities of the tested probiotic strains to human keratin. As can be seen, all of the tested probiotics were found to exhibit adhesion to keratin (with values ranging from 2.8 to 35%). Probiotic bacteria adhered in different percentages to human keratin and the adhesion ability differed significantly for each probiotic ($P < 0.0001$). *L. plantarum* 226v, *L. brevis* D-24, *L. salivarius* 20555, *L. rhamnosus* 20021, *L. casei* 01, *L. casei* 431 and *B. longum* showed the lowest values of adhesion with percentages below 5%, with no significant statistical differences between them ($P > 0.05$), while *P. innocua*, *L. acidophilus* LA-5, *L. delbrueckii*, *L. acidophilus* LA-10, *L. paracasei* LA-26, *B. lactis* B-94 and Bb12 exhibited the highest adhesion values. These microorganisms were selected as the best adherent probiotics. In particular, the probiotic strains exhibiting the highest adhesion to keratin were both bifidobacteria, *B. lactis* B-94 and *B. animalis* Bb12, with adhesion percentages of 31.1 and 35%, respectively and with significant differences ($P < 0.05$) with the other eleven tested strains. These results allowed the selection of the best adhering strains to keratin and these were used in a second assay to infer on the possible exclusion of pathogens. Table 2 shows the adhesion of the pathogenic bacteria to keratin, as well as the ability of selected probiotic strains to inhibit the adherence of pathogenic bacteria. Adhesion of pathogenic bacteria to human keratin also showed a high variability among microorganisms. For *Propionibacterium acnes*, only the probiotic *Propioniferax innocua* had the ability to significantly reduce its adherence ($P < 0.05$). The adherence capacity of *E. coli* to keratin significantly increased in the presence of *B. animalis* Bb12 ($P < 0.05$). *P. aeruginosa*'s adhesion to keratin also increased significantly in the presence of *B. animalis* Bb12 and *B.*

lactis B-94 ($P<0.05$). Finally, the probiotics *L. acidophilus* LA-10, *P. innocua*, *B. animalis* Bb12 and *B. lactis* B-94 caused a significant increase in the adhesion ($P<0.05$) of *S. aureus* (the most important pathogen associated with active lesions in AD), more noticeable in the case of the strain *B. animalis* Bb12 (Table 2). The rest of the probiotic strains did not significantly affect the pathogens' adhesion.

S. aureus (that showed the highest adhesion values – Table 2) in the presence of *B. animalis* Bb12 and *B. lactis* B-94 (the most adhering probiotics – Figure 1) exhibited a significant increase in keratin adhesion. This presumably occurs because since both have high affinity to keratin and the steric effects of probiotics not being enough to block all the binding sites since these are spatially distant. Therefore, the overall adhesion to keratin was increased.

Determination of antimicrobial activity

Taking into account the results from the previous assays, where we observed that all tested probiotics were able to adhere to human keratin (Figure 1) and in some cases decrease the attachment of pathogens to human keratin (Table 2), the second point to evaluate in this study was whether the best adhering probiotics produced any antimicrobial compounds.

Therefore, the supernatants produced by the probiotics (CFCS) were used to assess the potential antagonistic effect against a range of pathogenic bacteria. The agar well diffusion assay was used to evaluate the effect of potential antimicrobial substances in the CFCS against a set of pathogens (Figure 2). All of the tested lactobacilli, except *L. delbrueckii*, exhibited a significant zone of inhibition against *E. coli*, *P. aeruginosa* and *P. acnes* ($P<0.05$). The inhibition zones ranged from 0.8 to 2.3 mm. The probiotic *L. delbrueckii* was not able to inhibit the growth of *E. coli*; however, this probiotic showed a significant zone of inhibition in the presence of *P. acnes* (2.0 mm \pm 0.0) and *P. aeruginosa* (0.6 mm \pm 0.1). *B. animalis* Bb12 showed inhibition values ranging from 0.5 to 1.3 mm against *E. coli*, *P.*

aeruginosa and *P. acnes*. Regarding *B. lactis* B-94 and *P. innocua*, they were not able to inhibit any of the pathogens tested. *L. acidophilus* LA-10 and *L. paracasei* L-26 were the probiotic strains that showed a broad spectrum of inhibitory activities against all three pathogens.

To have a better understanding of the antimicrobial activity of CFCS from probiotic strains, the assays were repeated using the neutralized CFCS but no antimicrobial activity could be observed (data not shown). It is well known that lactic acid bacteria produce not only organic acids, which cause a decrease of pH in the culture medium, but also other antimicrobial substances known as bacteriocins, which possess antimicrobial activity. The fact that no antimicrobial activity from the neutralized CFCS was observed, suggests that the growth-inhibiting effect of the tested bacteria is mainly due to the production of organic acids. However, many of these are produced only after long incubation times or found at high concentrations of the extracts (Holo *et al.* 2002), which may explain why we did not observe any other antimicrobial activity after neutralizing the effect of the organic acids. Therefore, future studies should focus on the search of other antimicrobial substances produced by probiotic bacteria influencing its antimicrobial ability.

Determination of organic acids

Since the antimicrobial activity observed in the CFCS was mainly due to organic acid production (activity in the non-neutralized extracts), the next step was to identify and quantify the organic acids produced so these could be attributed to the observed antimicrobial activity. The pH of the extracts was registered. Table 3 lists data concerning organic acid production from glucose (the main carbohydrate source in MRS media).

Results indicated that all selected probiotic strains produced acetic and lactic acids as the main metabolites. In particular, concentrations of lactic and acetic acid varied from 18.7 to 160.2 mM and from 5.5 to 90.8 mM, respectively. The greatest producers of lactic acid were *L. acidophilus* LA-10 (160.2 mM) and *L. paracasei* L-26 (157.6 mM). The greatest amounts of acetic acid were produced by the two bifidobacteria: *B. lactis* B-94 and *B. animalis* Bb12 (90.8 and 82.3 mM, respectively). On the other hand, *P. innocua* was the probiotic producing the lowest quantity of these acids (18.7 (for lactic) and 5.5 (for acetic) mM, respectively). In general, lactic acid production was greater than acetic acid production for all tested probiotics. Moreover, the pH of the CFCS of each selected probiotic was examined and it varied between 6.1 and 3.8 (see Table 3). The relationship between pH, production of organic acids (Table 3) and zones of inhibition (Figure 3) of probiotics against each pathogen was studied; as expected, probiotics that lowered the medium pH the most also produced the highest amounts of organic acids and consequently displayed the greatest antimicrobial activities.

The probiotics that produced the higher amounts of organic acids (*L. acidophilus* LA-10 and *L. paracasei* L-26) with simultaneous reduction in the pH of the medium also displayed the largest zones of inhibition against pathogens. This is a consequence of the acidic environment created, being unsuitable for pathogen growth. However, *L. delbrueckii* although producing relatively low amounts of organic acids, revealed high antimicrobial activity against some pathogens, especially *P. acnes*. In fact, *L. delbrueckii* was the probiotic with greater antimicrobial activity against *P. acnes*. Additionally, the medium pH of *L. delbrueckii* was very similar to that of *B. lactis* B-94, even though, the latter did not produce any antimicrobial activity. Therefore, it is evident that the relationship between the medium pH and the antimicrobial activity of probiotics is not always direct.

Antibiofilm properties

The capacity to form biofilms represents one of the major virulence factors of the microorganisms since they allow microorganisms to trap nutrients and withstand hostile environmental conditions (Phillips and Schultz 2012). Therefore, the potential ability of probiotics to inhibit biofilm formation or destroy pre-formed biofilms represent a tool for reducing microbial colonization on epithelial mucosa which subsequently leads to infections (Bavington and Page 2005). Hence, this study assessed the capacity of the selected probiotic bacteria to inhibit the formation or destruction of pre-formed biofilms by four pathogens. The results disclosed (Figure 3) that most of the selected probiotic strains decreased biofilm formation by the pathogens. Particularly, five of the seven probiotics had the capability of reducing cell attachment (and therefore, biofilm formation) of *E. coli* (Figure 3C). The inhibition values varied from 22.9 to 13.1%. *L. delbrueckii* and *B. animalis* Bb-12 displayed the highest inhibition values.

The cell attachment of *S. aureus* (Figure 3D) was decreased in the presence of five probiotics, with no significant differences between them. The range of inhibition values was from 37.7 to 30.4%. Regarding the biofilm formation by *P. aeruginosa* (Figure 3B), it was reduced in the presence of six probiotics. *L. delbrueckii* exhibited the highest percentage of cell attachment inhibition (20.3%) and *L. acidophilus* LA-10 showed the lowest percentage (8.1%).

For *P. acnes* (Figure 3A), a reduction of cell attachment/biofilm formation from 20.5 to 9.8% was observed. In this case, all probiotics, showed ability to decrease *P. acnes* biofilm formation.

Once proved that the selected probiotics were able to reduce the cell attachment of pathogen (and therefore, biofilm formation), we evaluated their capacity to disrupt a pre-formed biofilm. The establishment of biofilms leading to the development of skin infection is

a main problem in the treatment of skin disorders. Despite most probiotics showing success on reducing cell attachment of pathogens, six of the seven tested probiotics were unable to break down the biofilms once formed. Exceptionally, *P. innocua* was able to destroy pre-formed biofilms of *E. coli*, *P. aeruginosa* and *S. aureus*. This was significantly evident for *S. aureus* (45.0%) (Figure 4). These results show that, inhibiting biofilm growth once established, is more difficult to achieve than disrupting cell attachment.

Quorum Sensing (QS) inhibitors

In order to elucidate the mechanism whereby the tested probiotics reduce biofilm formation (Figure 3 and 4) we carried out an assay for QS inhibition. In this assay, the anti-QS activity of the seven selected probiotics was screened using *C. violaceum* as the biosensor. This microorganism regulates the pigment production by N-hexanoyl-homoserine lactone (C6-HSL). The presence of QS inhibition is indicated by the lack of pigmentation of the biosensor (McLean *et al.* 2004). The results revealed that all probiotics, with the exception of *P. innocua* and *B. lactis* B-94, produced a reduction of the violet pigment production (Figure 5). Among the probiotics showing inhibition zones, the values ranged from 0.47 to 2.83 mm. The higher inhibition zone was observed for *L. acidophilus* LA-10 while the lowest was detected in *L. delbrueckii*. Thus, the described results suggest that probiotics may be able to inhibit the production of AHL at the level of gene expression of its synthase gene.

Discussion

The results obtained on the adherence capability of probiotics were consistent with a study where probiotic adhesion to keratin was found to be between 16 and 20% (Ouwehand *et al.* 2003). In previous studies, the strain Bb-12 exhibited a similar level of adhesion to human

intestinal mucous: 30.8% (Juntunen *et al.* 2001) and 23.2-29.8% (Kirjavainen *et al.* 1998).

Different mechanisms of adhesion to keratin by probiotics have been proposed, such as non-specific interactions (Ouwehand *et al.* 2002) or hydrophobic interactions (Ouwehand *et al.* 2003). However, the findings are not consistent and further studies are needed to identify other interactions that may be involved in the binding of these probiotic strains to keratin. *S. aureus* was by far the bacteria with the highest adhesion probably due to its known ability to adhere to human extracellular matrix and serum components due to the presence of adhesins (Clarke and Foster 2006). Despite the high values of adhesion to keratin exhibited by the bifidobacteria, they were not able to effectively prevent the adhesion of the pathogenic bacteria tested; in fact, they caused an increase in pathogen adhesion. This has been reported previously (Ouwehand *et al.* 2003, Collado *et al.* 2007) and its biological significance remains unknown; however the presence of specific adhesins or other receptors or coaggregation of both strains have been suggested as possible causes (Collado *et al.* 2007). The pathogenic bacteria are probably using different binding sites on keratin, which are not blocked by steric hindrance of the selected probiotics. This may suggest that the binding sites used are spatially separated from each other (Ouwehand *et al.* 2003). In fact, the failure of probiotics with high affinity to keratin to block the binding of pathogens has been already observed (Ouwehand *et al.* 2001). It is also plausible to speculate that by adhering to the probiotic bacteria, the pathogenicity associated with some of the pathogenic bacteria (like *P. acnes*) might be somehow compromised since direct adhesion to the skin would be diminished. However, this remains to be proved.

The antimicrobial activity results are also in line with the data presented in the literature; Shokryazdan *et al.* (2014) reported that several lactobacilli exhibited inhibition zones against *P. acnes* (ranging from 3.3 to 16.5 mm) and *E. coli* (11.3 to 14.7 mm), while

Ali *et al.* (2013) observed that the inhibition zones in *E. coli* against *Lactobacillus* spp. and *Bifidobacterium* spp. ranged from 0.8 to 6.9 mm.

Regarding the pathogenic bacterium *S. aureus*, no inhibition zones were found with any of the CFCS from probiotic strains. Nevertheless, in this case, around the wells we could observe zones of a yellow coloration, presumably due to some kind of antagonistic effect. Although the cause of this effect could not be elucidated, these findings are in accordance with previous reports that investigated the antimicrobial profile of CFCS from several probiotics against pathogens. Tejero-Sariñena and co-workers (2012) demonstrated that probiotics strains such as, *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp. and *Bacillus* spp. showed a broad spectrum of inhibitory activities towards all tested pathogens (*E. coli*, *Salmonella typhimurium*, *Enterococcus faecalis*, *Clostridium difficile*), except for *S. aureus*. Furthermore, the authors reported a production of 44 mM to 180 mM for lactic acid and 45 mM to 99 mM for acetic acid from lactobacilli and bifidobacteria (Tejero-Sariñena *et al.* 2012). Lactic acid is reported to be the main end product and that it is produced in greater amounts than acetic acid during 24 h of fermentation. Other researchers (Toba *et al.* 1991) report the production of antimicrobial peptides as responsible for the antimicrobial activity by *L. delbrueckii* subsp. *lactis* against other subspecies of *L. delbrueckii*. Thus, it is plausible that other mechanisms, such as the production of bacteriocins or QS inhibitors (Sifri 2008) might also be involved in antimicrobial activity of probiotic bacteria.

The antibiofilm activity has been vastly reported in the literature. Its activity against *E. coli* has been investigated mainly in medicinal plant extracts. Wojnicz *et al.* (2012) evaluated the impact on biofilm formation of six leaf extracts against uropathogenic *E. coli*; they reported that most of the plant extracts had the capacity to inhibit the biofilm formed by *E. coli*. Furthermore, the authors established that the extracts promoted the dysfunction of P fimbriae (or pyelonephritis-associated pili [PAP]), thereby preventing its attachment to the

host tissue. In fact, Kim and co-workers (2009) demonstrated that exopolysaccharides released from *L. acidophilus* A4 significantly repressed biofilm formation of *E. coli* by affecting genes related to curli production (*crl*, *csgA*, and *csgB*). Therefore, the activity of these probiotics in reducing cell attachment of *E. coli* may be explained by the presence of exopolysaccharides or other bacterial surface structures. These could be responsible for the reduction of pili that is correlated with the loss of the ability of *E. coli* strains to form biofilms (Aberg and Almqvist 2007). Recent findings indicate that chronic wound pathology may be caused by alterations in skin microbiota; in particular, *S. aureus* and *P. aeruginosa* are considered the two bacterial species mainly involved in biofilm-based wound infections (Wong *et al.* 2013), with the latter being difficult to eradicate mainly due to acquired antibiotic resistance (Hancock 1998). Therefore, probiotics may contribute to inhibit biofilm formation by *P. aeruginosa*. In accordance, Valdéz *et al.* (2005) investigated the ability of *L. plantarum* to inhibit the biofilm-capacity of *P. aeruginosa* and demonstrated that *L. plantarum* and/or its metabolites were able to inhibit *P. aeruginosa* biofilm formation. The inhibition of *S. aureus* biofilms by probiotics has also been previously investigated by Walencka *et al.* (2008) where they evaluated the effects of surfactants obtained from three *L. acidophilus* strains on *S. aureus*. The probiotic-derived surfactants were able to reduce the biofilm formation of *S. aureus*, most likely by influencing the staphylococcal cell surface hydrophobicity. Furthermore, *L. plantarum* has been demonstrated to be topically effective in preventing skin wound infections in mice, not only against *P. aeruginosa* (Valdéz *et al.* 2005) but also against *S. aureus* (Sikorska and Smoragiewicz 2013). Thus, these results may suggest that probiotics could be a promising tool to prevent and treat non-healing wounds.

P. acnes is predominant in sebaceous follicles and it is suspected to be of major importance in the pathogenesis of acne and also in a number of other opportunistic infections, such as postoperative mediastinitis, and infections of joint prostheses and of cerebrospinal

fluid shunts (Christensen and Brüggemann 2014). A previous report demonstrated that isolates of *P. acnes* from skin have the ability to form biofilm. Moreover, they reported that the ability to produce biofilm is a determinant virulence factor for *P. acnes*, and that biofilm formation is important for infection pathogenesis (Holmberg *et al.* 2009). Therefore, the probiotics' capacity to reduce the cell attachment of *P. acnes* may provide a useful tool to mitigate the harmful effects of *P. acnes* on skin or other infections. Accordingly, Sandasi *et al.* (2010) reported the success of plant extracts in inhibiting cell attachment of *Listeria monocytogenes*, however, inhibiting the growth of an already established biofilm was shown to be more difficult. The incapacity of most probiotics tested to reduce or destroy biofilm growth is in accordance with previous findings that postulate that cells in a biofilm structure are more resistant to antimicrobials when compared with free-floating cells (Krysinski *et al.* 1992). The properties that may explain this phenomena include (1) the three-dimensional architecture, i.e., the presence of several layers of bacteria promotes the development of nutrient and oxygen gradients (Walters *et al.* 2003); (2) the slower growth rate in biofilms compared to planktonic cells as a result of reduced nutrient and oxygen supply has been reported as another factor (Mah and O'Toole 2001); and (3) the matrix components, such as polysaccharides, proteins, nucleic acids and lipids, that can bind and/or neutralize antimicrobial agents (Mulcahy *et al.* 2008).

Biofilm formation by bacteria is, in fact, a main problem in medical clinical settings. Bacterial biofilm activity is regulated by QS. The QS is a system of cell-cell-communication where bacteria communicate via small molecules called autoinducers (AIs) to coordinate collective behaviours (Chen *et al.* 2011). It is therefore, a regulatory mechanism used by Gram-negative and Gram-positive bacteria in response to factors as varied as changing microbial population density and the expression of specific genes. QS inhibition has been suggested as a potential novel strategy for antimicrobial therapy to control infections,

particularly biofilm-based infections (Deep *et al.* 2011). A previous report evaluated the effect of the natural antimicrobial carvacrol on QS of *C. violaceum*. Carvacrol significantly reduced the production of violacein pigment in a concentration-dependent manner (Burt *et al.* 2014). Furthermore, the researchers suggested that the antibiofilm activity of carvacrol appeared to be related to interference with bacterial QS, since violacein production in *C. violaceum* was affected. Moreover, Valdéz *et al.* (2005) indicated that *L. plantarum*'s capacity to reduce *P. aeruginosa* biofilm was achieved by affecting the production of the QS signal molecules. These signal molecules in Gram-negative bacteria are called N-acylhomoserine lactone (AHL) (Sifri 2008). Despite a number of Gram-positive bacteria exhibiting QS systems, the nature of the signal molecules used are different from those of Gram-negative bacteria. In fact, it is not yet known any Gram-positive bacteria that produce AHL. Gram-positive QS systems typically use small post-translationally processed peptide signal molecules, called autoinducing peptides (AIPs) (Deep *et al.* 2011). Therefore, the reduction and/or destruction of *S. aureus* and *P. acnes* biofilms cannot be attributed to the presence of QS antagonists, since they are Gram-positive bacteria and *C. violaceum* is a biosensor capable of detecting AHLs, only produced by Gram-negative bacteria.

Nevertheless, the inhibition of biofilm attachment and/or growth of *P. aeruginosa* and *E. coli* could be explained by the presence of QS antagonists secreted by probiotics that interfere with AHLs production. Therefore, targeting QS systems represent a novel approach for treating bacterial infections. The discovery that a wide spectrum of microorganisms uses QS to control virulence factors' production makes it an attractive target for the discovery of new anti-virulence therapeutic strategies.

In conclusion, although all probiotic strains were able to adhere to human keratin, their ability to prevent the adhesion of some potential skin pathogenic bacteria was limited in some cases; antimicrobial activity of the CFCS of selected probiotics strains was observed towards *E. coli*, *P. acnes* and *P. aeruginosa*, however, none was able to inhibit the growth of *S. aureus*.

Most of the tested probiotics were able to prevent biofilm formation, which may be related to QS inhibition. However, with the exception of *P. innocua*, probiotics were unable to break down the biofilms once formed. In the future, more in-depth studies regarding production of other antimicrobial substances such as bacteriocins should be undertaken as well as studies elucidating the mechanisms behind the destruction of pre-formed biofilms. With this, a breakthrough in the treatment of skin-related infections could take place allying the use of probiotics as a natural alternative to a targeted treatment approach to alleviate symptoms associated with several skin disorders.

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Conflict of Interest

No conflict of interest declared.

References

- Aberg, V. and Almqvist, F. (2007) Pilicides-small molecules targeting bacterial virulence. *Org Biomol Chem* **5**, 1827–1834.
- Ali, F.S., Saad, O.A.O. and Salwa, A.H. (2013) Antimicrobial activity of probiotic bacteria. *Egypt Acad J Biolog Sci* **5**, 21-34.
- Bavington, C. and Page, C. (2005) Stopping Bacterial Adhesion: A Novel Approach to Treating Infections. *Respiration* **72**, 335–344.
- Burt, S.A., Ojo-Fakunle, V.T.A., Woertman, J. and Veldhuizen, E.J.A. (2014) The Natural Antimicrobial Carvacrol Inhibits Quorum Sensing in *Chromobacterium violaceum* and Reduces Bacterial Biofilm Formation at Sub-Lethal Concentrations. *PLoS ONE* **9**, 93414.
- Chase, E.P. and Armstrong, A. (2012) Advances in management of atopic dermatitis: new therapies and novel uses of existing treatments. *Semin Cutan Med Surg* **31**, 17-24.
- Chen, G., Swem, L.R., Swem, D.L., Stauff, D.L., O’Loughlin, C.T., Jeffrey, P.D., Bassler, B.L. and Hughson, F.M. (2011) A strategy for antagonizing quorum sensing. *Mol Cell* **42**, 199–209.
- Chen, Y.E. and Tsao, H. (2013) The skin microbiome: Current perspectives and future challenges. *J Am Acad Dermatol* **69**, 143-155.
- Christensen, G.J. and Brüggemann, H. (2014) Bacterial skin commensals and their role as host guardians. *Benef Microbes* **5**, 201-215.
- Clarke, S.R. and Foster, S.J. (2006) Surface Adhesins of *Staphylococcus aureus*. *Adv Microb Physiol* **51**, 187-224.
- Collado, M.C., Grzéskiwiak, L. And Salminen, S. (2007) Probiotic strains and their combination inhibit *in vitro* adhesion of pathogens to pig intestinal mucosa. *Curr Microbiol* **55**, 260-265.

Deep, A., Chaudhary, U. and Gupta, V. (2011) *Quorum sensing* and bacterial pathogenicity: From molecules to disease. *J Lab Physicians* **3**, 4-11.

Djordjevic, D., Wiedmann, M. and Mclandsborough, L.A. (2002) Microtitre plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol* **68**, 2950-2958.

Hancock, R.E. (1998) Resistance mechanisms in *Pseudomonas aeruginosa* and other non-fermentative gram-negative bacteria. *Clin Infect Dis* **27**, 93-99.

Holmberg, A., Lood, R., Mörgelin, M., Söderquist, B., Holst, E., Collin, M., Christensson, B. and Rasmussen, M. (2009) Biofilm formation by *Propionibacterium acnes* is a characteristic of invasive isolates. *Clin Microbiol Infect* **15**, 787-795.

Holo, H., Faye, T., Brede, D.A., Nilsen, T., Odegard, I., Langsrud, T., Brendehaug, J. and Nes, I.F. (2002) Bacteriocins of propionic acid bacteria. *Le Lait* **82**, 59-68.

Holzappel, W.H., Haberer, P., Snel, J., Schillinger, U. and Huis in't Veld, J.H. (1998) Overview of gut flora and probiotics. *Int J Food Microbiol* **41**, 85-101.

Juntunen, M., Kirjavainen, P.V., Ouwehand, C., Salminen, S.J. and Isolauri, E. (2001) Adherence of Probiotic Bacteria to Human Intestinal Mucus in Healthy Infants and during Rotavirus Infection. *Clin Diagn Lab Immunol* **8**, 293-296.

Kalliomäki, M., Salminen, S., Arvilomi, H., Koskinen, P. and Isolauri, E. (2001) Probiotics in primary prevention of atopic disease. A randomised placebo-controlled trial. *Lancet* **357**, 1076-1079.

Kim, H.-S., Park, S.-J. and Lee, K.-H. (2009) Role of NtrC-regulated exopolysaccharides in the biofilm formation and pathogenic interaction of *Vibrio vulnificus*. *Mol Microbiol* **74**, 436-453.

Kirjavainen, P.V., Ouwehand, A.C., Isolauri, E. and Salminen, S.J. (1998) The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol Lett* **167**, 185-189.

Krysinski, E.P., Brown, L.J. and Marchisello, T.J. (1992) Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *J Food Prot* **55**, 246-251.

Laparra, J.M., Corzo-Martinez, M., Villamiel, M., Moreno, F.J. and Sanz, Y. (2011)

Maillard-type glycoconjugates from dairy proteins inhibit adhesion of *Escherichia coli* to mucin. *Food Chem* **129**, 1435-1443.

Mah, T.F. and O'Toole, G.A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34-39.

Matilla-Sandholm, T. and Blum, S. (1999) Probiotics: towards demonstrating efficacy. *Trends Food Sci Technol* **10**, 393-399.

McLean, R.J.C., Pierson III, L.S. and Fuqua, C.A. (2004) simple screening protocol for the identification of quorum signal antagonists. *J Microbiol Methods* **58**, 351-360.

Mulcahy, H., Charron-Mazenod, L. and Lewenza, S. (2008) Extracellular DNA Chelates Cations and Induces Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms. *PLoS Pathog* **4**, e1000213.

Oelschlaeger, T.A. (2010) Mechanisms of probiotic actions - A review. *Int J Med Microbiol* **300**, 57-62.

Ouwehand, A.C., Tuomola, E.M., Tölkö, S. and Salminen, S. (2001) Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *Int J Food Microbiol* **64**, 119-126.

Ouwehand, A.C., Salminen, S. and Isolauri, E. (2002) Probiotics: an overview of beneficial effects. *Antonie van Leeuwenhoek* **82**, 279-289.

Ouwehand, A.C., Batsman, A. and Salminen, S. (2003) Probiotics for the skin: a new area of potential application? *Lett Appl Microbiol* **36**, 327-331.

Peral, M. C., Martinez, M.A., and Valdéz, J.C. (2009) Bacteriotherapy with *Lactobacillus plantarum* in burns. *Int Wound J* **6**, 73-81.

Phillips, P.L. and Schultz, G.S. (2012) Molecular mechanisms of biofilm infection: Biofilm virulence factors. *Adv Wound Care* **1**, 109-113.

Rosenfeldt, V., Benfeldt, E., Nielsen, S.D., Michaelsen, K.F., Jeppesen, D.L., Valerius, N.H. and Paerregaard, A. (2003) Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J Allergy Clin Immunol* **111**, 389-395.

Rosenthal, M., Goldberg, D., Aiello, A., Larson, E. and Foxman, B. (2011) Skin microbiota: microbial community structure and its potential association with health and disease. *Infect Genet Evol* **11**, 839-848.

Sandasi, M., Leonard, C.M. and Viljoen, A.M. (2010) The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Lett Appl Microbiol* **50**, 30-35.

Scharschmidt, T.C. and Fischbach, M.A. (2013) What lives in our skin: ecology, genomics and therapeutic opportunities of the skin microbiome. *Drug Discov Today Dis Mech* **10**, e83-e89.

Shokryazdan, P., Sieo, C.C., Kalavathy, R., Liang, J.B., Alitheen, N.B., Jahromi, M.F. and Ho, Y.W. (2014) Probiotic potential of *Lactobacillus* strains with antimicrobial activity against some human pathogenic strains. *Biomed Res Int* **2014**, 1-16.

Sifri, C.D. (2008) Quorum Sensing: Bacteria Talk Sense. *Clin Infect Dis* **47**, 1070-1076.

Sikorska, H. and Smoragiewicz, W. (2013) Role of probiotics in the prevention and treatment of meticillin-resistant *Staphylococcus aureus* infections. *Int J Antimicrob Agents* **42**, 475-481.

Tejero-Sariñena, S., Barlow, J., Costabile, A., Gibson, G.R. and Rowland, I. (2012) *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe* **18**, 530-538.

Toba, T., Yoshioka, E. and Ithoh, T. (1991) Lacticin, a bacteriocin produced by *Lactobacillus delbrueckii* subsp. *lactis*. *Lett Appl Microbiol* **12**, 43-45.

Valdéz, J.C., Peral, M.C., Rachid, M., Santana, M. and Perdigón, G. (2005) Interference of *Lactobacillus plantarum* with *Pseudomonas aeruginosa* *in vitro* and in infected burns: the potential use of probiotics in wound treatment. *Clin Microbiol Infect* **11**, 472-479.

Walencka, E., Rózalska, S., Sadowska, B. and Rózalska, B. (2008) The influence of *Lactobacillus acidophilus*-derived surfactants on staphylococcal adhesion and biofilm formation. *Folia Microbiol Praha* **53**, 61-66.

Walters, M.C., Roe, F., Bugnicourt, A., Franklin, M.J. and Stewart, P.S. (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* **47**, 317-323.

Weston, S., Halbert, A. and Richmond, P. (2005) Effect of probiotics on atopic dermatitis: a randomized controlled trial. *Arch Dis Child* **90**, 892-897.

Wojnicz, D., Kucharska, A.Z., Sokót-Lętowska, A., Kicia, M. and Tichaczek-Goska, D. (2012) Medicinal plants extracts affect virulence factors expression and biofilm formation by the uropathogenic *Escherichia coli*. *Urol Res* **40**, 683-697.

Wong, V.W., Martindale, R.G., Longaker, M.T. and Gurtner, G.C. (2013) From germ theory to germ therapy: skin microbiota, chronic wounds, and probiotics. *Plast Reconstr Surg* **132**, 854–886.

Figure Legends:

Figure 1. Probiotic adhesion (%) to human keratin. Error bars indicate standard deviations (n= 6). Multiple comparisons were done using the Tukey's HSD test (P<0.05). Means sharing the same letter are not significantly different from each other.

Figure 2. Inhibitory growth zones of CFCS against a panel of Gram-positive and Gram-negative pathogenic bacteria, namely (A) *P. acnes*, (B) *P. aeruginosa* and (C) *E. coli*. Error bars indicate standard deviations (n=6). All experiments were replicated three times (with duplicates). *,**,*** represent significant differences (P<0.05, P<0.01 and P<0.001, respectively) with respect to the control (pathogen only).

Figure 3. Effect of probiotics on cell attachment and biofilm formation/growth by (A) *P. acnes*, (B) *P. aeruginosa*, (C) *E. coli* and (D) *S. aureus*, expressed as percentage of cell attachment inhibition. Error bars indicate standard deviations (n=6). Means sharing the same letter are not significantly different from each other (P<0.05).

Figure 4. Effect of *P. innocua* upon the growth and development of pre-formed biofilms by *E. coli*, *P. aeruginosa* and *S. aureus*, expressed as percentage of destruction. Error bars indicate standard deviations (n=6). Means sharing the same letter are not significantly different from each other (P<0.05).

Figure 5. Inhibition zones by *C. violaceum* in the presence of the CFCS of selected probiotic strains. Error bars indicate standard deviations (n=9). *,**,*** represent significant differences (P<0.05, P<0.01 and P<0.001, respectively) with respect to the control.

Bacterial species	Strain	Incubation conditions
PROBIOTICS		
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	DSMZ 20081	MRS, Anaerobic, 37 °C
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> Bb12	CHR Hansen Bb 12	MRS, Anaerobic, 37 °C
<i>Lactobacillus acidophilus</i> LA-5	CHR Hansen LA-5	MRS, Anaerobic, 37 °C
<i>Bifidobacterium lactis</i> B-94	DELVO PRO LAFTI B-94	MRS, Anaerobic, 37 °C
<i>Bifidobacterium longum</i> subsp. <i>infantis</i>	DSMZ 20088	MRS, Anaerobic, 37 °C
		MRS, Facultative
<i>Lactobacillus acidophilus</i> LA-10	DELVO PRO LAFTI LA-10	Aerobe, 37 °C
<i>Lactobacillus acidophilus</i> L-26	DELVO PRO LAFTI L-26	MRS, Aerobic, 37 °C
<i>Propioniferax innocua</i>	DSMZ 8251	YTSB, Aerobic, 37 °C
<i>Lactobacillus plantarum</i>	DELVO PRO LAFTI 226v	MRS, Aerobic, 37 °C
<i>Lactobacillus brevis</i>	DELVO PRO LAFTI D-24	MRS, Aerobic, 37 °C
<i>Lactobacillus salivarius</i>	DSMZ 20555	MRS, Aerobic, 37 °C
<i>Lactobacillus casei</i> subsp. <i>rhamnosus</i>	DSMZ 20021	MRS, Aerobic, 37 °C
<i>Lactobacillus casei</i> 01	CHR Hansen 01	MRS, Aerobic, 37 °C
<i>Lactobacillus casei</i> 431	DELVO PRO LAFTI 431	MRS, Aerobic, 37 °C
PATHOGENS		
<i>Propionibacterium acnes</i>	DSMZ 1897	RCM, Anaerobic, 37 °C
<i>Staphylococcus aureus</i> (MRSA)	Internal Collection CINATE	MH, Facultative anaerobe, 37 °C
<i>Escherichia coli</i>	Internal Collection CINATE	MH, Aerobic, 37 °C
<i>Pseudomonas aeruginosa</i>	Internal Collection CINATE	MH, Aerobic, 37 °C
OTHERS		
<i>Chromobacterium violaceum</i>	CCUG 37577	PCA, Aerobic, 37 °C

Table 1: Bacterial strains used and their culture conditions.

Culture media: **MRS:** de Man, Rogosa and Sharp (Biokar Diagnostics, Beauvais, France); **YTSB:** Yeast Tryptic Soy Broth (Biokar Diagnostics); **NB:** Nutrient Broth (Biokar Diagnostics); **RCM:** Reinforced Clostridial Medium (Biokar Diagnostics); **MH:** Muller-Hinton (Biokar Diagnostics);

Culture Collections: DSMZ: German Collection of Microorganisms and Cell Cultures; CHR Hansen: DELVOPRO LAFTI; CCUG: Culture Collection, University of Gothenburg; CINATE: Center for Innovation and Technological Support, Portuguese Catholic University, Porto, Portugal.

Table 2: Adhesion of *E. coli*, *S. aureus* (MRSA), *P. acnes* and *P. aeruginosa* to human keratin without and in the presence of selected probiotics: *L. delbrueckii*, *B. animalis* subsp. *lactis* Bb12, *L. acidophilus* LA-5, *L. acidophilus* LA-10, *L. paracasei* LA-26, *P. innocua* and *B. lactis* B-94. Values are expressed as the mean of duplicates in independent triplicate assays (n=6). Comparisons with the control were done using the Dunnett's multiple comparison test (P<0.05). * represents a significant difference with P<0.05; ** represents a significant difference with P<0.01; *** represents a significant difference with P<0.001 and **** represents a significant difference with P<0.0001.

	Pathogen adhesion without the presence of probiotic strains (%)			
	<i>E. coli</i>	<i>S. aureus</i>	<i>P. acnes</i>	<i>P. aeruginosa</i>
	5.4 ± 1.98	19.33 ± 2.97	6.27 ± 2.02	1.23 ± 0.26
	Pathogen adhesion in the presence of each probiotic strain (%)			
	<i>E. coli</i>	<i>S. aureus</i>	<i>P. acnes</i>	<i>P. aeruginosa</i>
<i>L. delbrueckii</i>	2.91 ± 0.50	27.41 ± 0.72	4.18 ± 1.18	1.30 ± 0.14
<i>B. animalis</i> Bb12	16.55 ± 2.77***	55.62 ± 1.03****	2.50 ± 0.44	6.33 ± 0.52****
<i>L. acidophilus</i> LA-5	3.12 ± 0.36	26.38 ± 4.05	2.53 ± 0.33	1.70 ± 0.30
<i>L. acidophilus</i> LA-10	4.02 ± 0.40	28.89 ± 3.05*	2.75 ± 0.9	1.62 ± 0.034
<i>L. paracasei</i> L-26	2.89 ± 0.21	25.48 ± 3.95	3.73 ± 0.21	1.23 ± 0.21
<i>P. innocua</i>	9.06 ± 2.35	28.94 ± 3.80*	1.05 ± 0.08*	2.60 ± 0.39
<i>B. lactis</i> B-94	9.33 ± 0.35	31.73 ± 1.31**	2.63 ± 0.00	4.46 ± 1.18***

Table 3: The pH value and the organic acid concentrations (mM) in the medium produced by the selected probiotic strains.

	pH	Organic acid concentrations (mM)		
		Lactic acid	Acetic acid	Total
<i>L. delbrueckii</i>	4.60	70.94	64.45	135.38
<i>B. animalis</i> Bb12	4.50	88.03	82.26	170.30
<i>L. acidophilus</i> LA-5	4.15	100.69	59.45	160.14
<i>L. acidophilus</i> LA-10	3.80	160.19	62.61	222.81
<i>L. paracasei</i> L-26	3.98	157.64	57.45	215.09
<i>P. innocua</i>	6.13	18.65	5.50	24.15
<i>B. lactis</i> B-94	4.64	22.98	90.76	113.74

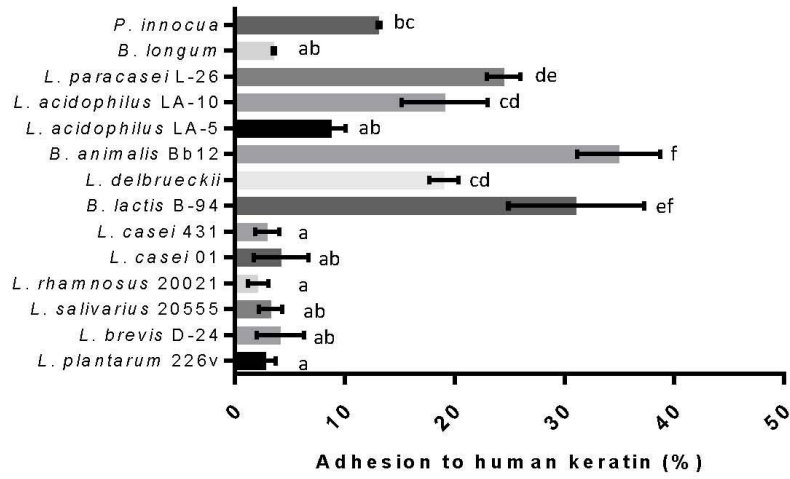


Figure 1

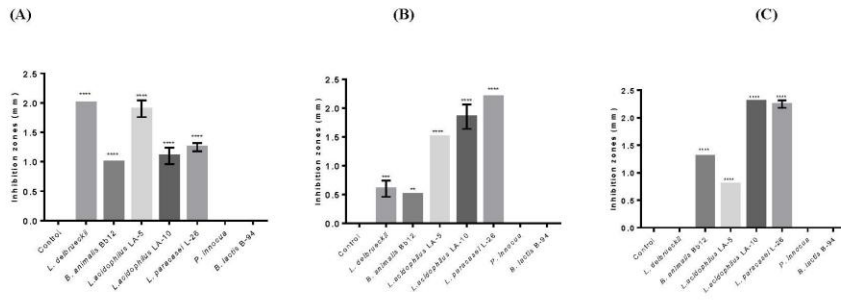


Figure 2

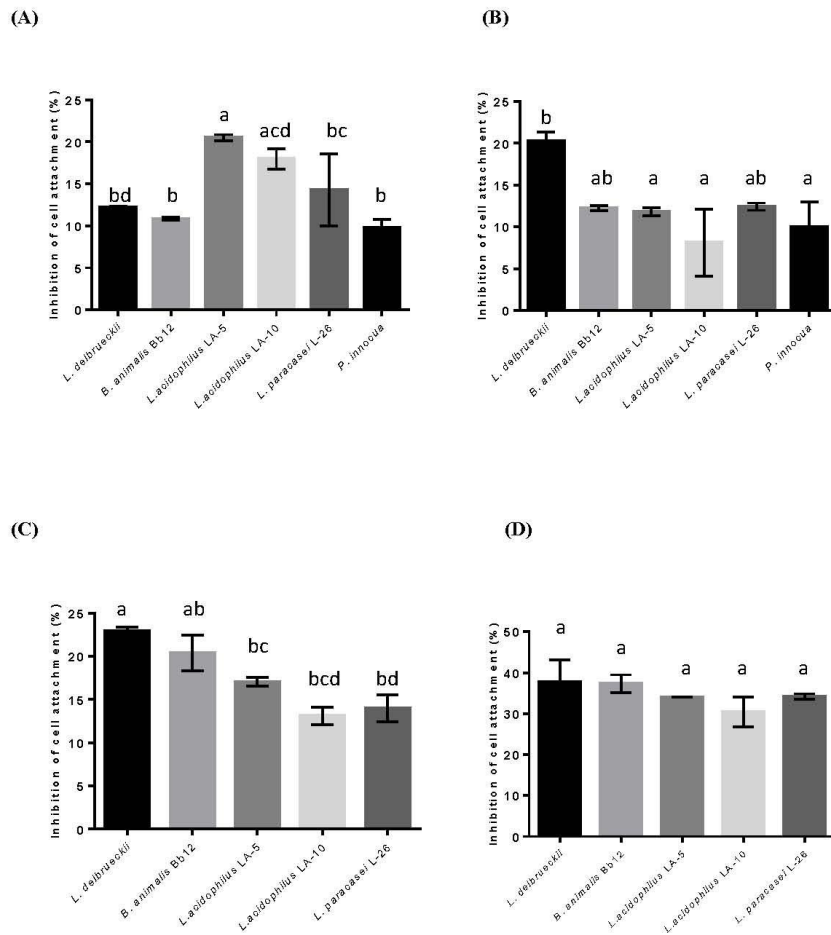


Figure 3

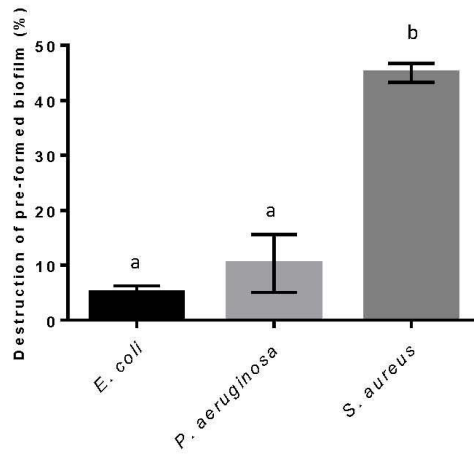


Figure 4

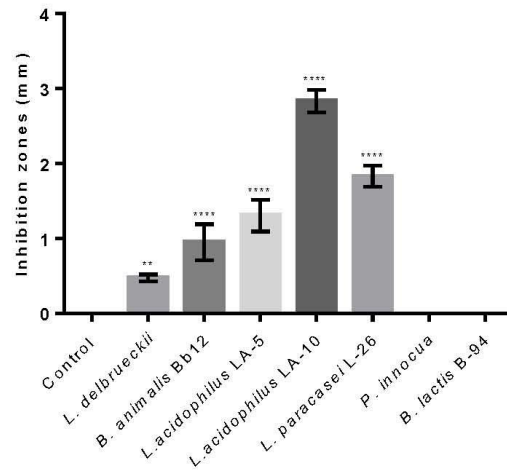


Figure 5