Assessment of the effect of stress-tolerance acquisition on some basic characteristics of specific probiotics

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Abstract

The production of viable functional probiotics presupposes stability of strain features in the final product. In this study it was evaluated the effect of acquisition of heat-tolerance and freeze-drying on the adhesion properties of *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, *Bifidobacterium lactis* Bb-12 and *Bifidobacterium animalis* IF20/1 and on their ability to inhibit the adhesion of pathogens in a mucus model. Both fresh and freeze-dried cultures were evaluated. Significant differences were observed between fresh, freeze dried, fresh heat-tolerant and freeze dried heat- tolerant strains, especially in the ability of the freeze dried probiotics to exclude, displace or outcompete pathogens.

Based on our study characterizing probiotic properties such as adhesion and competitive exclusion, it seems possible to adapt probiotics to processing stresses, such as heat, without significantly changing the probiotic properties of the strains assessed. This may provide new options for future probiotic production technology. However, our results also emphasize that

the properties of the stress-adapted strains, as well as the effect of the production processes should always be assessed as these are strain-specific.

Keywords: probiotics, adhesion, heat-tolerant, exclude, displace, outcompete.

1.1. Introduction

Probiotics have been defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO; 2001) and usually belong to the genus *Lactobacillus* or *Bifidobacterium*. Temporary colonization of the intestinal tract is a critical property of a probiotic (Alander et al. 1999). Colonization should be a probiotic property by which the intestinal mucosa may provide the host with protection by competitively excluding pathogens through competition for binding sites and nutrients, or by production of antimicrobials (Collado et al. 2005).Probiotic properties have been defined on a strain to strain basis and quality control should be focused on these properties and care should be taken to keep the original properties intact (Tuomola et al. 2000). However, recently Grzeskowiak and coworkers (2011) demonstrated that even the same strain properties may change depending on the manufacturing and processing conditions.

The definition of a probiotic includes the clause "in adequate amounts" and although this is not a known exact number, high levels of viable microorganisms are recommended for a beneficial effect to be seen (Knorr, 1998). The "adequate amount" of each probiotic varies and may also depend on the production technology used i.e. freeze dried in milk or encapsulated in calcium alginate, for example (Saxelin et al. 2010). Currently there is not a uniform dosing or frequency of dosing recommendation, with ranges of 2×10^{7} – 3.2×10^{12} colony forming units being used (Caglar et al. 2006, Gionchetti et al. 2007, Wallace et al. 2011). Consequently, the retention of high viability during drying and storage is imperative and challenging and the need to study each potential probiotic strain or mix of strains separately is necessary.

The production of viable functional probiotics presupposes stability of strain features in the final product. To achieve this, pre-exposing the probiotics to stress has been reported potentially beneficial in gradually modifying heat-, bile- and acid-tolerance (Berger et al.

2010; Collado and Sanz 2006; Margolles et al. 2003). However, as it has been previously shown in bile and acid adapted strains this may influence the strain properties significantly (Collado et al. 2006; Gueimonde et al. 2005; 2007). The aim of this study was to produce process stable probiotic strains, specifically heat resistant strains, and to study the effect of heat resistance acquisition and freeze-drying on the adhesion of the strains to human colonic mucus, as well as their ability to inhibit, compete with and displace the adhesion of five common model pathogens.

For this purpose we obtained heat-tolerant derivatives from common probiotic strains and evaluated their adhesion properties and their ability to exclude, displace or outcompete pathogens in a mucus model. To this end both fresh and freeze-dried bacteria were tested. We hypothesised that the strain properties stay similar to those of the original isolates when gradually adapted to heat.

1.2. Material and Methods

1.2.1. Bacterial strains and culture conditions

Lactobacilli and bifidobacteria used in this study were *Lactobacillus rhamnosus* GG (ATCC 53013), *Lactobacillus casei* Shirota (isolated from Yakult®, Tokyo, Japan), *Bifidobacterium lactis* Bb-12 (Christian Hansen, Hørsholm, Denmark) and *B. animalis* IF20/1, isolated from the feces of a 2-day old, vaginally delivered, breast-fed infant. The microorganisms were cultured from a frozen stock by inoculating Man Rogosa Sharpe (MRS) broth (Difco, Detroit, MI, USA) with a 1 % inoculum and incubated at 37 °C for 48 h under anaerobic conditions. Heat-shocked (HS) derivatives of these strains were obtained by successive 10 min heat-shocks, of resuspended cells in Phosphate buffered saline (PBS, pH 7,2), at increasing temperatures starting from 60 °C and incrementing the temperature by 5 °C in each successive shock. After each shock cells were inoculated to fresh media followed by recovery at 37 °C until the stationary phase of the growth was obtained as earlier studies have shown that stationary phase cultures are more resistant to heat stress (Kim et al. 2001; Saarela et al. 2004). The heat-treatment was stopped when no colonies were able to survive after heat treatment. With this selection process, the *Lactobacillus rhamnosus* GG derivative strain was obtained at temperature 85 °C, the *Lactobacillus casei* Shirota derivative strain was obtained at temperature 80 °C, the *Bifidobacterium lactis* Bb-12 derivative strain was obtained at

temperature 70 °C and the *Bifidobacterium animalis* IF20/1 derivative strain was obtained at temperature 85 °C. All bacteria were metabolically labelled by addition to the media of 10 µl ml^{-1} tritiated thymidine (5-^{3}H-thymidine 1.0 mCi/ml; Amersham Biosciences, UK) and incubated overnight under anaerobic conditions at 37 °C.

Fresh and freeze-dried cultures from the different strains were assessed. To obtain fresh cultures, MRS media containing 10 µl ml^{-1} tritiated thymidine was inoculated at 1 % with a preinoculum and incubated overnight for labelling. For freeze-drying, bacterial biomass grown in MRS was freeze-dried by a commercial culture company (Fermlabs Ltd, Finland) using maltodextrins as cryoprotectant. Then, 0.1 g freeze-dried bacteria were used to inoculate 10 ml of the MRS media containing 10 µl ml^{-1} tritiated thymidine to obtain, after overnight growth, labelled bacteria.

The model pathogens for the study, *Clostridium difficile* DSM 1296, *Escherichia coli* NTCT 9001, *Salmonella enterica* serovar Typhimurium ATCC 12028, *Streptococcus mutans* ATCC 2517S, and *Enterobacter sakazakii* ATCC 29544 were grown in Gifu Anaerobic Medium (GAM) (Nissui Pharmaceutical, Tokyo, Japan) for 18 h at 37 °C. All strains were incubated under anaerobic conditions (10 % H_2, 10 % CO_2 and 80 % N_2; Concept 400 anaerobic chamber, Ruskinn Technology, Leeds, UK).

1.2.2. Heat-tolerance assay

Heat-tolerance of derivative strains was tested in PBS after incubation of strains at 60 ^{0}C, 70 ºC and 80 ºC. First, strains were cultured in GAM broth in anaerobic conditions at 37 °C (24 h for lactobacilli and 48 h for bifidobacteria); bacteria were harvested by centrifugation, washed twice with PBS and resuspended into PBS for heat-tolerance tests. Samples were taken during incubation (at 1, 2, 5 and 10 min) and 1 ml plated on GAM agar. In order to verify that the obtained derivative strains belonged to the expected species, to exclude contaminations with other microorganisms, original and heat-tolerant strains were identified by sequencing the partial 16S ribosomal DNA (rDNA) gene (Kullen et al. 2000).

1.2.3. Mucus adhesion assay

Human colonic mucus was collected as previously described (Ouwehand et al. 2002) and stored at -80 °C until use. Before use the protein concentration of the mucus was determined and diluted to 0.5 mg/ml in HEPES (N-2-hydroxyethylpiperazine-N-2-ethanosulphonic acid)-Hanks buffer (HH; 10 mM HEPES, pH 7.4). 100 µl of mucus was immobilized into

polystyrene microtitre plate wells (Maxisorp, Nunc, Roskilde, Denmark) by incubation at 4 °C for 18 h.

Radiolabelled pathogenic bacteria were collected by centrifugation at 1500 g for 7 min and washed twice with PBS and the absorbance (A_{600}) was normalised to 0.25 (aprox. 10^8 cfu/ml). Of this, 100 µl was added to the wells and incubated at 37 °C for 1 h. The wells were washed twice with 200 µl HH buffer to remove unattached bacteria and the adhered bacteria were released and lysed by incubation at 60 °C for 1 h in 1 % (wt/vol) sodium dodecyl sulphate in 0.1 M NaOH (200 µl per well). The contents of each well were added to 1 ml of scintillation liquid (OptiPhase "HiSafe 3", Wallac, Milton Keynes, UK) in a microcentrifuge tube and the radioactivity was measured by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilized mucus. Adhesion was determined in three independent experiments, and each assay was performed in triplicate.

1.2.4. Inhibition of pathogen adhesion to colonic mucus

To test the ability of the probiotic strains to inhibit the adhesion of pathogens the procedure described by Collado et al. (2005) was used. In brief, unlabelled probiotic bacteria (100 µl, 10^8 cells ml^{-1} approximately) were added to the wells and incubated for 1 h at 37 °C. Non-bound probiotic bacteria were removed by washing twice with HH-buffer and then, radiolabelled pathogens (100 µl, 10^8 cells ml^{-1} approximately) were added to the wells and incubated at 37 °C for 1 h. After this second incubation, unbound bacteria were washed and bound bacteria were lysed as described in adhesion assays. Radioactivity was assessed by liquid scintillation. The adhesion inhibition was calculated as the difference between the adhesion of the pathogen in the absence (control) and presence of probiotic strains. Inhibition was determined in three independent experiments and each assay was performed in triplicate.

1.2.5. Displacement of pathogens adhered to colonic mucus.

The ability of the studied probiotic strains to displace already adhered pathogens was assessed according to Collado et al. (2005). Radiolabelled pathogens were added to the wells containing mucus and incubated for 1 h at 37 °C. After the washing and removing of unbound pathogens, unlabelled probiotic strains were added, and the plate was incubated for 1 h at 37 °C. After that, the wells were washed and the bound bacteria were recovered after lysis and radioactivity was measured. Displacement of pathogens was calculated as the

difference in adhesion before versus after addition of probiotics. Three independent experiments were carried out. Each assay was performed in triplicate to calculate intra-assay variation.

1.2.6. Competitive exclusion of pathogens adhered to colonic mucus.

Equal quantities of bacterial suspension of probiotic and radiolabeled pathogens were mixed and added to the immobilized mucous. This was incubated for 1 h at 37°C. The cells of the pathogen bound to the mucous were released and lysed as above. Exclusion of pathogens was calculated as the difference between the adhesion of pathogens before and after the addition of the probiotic strains.

1.2.7. Statistics

Statistical analysis was performed using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Data were subjected to one-way analysis of variance (ANOVA) and the Student-Newman-Keuls (S-N-K) test was used for comparison of the means, where appropriate. $P < 0.05$ was considered statistically significant.

1.3. Results

1.3.1. Heat-tolerance of the strains

Heat-tolerance of the derivative strains obtained in the study was compared to the corresponding parental strain by incubating strains in PBS at different temperatures (60 °C, 70 °C and 80 °C). Enhanced heat-tolerance (1700-fold) of the derivative strain was obtained for *Lactobacillus rhamnosus* GG (after 20 min heat-treatment at 60 °C) and *Bifidobacterium lactis* Bb-12 (after 20 min at 60 °C), with viability increasing 1700-fold ($P = 0.0002$) and 16-fold ($P = 0.0003$), respectively (Figure 1). Regarding *Bifidobacterium animalis* IF20/1 and *Lactobacillus casei* Shirota the stress adaptation did not enhance the thermo-tolerance of the strains (Figure 1). Increased tolerance was obtained also at temperatures 70 °C and 80 °C, although the temperatures were already lethal also for derivative strains, thus the results from 60 °C are only shown.

1.3.2. Adhesion of probiotics to human colonic mucus

The adhesion of each strain was tested, and the effect of the heat-shock procedure as well as freeze drying on this was observed (Fig. 2).

The fresh cultures of heat treated probiotic strains did not show any changes in their adhesion abilities to human colonic mucus, except for strain Bb12 that significantly increased its adhesiveness from approximately 6 % to approximately 10 % (Fig. 2B).

Freeze-drying, however, caused a significant decrease of the adhesion capabilities for all strains tested except SHI (Fig. 2).

When the heat treated variant of LGG was freeze-dried (LGG HS FD), a further significant decrease was observed in its adhesiveness (Fig. 2A). Heat treated, freeze-dried strains Bb12 and IF20/1 (Bb12 HS FD and IF20/1 HS FD) showed no significant differences to their original freeze dried counterparts (Fig. 2B and D). Again, SHI showed no significant differences between the original and any of its derivatives (Fig. 2C).

No protective mechanism was noted by heat treating the bacteria prior to freeze drying (Fig. 2). This may be due to the pre-culturing of the strains for this experiment where all derivatives are grown at 37°C which may result in the loss of heat-tolerance and hence no differences may be seen in their stability during freeze-drying. The heat-tolerant strains should rather constantly be exposed to the heat stress prior to performing this assay.

Adhesion of the pathogens was also studied and the tested strains showed low adhesion potential. *S. typhimurium* showed the lowest adhesion of 0.25 %, followed by *E. sakazakii* at 0.4 %. *E. coli* and *S. mutans* showed an adhesion of 0.8 % and *C. difficile* displayed the most marked adhesion to colonic mucus at 1 %.

1.3.3. Adhesion mechanisms

1.3.3.1. Inhibition by exclusion

The effect of heat-tolerance acquisition and freeze drying on ability of the four probiotic strains to inhibit the adhesion of the pathogens was tested (results not shown). We only found a significant decrease in the ability of freeze-dried derivatives of Bb12 HS, SHI and SHI HS and IF20/1 in excluding *E. coli*. Heat tolerance and freeze drying of LGG HS FD showed no

significant effect on its ability to exclude this pathogen and no significant changes were observed for any of the other pathogens tested.

1.3.3.2. Displacement of pathogens adhered to intestinal mucus

All original and heat-tolerant probiotic strains were capable of displacing all pre-adhered tested pathogens (Table 1). However, freeze drying the probiotics has a largely damaging effect on the displacement abilities of these strains, apart from IF20/1 whose displacement ability is not changed significantly. The displacement of *E. coli* by SHI was significantly reduced (P = 0.0001) by freeze drying the original and the heat treated derivatives, the latter losing its displacement ability fully. The same derivatives of SHI significantly reduce their abilities to displace *S. typhimurium* (P = 0.002), *C. difficile* (P = 0.002) and *E. sakazakii* (P = 0.001). Bb12 FD and its derivative Bb12 HS FD also significantly decrease the displacement of *C. difficile* and *S. mutans,* and only Bb12 HS FD strain loses its displacement ability towards *E. sakazakii.* LGG FD shows a significant decrease (P = 0.033) and a further significant decrease (P = 0.0001), when the heat treated strain is freeze dried, losing its displacement capacity (-58.7 %) against *E. sakazakii*.

1.3.3.3. Competitive exclusion to intestinal mucus

The results of the inhibition by competition are shown in table 1. The freeze dried derivatives differed significantly in their ability to competitively exclude the pathogens. No significant differences in inhibition of *E. coli* were observed for any strain. With respect to *S. typhimurium,* only IF20/1 had an effect, with IF20/1 FD showing a significant reduction (P = 0.002) in inhibition to this pathogen when compared to the fresh cultures or IF20/1 HS FD. *C. difficile* was not outcompeted by LGG original, but the strains ability to outcompete this pathogen was significantly increased (P = 0.014) by LGG FD and LGG HS FD variants. This was repeated for Bb12 against *C. difficile* where the freeze dried variants increased the ability of Bb12 to outcompete *C. difficile* significantly (P = 0.021). SHI FD showed a significant decrease (P = 0.011) in its ability to outcompete the same pathogen without observing the same effect for the SHI HS derivative strain. However, no statistically significant differences were observed for the IF20/1 strains. The treatment of all probiotics resulted in no significant differences observed in their ability to outcompete *E. sakazakii* except LGG FD, where a significant increase (P = 0.037) was seen. Finally, the only significant difference observed for the competition with *S. mutans* was again seen for IF20/1 FD, where a significant decrease (P = 0.032) was observed.

1.4. Discussion

There is increasing interest in the incorporation of probiotics into foods other than fermented dairy products due to consumer demand for a wider range of products. However, there are technological challenges in the incorporation of probiotics to non-dairy products (Grześkowiak et al. 2011). Probiotics should remain viable in the end product. The acidity of the product as well as the high temperatures used for processing of the products can cause challenges in maintaining the viability. The ability to withstand stress during processing and storage of products is important and thermotolerant probiotics are of great interest in this area (Saarela et al. 2004). However, the probiotic properties such as adhesion to the intestinal mucus should not be altered by stress adaptation. In this work, heat adaptation was used to enhance heat-tolerance of probiotic strains and the effect of stress adaptation as well as freeze drying on the mucus adhesion and the ability to exclude, displace or outcompete pathogens were evaluated.

Stress adaptation of strains can be done by exposing strains to lethal or sub-lethal conditions ((Alvarez-Ordonez et al. 2008; Annous et al. 1999). Several factors can affect the heat-tolerance of a strain including growth medium and pH, however other stresses such as use of ethanol, hydrogen peroxide, oxygen, pressure, starvation or osmotic environment can lead to increased heat- or acid stress response by cross protection. The stress adaptation is a species- or even strain-specific characteristic involving the fatty acid composition of the membrane ((Alvarez-Ordonez et al. 2008; Annous et al. 1999).

The effects of heat stress response have been studied in probiotic bacteria to improve their performance during food processing. Desmond et al. (2001) showed that heat adapted (at 52 °C for 15 min) *L. paracasei* NFBC 338 exhibited a 300- and 700-fold increase in thermotolerance at 60 °C in MRS and reconstituted skim milk, respectively. Pressure pre-treatment of *L. rhamnosus* GG increased survival of the strain when exposed to 60 °C indicating cross protection (Ananta et al. 2004) while heat and acid responses did not present significant cross-protection in *Propionibacterium freudenreichii* (Leverrier et al. 2004). In this work, a considerably enhanced heat-tolerance (1700-fold) was obtained with *Lactobacillus rhamnosus* GG and enhanced heat-tolerance (16-fold) was obtained with *Bifidobacterium lactis* Bb-12 when strains were exposed to sublethal heat. However, for two other strains, *B.*

animalis IF20/1 and *L. casei* Shirota, heat adaptation did not lead to significantly increased heat-tolerance.

The impact of the freeze-drying process on probiotic adhesion properties was studied and compared to the original strains. The stress adapted strains obtained from exposure to increasing heat were expected to show increased survival after freeze-drying as this adaption is thought to protect the bacterial membrane which is the primary site for dehydration damage (Castro et al. 1997). Bacterial death during freeze-drying is due to osmotic shock and formation of intracellular ice and recrystallisation (Heckl, 1985). During freeze-drying the frozen water is removed by sublimation, in an effort to reduce damage to biological structures. However, the viability of the freeze-dried microorganism depends on several factors such as the strain used, bacterial cell size, and efficacy of the cryoprotectant (Fonseca et al. 2000). Maltodextrins have previously been shown to increase survival of lactic acid bacteria during freeze-drying due to direct interactions with proteins and membranes (De Guilio et al. 2005).

In this study, freeze drying of probiotics was found to have an effect on adhesion potential, showing an overall significant decrease in the ability of the freeze dried probiotic to exclude, displace or outcompete certain pathogens. Only in isolated cases a significant increase was noticed. These increases were observed for *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 in inhibition by competition assays. Out of all the combinations of probiotic derivatives and pathogen tested here, there does not seem to be a trend in effect of freeze drying on adhesion potential. The effect of freeze drying appeared to be strain dependent and *L. casei* Shirota properties appeared more sensitive to freeze drying than the other strains as it showed more frequent decreases in ability to adhere.

Taken together, it appears that the tolerance to processing conditions and stress induced during processing and storage can be enhanced by traditional conditioning methods. However, manufacturing processes, such as freeze-drying, may affect strain properties and should be assessed independently. Stress-tolerant strains may be new options for future probiotic technology, providing that there are no changes in their original properties, to enable the use of derived probiotics in same target groups and populations as earlier reported.

Acknowledgements

This work was supported by Technology Development Agency of Finland.

1.5. References

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Table 1. The percentage inhibition of model pathogens by the study strains in a human intestinal mucus model. Results are shown as mean ± standard deviation and they are represented as percentages compared to adhesion inhibition of pathogen strains without probiotic strains

Probiotic	% pathogen inhibition by exclusion					% pathogen displacement					% pathogen inhibition by competition				
	E. coli	*S. typhimurium*	*C. difficile*	*E. sakazakii*	*S. mutans*	*E. coli*	*S. typhimurium*	*C. difficile*	*E. sakazakii*	*S. mutans*	*E. coli*	*S. typhimurium*	*C. difficile*	*E. sakazakii*	*S. mutans*
LGG	22,0 ± 6,4	-0,54 ± 1,9	-95,9 ± 10,0	-17,9 ± 2,7	-3,3 ± 1,5	36,3 ± 2,4	58,3 ± 9,4	58,4 ± 1,4	63,8 ± 1,5^{a}	62,6 ± 1,0	-6,5 ± 1,5	16,3 ± 4,3	-14,0 ± 1,4^{a}	-19,9 ± 1,4^{a}	-7,2 ± 3,4
LGG HS	10,7 ± 6,1	-38,3 ± 5,3	-53,2 ± 5,6	-53,2 ± 5,6	-29,7 ± 3,7	49,0 ± 1,3	57,6 ± 1,1	40,6 ± 2,9	59,0 ± 1,6^{a}	62,1 ± 1,1	14,4 ± 1,5	16,4 ± 0.8	6,1 ± 3,9^{a}	-5,0 ± 1,5^{a}	13,6 ± 2,4
LGG FD	-18,6±10,3	33,0 ± 6,3	-23,1 ± 9,5	-23,1 ± 9,5	28,0 ± 3,0	18,4 ± 1,1	26,2 ± 1,0	21,4 ± 1,0	11,6 ± 5,8^{b}	32,2 ± 9,3	11,0 ± 3,0	24,6 ± 6,0	39,4 ± 6,8^{b}	35,9 ± 8,0^{b}	22,1 ± 3,7
LGG HS FD	-20,8 ± 14,2	35,9 ± 3,9	-19,2 ± 1,5	-19,2 ± 1,5	42,0 ± 11,0	23,9 ± 6,5	16,2 ± 1,3	25,5 ± 3,1	-58,7 ± 1,0^{c}	29,3 ± 7,4	21,6 ± 3,2	34,3 ± 3,5	25,8 ± 0,73^{b}	-14,3 ± 0,8^{a}	22,13 ± 3,2
P value	**0,23**	**0,29**	**0,771**	**0,834**	**0,142**	**0,519**	**0,057**	**0,456**	**0,0001**	**0,065**	**0,379**	**0,166**	**0,014**	**0,037**	**0,738**
Bb12	32,3 ± 11,1^{a}	-18,5 ± 4,5	-35,3 ± 5,0	8,4 ± 1,5	-22,2 ± 4,6	57,3 ± 1,2	61,6 ± 8,1	65,7 ± 1,2^{a}	61,8 ± 17,9^{a}	68,3 ± 7,1^{a}	19,5 ± 1,0	36,8 ± 9,7	-0,6 ± 1,5^{a}	-5,1 ± 2,1	19,1 ± 2,2
Bb12 HS	21,2 ± 1,5^{a}	-17,8 ± 4,7	-49,3 ± 5,1	0,8 ± 2,6	2,2 ± 3,3	54,1 ± 1,4	50,0 ± 1,7	56,8 ± 1,7^{a}	61,2 ± 19,2^{a}	58,9 ± 13,5^{a}	21,6 ± 2,0	34,8 ± 3,6	6,8 ± 0,9^{a}	-9,4 ± 5,6	3,7 ± 3,3
Bb12 FD	14,5 ± 3,7^{a}	19,6 ± 2,6	-11,5 ± 1,1	-34,9 ± 1,2	10,2 ± 1,1	27,3 ± 6,7	32,2 ± 6,8	23,0 ± 1,3^{b}	21,6 ± 1,3^{a}	31,4 ± 3,4^{b}	20,1 ± 1,7	32,1 ± 3,1	51,9 ± 6,2^{b}	24,7 ± 1,8	17,1 ± 5,4
Bb12 HS FD	-41,3 ± 1,5^{b}	24,6 ± 11,0	-25,3 ± 6,2	-25,3 ± 6,2	-12,7 ± 1,5	28,2 ± 5,9	19,6 ± 8,8	-0,4 ± 0,4^{b}	-30,3 ± 6,2^{b}	17,4 ± 7,7^{b}	23,3 ± 2,3	28,8 ± 2,2	43,2 ± 1,1^{b}	33,2 ± 7,0	39,2 ± 6,7
P value	**0,01**	**0,691**	**0,899**	**0,262**	**0,856**	**0,126**	**0,1**	**0,008**	**0,007**	**0,011**	**0,958**	**0,759**	**0,021**	**0,171**	**0.686**
SHI	31,5 ± 8,0^{a}	19,1 ± 2,1	-42,1 ± 3,7	15,1 ± 2,1	16,9 ± 1,1	55,2 ± 7,8^{a}	60,9 ± 7,0^{a}	62,2 ± 1,0^{a}	59,1 ± 13,4^{a}	33,7 ± 1,8	20,5 ± 7,4	31,0 ± 3,7	42,9 ± 2,9^{a}	-4,1 ± 0,5	18,6 ± 2,8
SHI HS	29,8 ± 1,3^{a}	19,5 ± 1,8	-47,8 ± 4,4	10,1 ± 1,8	-8,7 ± 0,3	53,2 ± 8,5^{a}	58,0 ± 9,77^{a}	56,8 ± 1,4^{a}	62,8 ± 11,6^{a}	60,1 ± 6,7	5,7 ± 0,5	12,2 ± 4,4	2,8 ± 1,3^{a}	-6,7 ± 3,8	18,7 ± 2,1
SHI FD	-24,1 ± 1,0^{b}	35,0 ± 7,7	-9,5 ± 1,5	11,7 ± 0,9	-25,4 ± 1,1	8,5 ± 3,3^{b}	-4,1 ± 0,4^{b}	10,0 ± 1,8^{b}	-15,5 ± 6,2^{b}	23,3 ± 3,1	18,7 ± 0,8	16,1 ± 4,0	-47,7 ± 3,1^{b}	20,5 ± 0,9	27,3 ± 9,2
SHI HS FD	-38,3 ± 1,6^{b}	33,2 ± 4,7	-40,8 ± 1,6	-45,7 ± 1,3	8,8 ± 0,5	-4,1 ± 0,5^{b}	12,9 ± 1,3^{b}	-2,6 ± 0,4^{b}	12,6 ± 1,1^{b}	32,5 ± 3,0	15,6 ± 4,0	32,6 ± 1,2	49,1 ± 8,3^{a}	23,4 ± 5,2	35,7 ± 2,5
P value	**0,006**	**0,791**	**0,8**	**0,079**	**0,519**	**0,0001**	**0,002**	**0,002**	**0,001**	**0,133**	**0,229**	**0,173**	**0,011**	**0,613**	**0,893**
IF20/1	36,8 ± 1,7^{a}	30,3 ± 2,1	-36,8 ± 4,6	28,2 ± 2,3	17,5 ± 2,1	49,0 ± 8,0	57,4 ± 1,4	36,9 ± 3,5	59,6 ± 1,8	58,7 ± 13,5	22,4 ± 2,2	26,1 ± 1,1^{a}	-9,5 ± 0,7	-15,4 ± 1,8	19,6 ± 1,3^{a}
IF20/1 HS	19,1 ± 2,9^{a}	-26,4 ± 5,8	-33,8 ± 4,1	8,2 ± 0,2	-8,9 ± 0,4	53,5 ± 7,0	57,1 ± 1,6	57,6 ± 1,5	54,6 ± 2,1	65,5 ± 7,7	25,9 ± 6,0	31,9 ± 7,6^{a}	18,8 ± 2,5	-6,6 ± 1,4	15,0 ± 1,5^{a}
IF20/1 FD	-44,9 ± 4,0^{b}	-7,5 ± 0,5	-22,9 ± 0,9	33,1 ± 7,9	-44,3 ± 1,1	28,6 ± 3,5	44,6 ± 6,8	24,7 ± 5,1	32,8 ± 8,3	35,0 ± 4,3	17,6 ± 5,9	-6,2 ± 0,7^{b}	35,0 ± 1,6	-16,5 ± 2,7	-24,5 ± 0,7^{b}
IF20/1 HS FD	21,1 ± 0,7^{a}	38,5 ± 1,4	-20,4 ± 0,92	-37,1 ± 1,0	-45,6 ± 7,2	35,5 ± 4,1	49,6 ± 7,9	23,6 ± 1,0	30,5 ± 1,1	39,7 ± 7,4	27,3 ± 7,5	19,4 ± 5,7^{a}	17,1 ± 0,9	32,0 ± 6,9	31,9 ± 7,5^{a}
P value	**0,043**	**0,451**	**0,977**	**0,084**	**0,201**	**0,059**	**0,841**	**0,583**	**0,472**	**0,112**	**0,656**	**0,002**	**0,071**	**0,061**	**0,032**

presence (taken as 0 %).

[a, b] Columns with different superscripts differ significantly ($P < 0.05$)

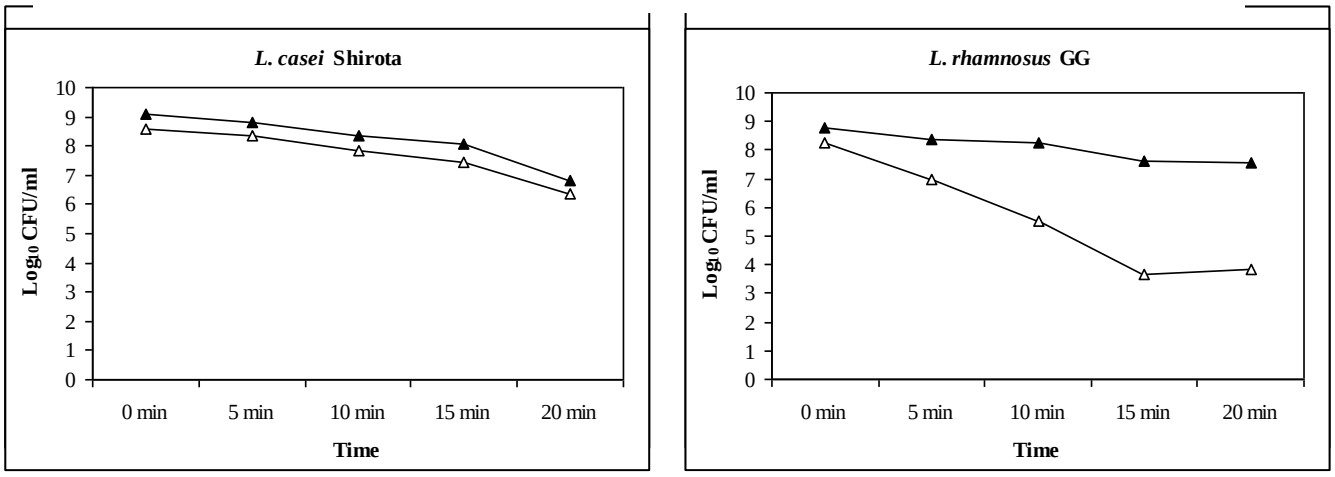
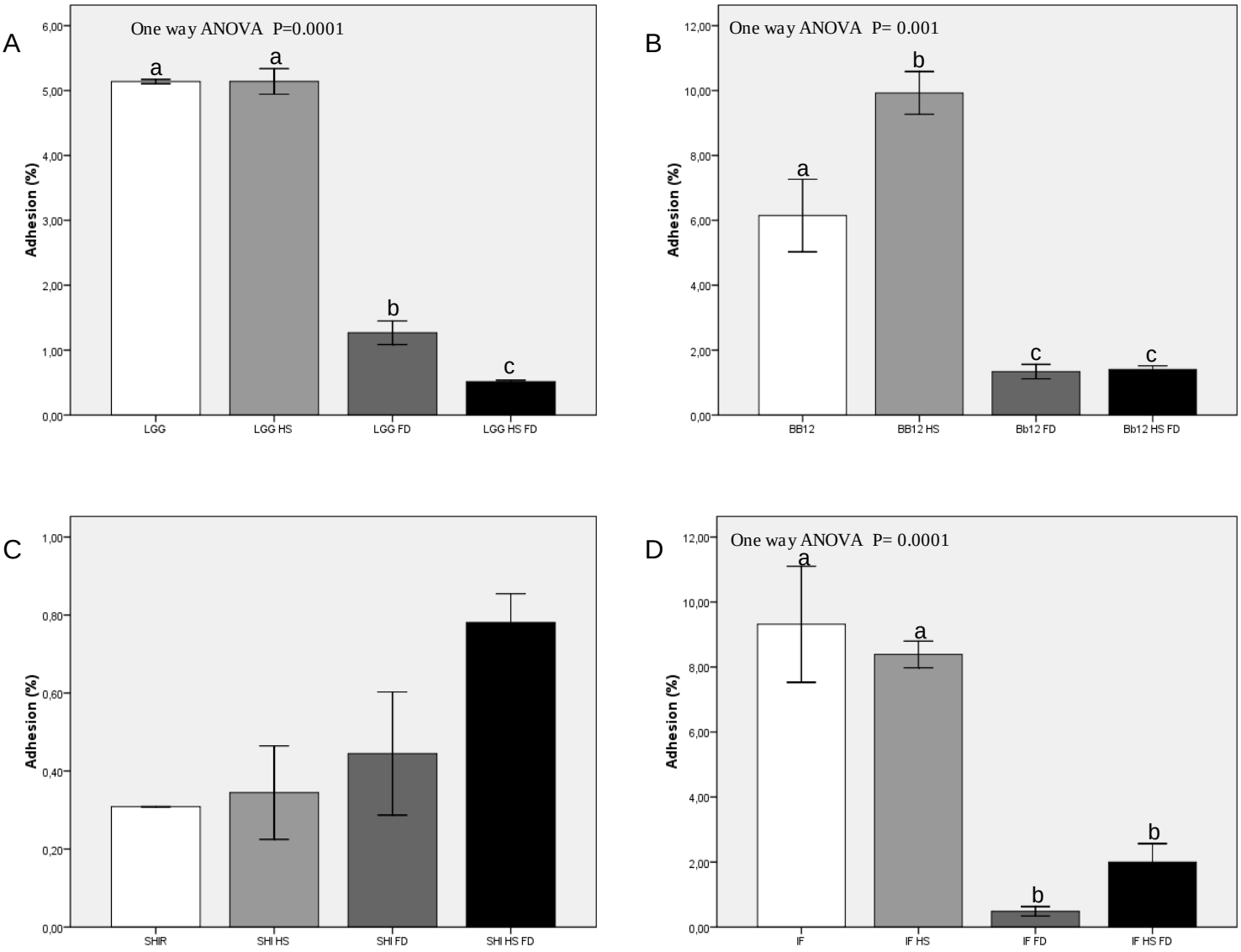

L. casei Shirota
Log10 CFU/ml
10
9
8
7
6
5
4
3
2
1
0
0 min
5 min
10 min
15 min
20 min
Time
L. rhamnosus GG
Log10 CFU/ml
10
9
8
7
6
5
4
3
2
1
0
0 min
5 min
10 min
15 min
20 min
Time

Figure 1



443 **Figure 2**

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Figure 1. Heat-tolerance of the derivative strains when compared to parental strains during incubation at 60 ^{0}C. Parental strains marked as open triangle, derivative strains marked with closed triangles. Each value is a mean ± SD for 3 determinations.

Figure 2. Adhesion of probiotic strains LGG (A), BB12 (B), SHI (C) and IF (D) to human colonic mucus in their original form, the heat-shocked derivative (HS), the freeze dried form (FD) and the heat-shocked derivative freeze dried (HS FD). Results are expressed as the percentage of radioactivity recovered from immobilised mucus compared to radioactivity added to mucus (mean and SD).