EFFECT OF HYDROLYZED MILK ON THE ADHESION OF *LACTOBACILLI* TO INTESTINAL CELLS*

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Milk is an essential part of the human diet and is undoubtedly a major calcium source in human nutrition, accepted well by most individuals. Knowledge on how the components from dairy products support or reduce the adherence of probiotics to the intestinal epithelium is limited. The purpose of this study was to investigate the effect of acid-hydrolyzed milk on the adhesion ability of two potentially probiotic strains (*Lactobacillus plantarum* S2*, Lactobacillus gasseri* R) to *in vitro* human intestinal epithelial model consisting of Caco-2 and mucus-secreting HT29-MTX co-culture. The adhesion of our tested strains *L. gasseri* and *L. plantarum* was 4.74 and 7.16%, respectively, when using inoculum of 2×10^8 CFU ml^{-1}. Addition of acid-hydrolyzed milk to co-culture decreased the adherence by 53.7% for *L. gasseri* R and by 62.2% for *L. plantarum* S2. The results of this study evidently indicate the potential importance of the food matrix as a factor influencing probiotic colonization of the gut.

bacterial adhesion; acid-hydrolyzed milk; *Lactobacillus gasseri* R; *Lactobacillus plantarum* S2; cells of the small intestine; Caco-2; HT29-MTX

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INTRODUCTION

Milk is a complex food whose consumption confers a number of nutritional and physiological benefits (Phelan et al., 2009). Milk and a wide range of fermented milk products are considered as one of sources of probiotic bacteria which confer a health benefit on the host (Ouwehand, Salminen, 1998; Ouwehand et al., 2002). Besides, milk is considered as optimal system for delivery of probiotic bacteria. Most commercially available probiotics include lactic acid bacteria (LAB) predominantly selected from the *Lactobacillus* genera (Bejar et al., 2011).

Studies suggest that bacteria are not simply suspended in milk, but possibly due to hydrophobic nature of their cell wall, they adhere to hydrophobic surface components of the milk fat globules (Brisson et al., 2010). Interestingly, bacteria grown in fat-containing milk, instead in growth media, show lower adherence to human epithelial cells (Ouwehand et al., 2001). Milk components might thus interact with bacteria and affect their biological properties.

Probiotics are believed to temporarily colonize the intestine by adhering to intestinal surface and therefore, adherence to the intestinal mucosa has been considered as one of the criteria for selection of potential probiotic strains (Tuomola, Salminen, 1998). Autoaggregation assays have been developed to predict these properties (Del Re et al., 2000; Collado et al., 2008), however, cell culture models based on Caco-2 and HT-29 cell lines, which use isolated colon adenocarcinoma cells, are closer to *in vivo* conditions. Co-cultures of Caco-2 and mucin-secreting HT29-MTX cells are believed to better represent the complex mucosa (Laparra, Sanz, 2009).

The adhesive mechanism of surface molecules mediating the adhesion of lactobacilli to the intestinal epithelium is scant, as only few of them have been identified and characterized (Dhanani et al., 2011). It is also known that in addition to host-bacteria and

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bacteria–bacteria relationship, diet has a strong impact in establishing a well-balanced intestinal microbiota on the extent to which different intestinal bacteria colonize the intestine (Bustos et al., 2012). Diet may shape microbial culture by either providing selective substrates for microbial growth, but also by changing adherence properties of individual strains or bacterial groups as it has been suggested by recent *in vitro* studies (Ouwehand et al., 2001; Coppa et al., 2006; Parkar et al., 2008). Getting back to the possible role of dietary milk constituents, recent study of Clarke et al. (2014) suggests that professional rugby players with high whey protein supplements intake show different microbial patterns from the normal population. The evidence is lacking, but we may hypothesize about if whey or generally a milk protein has an effect on the adherence of certain bacterial groups in these individuals.

The aim of this study was to determine the effect of acid-hydrolyzed milk, resembling the gastric milk digesta, on the adherence of two potential probiotic *Lactobacillus* strains in the epithelial model based on co-culture of Caco-2 and HT29-MTX cells. Selection of both strains (*Lactobacillus gasseri* R, *Lactobacillus plantarum* S2) for this study was based on their autoaggregation ability as we wanted to proof the concept with low and high aggregating strains.

MATERIAL AND METHODS

Bacterial strains

The strain *Lactobacillus gasseri* R was isolated from a faecal sample of one-month-old infant; strain of *Lactobacillus plantarum* S2 was isolated from colon of pig aged 5 months. Both were isolated using Rogosa agar (Oxoid Ltd., Basingstoke, UK) after 72 h of microaerophilic cultivation at 37°C. Strains were identified by MALDI-TOF Mass Spectrometry using the MALDI BioTyper (TM) system (Bruker Daltonik, Bremen, Germany) according to Kmeť, Drugdová (2012). They were further characterized by a biochemical test API 50CHL (bioMérieux, Marcy l'Etoile, France) and their autoaggregation properties were determined according to Reniero et al. (1992) later adapted by Vlková et al. (2008).

Cell cultures

The human epithelial intestinal cell lines, colorectal adenocarcinoma (Caco-2), and the mucin-producing cell line (HT29-MTX) were used to assess the adhesion abilities of two different bacterial strains in the presence or absence of acid-hydrolyzed milk. Both cell lines were originally from the American Type Culture Collection (Rockville, Maryland, USA). Caco-2 and HT29-MTX cell lines were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% nonessential amino acids, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin. The cell lines were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 and 95% air. The medium was changed every two days, and the cells were sub-cultured at 80% confluence every week (Bustos et al., 2012; Jensen et al., 2012). Medium and reagents were purchased from Sigma-Aldrich and Invitrogen (Waltham, USA).

Preparation of bacterial suspension

Prior the adherence test, bacteria were grown anaerobically on Man, Rogosa, and Sharpe (MRS) broth (Oxoid) at 37°C for 24 h, diluted in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich). Bacteria were centrifuged (2 000 × *g*, 10 min); the pellet was washed twice with phosphate-buffered saline (PBS; pH 7.0). The bacterial suspension was diluted in PBS to a final concentration of 2×10^8 CFU ml^{-1} by measuring the optical density at 420 nm (Infinite M200; Tecan Austria GmbH, Grödig, Austria).

Preparation of milk hydrolyzate

Milk (UHT treated milk, Pilos brand) was hydrolyzed by adding equal volume of 2% HCl to the milk sample. Hydrolysis was done at room temperature and terminated after 30 min by adjusting pH to pH 7.0 with 1M NaOH.

Adhesion assays

This assay was carried out according to the study by Jensen et al. (2012) with a slight modification. For adhesion, the cell lines (combined co-culture Caco-2/HT29-MTX) were seeded in 24-well culture plates at concentration of 3.6×10^4 cells per well (Caco-2) and 4×10^3 cells per well (HT29-MTX) and grown 14 ± 1 days past confluence at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, prior to the adhesion assays. The culture medium was changed every two days (Gagnon et al., 2013). Before experiments, the cell layers were washed with DPBS to remove antibiotics from the original cell media.

Bacterial suspension of 100 µl volume was added to previously washed cell monolayers. After that, 100 µl of dilute milk hydrolyzate (containing 5% of the original milk sample) were added whereas 100 µl of PBS were added to control wells. For each strain, controls and treated wells were set in triplicate. Then, plates were incubated at 37°C for 1 h under 5% CO_2. After the incubation period, supernatants were removed and the cell layers were softly washed three times with Dulbecco's PBS to remove non-attached bacteria. Finally the cell layers were trypsinized by addition of 300 µl 1% Triton-X100 (Sigma-Aldrich)

per well for 3 min followed by addition of 700 µl PBS. The remaining suspensions with viable adhered bacteria were diluted and plated on MRS agar (Oxoid) in Petri dishes. Bacterial counts were determined after aerobic incubation for 48 h at 37°C. Adhesion data were expressed as the percentage of bacteria adhered compared to the total of bacteria added. Each of the tested lactobacilli strains was analyzed in triplicate.

Statistical analysis

Mean values, standard deviations and standard errors and correlation coefficients were calculated based on the values for the different variables studied. Statistical analysis was performed using the IBM SPSS Statistics ver. 19.0 (IBM, Armonk, NY, USA). Student's *t*-test was used for comparisons at $P < 0.05$. Means were given ± 1 SD (standard deviation).

RESULTS

Both strains showed autoaggregation phenotype and formed sand-like particles in liquid cultivation media. Our tested strain of *L. gasseri* showed high autoaggregation properties (71.61%) after 1 hour, while at the same time point tested strain of *L. plantarum* showed autoaggregation properties close to zero and 7.15% after 4 h. Accordingly, *L. gasseri* R strain exhibited higher relative adhesion ability in the adhesion assay. A significant difference between the strains was seen even in their adhesion properties to cultured epithelial cells. From the initial 2×10^8 cells ml^{-1} applied on the monolayer, adherence of our tested strains *L. gasseri* was 7.16% and that of *L. plantarum* 4.74% (Fig. 1). Addition of acid-hydrolyzed milk in the concentration of 70 µg ml^{-1} to the assay reduced the adhesion by 53.7% for *L. gasseri* R and by 62.2% for *L. plantarum* S2.

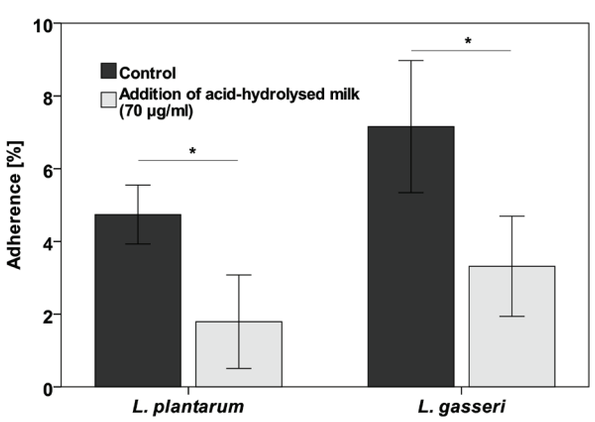

Fig. 1. Adhesion of *Lactobacillus gasseri* R and *Lactobacillus plantarum* S2 to the co-culture (Caco-2 and HT29-MTX) cell lines after hydrolyzed milk treatment. Values are expressed as percentage of bacterial adhesion, after 1 h of treatment with adding of hydrolyzed milk, compared to the control. Values are means ± SD of three independent assays. *significantly different ($P < 0.05$)

DISCUSSION

The adhesion to the intestinal mucosa is one of the required properties to select adequate probiotic microorganisms and regarded as a necessary assessment for colonization. A significant characteristic of the adhesion is the ability of probiotic strains to confer a health benefit. *Lactobacillus gasseri* R was shown to exhibit preferably adherence properties to co-culture compared to *Lactobacillus plantarum* S2, which was previously stated to possess significant adhesion abilities (García-Cayuela et al., 2014). Generally, the two lactobacilli strains selected for this study adhered quite well to the co-culture Caco-2 and HT29-MTX cells.

The effect of acid-hydrolyzed milk on reducing the adhesion might be caused by various mechanisms, starting from non-specific changes in pH, cationic bridging, to specific competence between the milk protein and glycoprotein on cellular surface based on hydrophobic interactions (Oliveira, 1997). Although the mechanism remains unknown, we suppose that similar interactions might be found in the lumen of the digestive tract and that the food matrix influences adhesion of food-derived probiotic cultures and thus reduces their potential *in vivo*.

The adhesion is mediated to a large extent by the presence of large molecular weight surface layer proteins (Rojas et al., 2002; Roos, Jonsson, 2002; Wang et al., 2008,) such as the fibronectin-binding protein (FpbA), mucin binding protein (Mub), and surface-layer protein (SlpA) or their homologs. These proteins are known to be expressed by *Lactobacillus* spp. and mediate the adhesion to epithelial cells to some extent and might interact with some hydrophobic milk proteins. However, this hypothesis needs to be confirmed in further studies. On the cellular site, adhesion may be affected by the expression of mucin. It is known that the presence of some lactobacilli upregulates genes for mucin expression (Mattar et al., 2002, Di Caro et al., 2005) and so does the presence of some milk constituents (Martínez-Maqueda et al., 2013). It is known that milk and some fraction of the bovine milk proteins affect the bacterial adhesion. This is of high interest during milk processing and several milk protein components, such as casein, caseinomacropeptide, albumin, etc. have been reported to reduce pathogen adhesion on stainless steel surfaces (Barnes et al., 1999) and to polystyrene surface (Janer et al., 2004). Not only surface-bound structures but also some other molecules present as part of the adhesion mechanism might either support or suppress adhesion. In the past, presence of adhesion-promoting factors of proteinaceous nature,

e.g. some bridging proteins, was proposed by some studies (Coconnier et al., 1992), but their action still remains dubious (Greene, Klaenhammer, 1994). Further studies confirm the possible role of polyphenols (Parkar et al., 2008) or oligosaccharides (Coppa et al., 2006). These compounds promote adhesion of some bacterial strains to epithelium or cultured epithelial cells, however, the mechanism also remains unknown.

Milk and milk proteins have been subject in many studies where adherence was considered as an initial step to pathogenesis (Barnes et al., 1999; Janer et al., 2004). Human κ-casein was shown to reduce the adhesion of bacteria to the tissue, namely, inhibition *Helicobacter pylori* adhesion to its target (Strömqvist et al., 1999) and BSA to prevent adherence of pathogenic *Listeria monocytogenes* (Almakhlafi et al., 1994). However, this preliminary study is focused on different aspects of the topic, showing possible role of milk proteins in the interactions between beneficial bacteria and epithelial model.

Human intestinal epithelial cell lines HT-29 and Caco-2 and mucus-secreting HT29-MTX cells provide an excellent system for characterizing how lactobacilli interact with a well-defined brush border and mucus and what constituents of food matrix may interfere.

CONCLUSION

Our results demonstrated that the acid-hydrolyzed milk has the potential to alter gut microbiota by modifying adhesion of selected probiotic *Lactobacillus* spp. strains to intestinal cells. The addition of acid-hydrolyzed milk to the assay reduced the adhesion in both used strains of lactobacilli. Consequently, the consumption of a diet rich in milk could affect the intestinal microbiota and improve microbiota imbalances. Further studies on the effect of hydrolyzed milk on the adhesion ability and viability of other bacteria will help better understand the interaction of its hydrolyzates with gut microbiota. Determining how these components contribute to probiotic action could lead to improved and more effective probiotic formulas and specific dietary recommendations for consumer's health.

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