Analysis of biofilm formation by intestinal lactobacilli

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Abstract

In this study, biofilm forming potential of intestinal *Lactobacillus reuteri* strains under different culture conditions was characterized by microtiter plate biofilm assays. Moreover, the spatial organization of exogenously applied *L. reuteri* L2/6 (a pig isolate) at the specific locations in gastrointestinal tract of monoassociated mice was investigated by FISH. We did not detect biofilm formation by tested strains in nutrient-rich MRS medium. On the contrary, a highly positive biofilm formation was observed in medium with lower accessibility to the carbon sources and lack of salts. The results obtained confirmed the significant role of Tween 80 and the quantity and nature of the sugars in the growth medium on biofilm formation. The omission of Tween 80 in MRS medium favored the formation of biofilm. Abundant biofilm formation was detected in the presence of lactose, galactose and glucose. However, gradual increase in sugars concentration triggered significant decrease in biofilm formation. In addition, conditions related to the gastrointestinal environment such as low pH, the presence of bile and mucins highly modulated biofilm production. This effect seems to be dependent on the specificity and properties of the medium used for cultivation. From the evidence provided by this study we conclude that the biofilm formation capacity of *L. reuteri* is strongly dependent on the environmental factors and culture medium used.

Key words: biofilm, *Lactobacillus*, probiotic, GIT, FISH

55 Introduction

56 The use of probiotics in the prevention and treatment of diarrheal diseases has been
57 proposed for many years (Corcionivoschi et al. 2010). The *Lactobacillus* species found in the
58 gastrointestinal tract (GIT) have received an important attention due to the possession of
59 health-promoting properties. They are commonly used as probiotics, which according
60 FAO/WHO are defined as live microorganisms, and when administered in adequate amounts
61 confer a health benefits to the host. The proposed mechanism of probiosis include effects on
62 composition, diversity and function of the intestinal microbiota by competition for the
63 nutrients, production of growth substrates or inhibition and modulation of intestinal immunity
64 (Preidis and Versalovic, 2009; Thomas and Versalovic, 2010). Preidis et al. (2012)
65 demonstrated a transient increase in phylogenetic diversity and evenness between taxa of the
66 fecal microbiome in mice 24 hrs after a single probiotic *L. reuteri* gavage. The diversity in
67 microbial communities was shown to be associated with increased ecological stability
68 (Eisenhauer et al. 2012).

69 One of the frequently exploited activities used to screen probiotic candidates is
70 adhesion to the mucus and epithelial cells or co-aggregation what is presumed to be essential
71 for the immune modulation, pathogen exclusion and enhanced contact with the gut mucosa
72 (Ouweland et al. 2002). In order to study the process of adhesion a variety of methods for the
73 quantitative measurement of adhesion such as quantitative culturing, radioactive labeling,
74 fluorescence *in situ* hybridization – FISH (Bernet et al. 1993; Mack et al. 1999; Mare et al.
75 2006), or *in vitro* model systems e.g. immobilized mucus models and cell-culture models
76 (Tuomola et al. 1998; Jonsson et al. 2001; Li et al. 2008) have been developed. However,
77 most studies show that probiotic lactobacilli do not permanently colonize GIT and are
78 beneficial to the hosts only for a brief periods after they have stopped being administered
79 (Tannock et al. 2000; Garrido et al. 2005).

Bacteria of the genus *Lactobacillus* in mice, rats, chickens and pigs are clearly autochthonous to the proximal GIT regions (Tannock 1992; Walter 2005a). The epithelial associations formed by lactobacilli in parts of the stomach, esophagus and crop show characteristics typical for the bacterial biofilms formation (Abee et al. 2011). These bacteria are firmly adhered to the surface of nonglandular, squamous stratified epithelium and embedded in a self-produced matrix of extracellular polymeric substance (Tannock et al. 2005; Walter et al. 2007). From an ecological point of view, living in a biofilm is a selective advantage that allows microbes to live in a protected niche allowing to interact directly with the host, and to prolong their survival in the GIT with higher metabolic and beneficial efficiency (Macfarlane 2008; Jones and Versalovic 2009). The findings suggest that several genetic and environmental factors influence the formation of these microbial structures within the GIT (Lebeer et al. 2007; Monds and O'Toole 2009). The numbers of hierarchically ordered genetic factors control the temporal development of biofilm formation and these genetic switches are normally activated in response to the changes of external stimuli i.e. shear stress, microbe–microbe interactions, presence of oxygen, host–microbe interactions (Marzorati et al. 2011).

Lactobacillus reuteri, a large component of GIT biofilm, has been very frequently used to identify bacterial factors that allow lactobacilli to persist in the GIT. Its genome harbor many genes encoding large cell surface proteins putatively involved in adhesion to the epithelium and biofilm formation (Walter et al. 2011; Frese et al. 2011). The cell surface proteins MucBP and Lar 0958 are involved in binding of *L. reuteri* to the mucus (Roos and Jonsson 2002; MacKenzie et al. 2010; Etzold et al. 2014). A large surface protein (Lsp) binding to the epithelium of forestomach have also been functionally characterized (Walter et al. 2005b). The EPS-producing enzymes, GtfA and Inu, of *L. reuteri* TMW1.106 have been shown to contribute to the cell aggregation, *in vitro* biofilm formation and colonization of the

mouse gastrointestinal tract (Walter et al. 2008; Sims et al. 2011). *L. reuteri* also transcribed with high frequency genes encoding pathways enhancing acid tolerance (urea degradation, arginine pathway, γ -aminobutyrate) and oxidative stress (glutathione synthesis) (Walter et al. 2011; Su et al. 2011, 2012). In addition, expression of pathways modifying the structure of the bacterial cell wall (DltA, cyclopropane-fatty-acyl-phospholipid synthase) had been correlated with acid resistance (Schwab et al. 2014). Walter et al. (2007) inactivated the *dltA* gene of *L. reuteri* 100-23 and consequently observed the reduction in strain competitiveness *in vivo*. Nevertheless the adherence was not affected. The LysM/YG proteins of *L. reuteri* show characteristics of proteins that can induce aggregation in lactobacilli, possibly by the N-terminal LysM domain binding to the peptidoglycan and C-terminal YG-motif to carbohydrate moieties (Frese et al. 2013).

Comparative genomics revealed that the evolution of *L. reuteri* resulted in host-confined phylogenetic lineages with a specialization to the particular hosts (Oh et al. 2010; Frese et al. 2011). Analyses performed suggest a fundamentally different genome evolution in the rodent isolate *L. reuteri* 100-23 and human isolate *L. reuteri* F275 (Frese et al. 2011). The ability of *L. reuteri* 100-23 to form epithelial biofilms in the mouse forestomach is strictly dependent on the strain's host origin. The host specificity of this strain appears to be mediated by a serine-rich surface adhesin Lr70902 (*FapI-like protein*) (Frese et al. 2013). Genome hybridization revealed that the sourdough isolate *L. reuteri* LTH2584 model had genome content very similar to that of model rodent isolate 100-23. Transcripts for the proteins that assured competitiveness of *L. reuteri* in cereal fermentation were also highly transcribed in biofilms, what corroborated with the proposed model of shared intestinal origin for the rodent and sourdough isolates (Su *et al.*, 2012). These observations strengthen the importance of habitat adaption when selecting lactobacilli for the health applications e. g. appropriate use of probiotics.

The present study evaluates the environmental conditions affecting biofilm formation of intestinal lactobacilli strains *in vitro* and subsequently investigates the spatial organization of exogenously applied *L. reuteri* L2/6 (a pig isolate) at specific locations in the gastrointestinal tract of monoassociated mice

Materials and methods

Micro-organisms and growth conditions

The strains of lactobacilli, used in the present study, were isolated in our Laboratory from the gut contents of healthy suckling piglets and pheasants. In the previous study the strains were identified by Maldi-Tof MS bacterial identification as *Lactobacillus reuteri*: L2/6, B2/1, B10/1, B1/1 and L26. The strains were characterized by inhibitory activity against pathogenic strains *in vitro* and by production of exopolysaccharides (Ryznerová 2013). Lactobacilli were routinely grown at 37°C in de Man-Rogosa-Sharpe agar (MRS; Carl Roth GmbH + CO. KG, Karlsruhe, Germany). Plates were incubated anaerobically (Gas Pak Plus, BBL Microbiology systems, Cockeysville, USA) at 37 °C for 48 h.

Genotypic identification of strains

Amplification of DNA was carried out using of species-specific primers: L-reu-1f (5-CAGACAATCTTTGATTGTTTAG-3) and L-reu-1r 5-CTTGTTGGTTTGGGCTCTTC-3 according to Dommels et al. (2009) and Garg et al. (2009). Aliquots of the PCR products were separated by horizontal 1 % agarose gel electrophoresis in TAE buffer pH 7.8 (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). A 100-bp DNA ladder (BioLabs, UK) was used as a reference. Gel was stained with GoodViewTM and visualized under UV light. For a positive control *Lactobacillus reuteri* DSM 17938 strain (BioGaia AB, Sweden) was used.

155 In vitro biofilm assay

156 The culture media assayed were standard MRS and modified PYG (peptone yeast
157 glucose) broths. PYG broth, by contrast to MRS medium, contains the lower amount of
158 glucose and no salts. Consists of (g/l): 15, bacteriological peptone; 5, enzymatic digest of
159 casein; 10, glucose; 10, yeast extract, pH 6.5. Other compounds tested in this study were:
160 Tween 80, glucose, sucrose, lactose, fructose, mannose, galactose, mucin from porcine
161 stomach type III and bile (bovine, minimum 50% bile acids, mixture of free and conjugated
162 bile acids, Sigma). The pH of the media was adjusted with 1.0 M HCl or 1.0 M NaOH before
163 autoclaving. One colony of the strain was transferred to a 5 ml of appropriate broth and
164 incubated for 18 h at 37 °C. Then the culture was centrifuged (10000 x g, 10 min), and the
165 sediment was resuspended in phosphate-buffered saline (PBS; g l⁻¹: 8 NaCl, 0.0002 KCl, 1.15
166 Na₂HPO₄, 0.2 KH₂PO₄; pH 7.4) to reach the McFarland standard 1 suspension that
167 corresponded to 1 x 10⁸ CFU ml⁻¹. A modified version of a previously described method
168 O'Toole et al. (1999) was used for assaying biofilm formation. A volume of 200 µl of the
169 culture was inoculated in a well of a polystyrene microtitre plate (Greiner ELISA 8 Well
170 Strips, 350µl, Flat Bottom, Medium Binding; Cruinn Diagnostics Ltd., Dublin, Ireland) and
171 incubated without shaking for 72 h at 37 °C. The biofilm formed in the well of the microtitre
172 plate was washed five times with 200 µl of PBS and dried at 25 °C for 40 min in an inverted
173 position. The remaining attached bacteria were stained for 30 min at 25 °C with 200 µl 0.1%
174 (W/V) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 V/V). The dye solution
175 was aspirated away, and the well was washed five times with 200 ml of distilled water. After
176 water removal and drying for 30 min at 25 °C the dye bound to the adherent biofilm was
177 extracted with 200 µl of the ethanol - acetone (80:20, V/V) mixture. Aliquot of 150 µl from
178 each well were taken and placed in a different microplate for OD determination at 570 nm
179 using a SynergyTM 4 Multi-Mode Microplate Reader (BioTek, USA). Each strain and/or

condition was tested in three independent experiments with eight biological replicates. Additionally, a sterile culture medium was always included as negative control to ensure that the influence on biofilm formation by mucin, and bile was not attributed to a nonspecific binding effect to crystal violet. Biofilm formation was categorized as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$) (Chaieb et al. 2007).

Animals and experimental design

The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol number 2860/12-221 for germ-free mice. The animals were handled and sacrificed in a humane manner in accordance with the guidelines established by the respective commission. Four-week-old female BALB/c germ-free mice were provided by the Institute of Microbiology, Nový Hrádok, Czech Republic. The mice were housed in gnotobiotic isolator for breeding of laboratory mice THF type 3271 101/97 (Ehret, Germany). Animals had continuous access to distilled autoclaved water in glass bottles and were fed ad libitum with complete sterile feed mixtures for mice holdings ST-1 (Velaz, Praha, Czech Republic). Twenty animals were divided into two groups. Experimental group was fed daily with 0.1 ml of strain *Lactobacillus reuteri* L2/6 containing 1×10^9 CFU/ml of bacteria by means of an intragastric feeding needle for a period of 14 days. Control group received 0.1 ml of sterile saline solution. At Day 14 mice were euthanized by sodium pentobarbital (86 mg/kg) followed by cervical dislocation. The GIT was aseptically removed and divided into sections consisting of stomach, duodenum and caecum.

Fluorescence in situ hybridization analysis

Samples of stomach, duodenum and caecum with contents were fixed immediately in Carnoy's solution for 4 hrs at 4°C to preserve the mucus layer. Prior to FISH analysis, serial paraffin sections of 7µm thickness were placed on positively charged slides (SuperFrost Plus, Braunschweig, Germany). FISH was performed as described by Lebeer et al. (2011).

Enzymatic mixture and hybridization buffer were used according to Czerwinski et al. (2012). For detection of *Lactobacillus* spp. probe Lab158 was used. Labeling was performed with TxRd (Texas red; sulforhodamine 101 acid chloride). Probe was HPLC purified and synthesized by Sigma Aldrich. For hybridization a probe at concentration 100µM diluted in ratio 1:25 into hybridization buffer was used. The hybridization was carried out overnight at 52°C in hybridization humid chamber. Samples were also stained with 4', 6-diamidino-2-phenylindole (DAPI) dye in concentration 2µl l⁻¹ during 10-15 min at 25°C for visualization of the other possible contaminant bacteria and tissue cells present in histological sections. Dried section slices were covered by long coverslips with Vectashield mounting medium to prevent photobleaching (Vector Laboratories, UK). The tissue sections were visualized by epifluorescence microscopy with Carl Zeiss Axio Observer Z1 microscope and images were analyzed with AxioVision Rel.4.6 software (Carl Zeiss, Belgium). For detection of probe Lab158 labeled with TxRd (excitation at 587nm and emission 647-670 nm - red color) filter set 64HE was used. To visualize tissue cells labeled with DAPI (excitation at 365 nm and emission at 445-450 nm - blue color) filter set 49 was used.

Statistical analysis

Data were analyzed with GraphPadPrism version 3.00 (GraphPad Software, San Diego California USA, www.graphpad.com.) by one-way analysis variance (ANOVA) followed by Tukey's multiple comparison test.

Results and Discussion

Bacterial biofilm formation is a step by step process by which the aggregation of bacteria form multicellular matrix consisting of a mixture of polymeric compounds including extracellular DNA, proteins and polysaccharides. Depending on the type of microorganisms,

age of biofilms and environmental conditions the EPS are main components determining the architecture of bacterial biofilms (Vu et al. 2009; Branda et al. 2005).

Microorganisms growing in biofilm frequently express phenotypes that are different from their nonadherent counterparts. For example, biofilm formed by lactobacilli cells exhibit greater resistance to the acetic acid and ethanol when compared to the planktonic cells (Kubota et al. 2008). Biofilm-growing probiotic strains have the ability to contribute to the enhanced thermotolerance and freeze-drying resistance (Cheow and Hadinoto 2013). *Lactobacillus reuteri* produce biofilms that retain functions potentially advantageous to the host such as modulation of cytokine output and production of antimicrobial agent reuterin (Jones and Versalovic 2009). When compared with the extensive studies performed on the biofilm formation of several microbial pathogens the factors and mechanisms controlling biofilm formation of probiotic bacteria have been so far poorly investigated. In fact, these above cited studies can provide insights how normal microbiota is maintained, what is a starting point and key for rational use of probiotics.

Several studies reported about the role of EPS production in biofilm formation by lactic acid bacteria. The EPS was described as a promoting factor for the intracellular interactions, autoaggregation and formation of microcolonies depending on the cellular and environmental conditions (Sturme et al. 2005; Lebeer et al. 2007, 2008; Walter et al. 2008a; Sims et al. 2011; Dertli et al. 2015). Strains of *Lactobacillus reuteri* can produce glucans and fructans of different linkage types (Kralj et al. 2004; Tieking et al., 2005; van Hijum et al., 2002, 2006). These compounds are synthesized from sucrose by a single action of extracellular enzymes termed glycosyltransferases, or in particular by glucosyltransferases and fructosyltransferases. Schwab et al. (2007) indicated that the impact of different glycosyltransferases on sucrose metabolism of *L. reuteri* is strain dependent and can be affected by the competitiveness of some *L. reuteri* strains in ecosystems where sucrose is

present. In strain *L. reuteri* TMW 1.106, GtfA accounts for sucrose utilization, metabolism, and growth of the organism. In contrast, FtfA of *L. reuteri* LTH5448 contributes to the sucrose turnover but alternative routes for sucrose metabolism are also functional for this strain.

Preliminary, *in vitro* investigations have shown that carbohydrate molecules are likely to be involved in biofilm formation by lactobacilli, while surface protein appears to initiate the adherence *in vivo* (Walter et al., 2005b; Frese et al. 2013). For example, it was reported that biofilm formation by *L. reuteri* TMW1.106 on glass surface was reduced after deletion of the *gtf* and *ftf* genes in comparison to wild type strain. However, the *in vivo* analysis of biofilm formation on forestomach epithelial cells revealed that there was no significant difference in biofilm formation properties of mutant or wild type strains (Walter et al., 2008b). In *L. reuteri* 100-21 the *ftf* mutant was able to form biofilms, on the forestomach epithelial surface, comparable to wild type strain (Sims et al. 2011). Biofilm formation analysis in *L. rhamnosus* GG demonstrated that EPS can be involved in this process but the effect of EPS on biofilm formation was more likely medium dependent (Lebeer et al. 2007). The level of EPS production as well as the structural composition can play a role in process of biofilm formation (Vu et al. 2009). Leber et al. (2007) observed increased *L. rhamnosus* GG biofilm formation by inulin due to stimulation of aggregation. These complex polysaccharides might be incorporated in the extracellular matrix, thereby enhancing biofilm formation.

To advance our understanding of how environmental factors may modify probiotic biofilm formation the EPS producing strains of *Lactobacillus reuteri*, isolated from the gut contents of healthy suckling piglets and pheasants, was tested. We determined how culture medium, some medium compounds and GIT simulated conditions (pH, bile, and mucin) may possibly influence biofilm formation. The strains subjected to the PCR analysis with species-specific primers for *Lactobacillus reuteri* (L-reu-1f and L-reu-1r) gave positive ~ 300 bp

amplicons (Fig. 1) what confirmed results obtained by Maldi-Tof MS from our previous study (Ryznerová 2013). Biofilm forming capacity was assessed with crystal violet dye, the standard stain for evaluation of biofilms formation (O'Toole et al. 1999). Crystal violet provides a good detection of biofilm mass. However, this dye does not identify biofilm viability due to the labelling both; the bacterial cells and extracellular matrix (Welch et al. 2012).

In tested strains of lactobacilli we have compared the capacity of biofilm formation in two culture media: the standard medium for lactobacilli (nutrient-rich MRS medium) and PYG medium (differs from MRS by lower availability of carbon sources and by salts deficiency). Strains showed similar rates of planktonic growth in the media used in the biofilm assay (data not shown). Fig. 2 shows production of biofilm by *Lactobacillus reuteri* strains in MRS broth and PYG broth under aerobic conditions. We did not detect biofilm formation of all tested strains in MRS broth. The absorbance levels were below 0.1 what were identical with negative controls readouts. Highly positive biofilm formation ($OD_{570} \geq 1$) was observed after 72 h incubation in PYG medium with lower availability of carbon sources and deficient in salts. However, the biofilm formation was different between the particular strains tested ($p < 0.001$; $p < 0.01$). Similar variability in biofilm formation on abiotic surfaces was also observed by Emanuel et al. (2010) in *L. rhamnosus*, Lebeer et al. (2007) in *L. rhamnosus* a *L. casei*, Jones a Versalovic (2009) in *L. reuteri*, and Ibarreche et al. (2014) in *L. sakei* a *L. curvatus* strains. We found that the quantity and nature of the sugars in the growth medium influenced biofilm formation in tested lactobacilli (Fig. 3). *L. reuteri* L2/6 strain formed biofilms in the presence of all tested sugars. However, *L. reuteri* B10/1 strain did not form biofilm in the presence of sucrose. Highly positive biofilm formation ($OD_{570} \geq 1$) was detected in the presence of lactose, galactose and glucose. The presence of fructose and mannose

303 resulted in less sizeable biofilm formation ($0.1 \leq OD_{570} < 1$). Gradual increase in sugars
304 concentration instigated significant decrease in biofilm formation ($P < 0.001$; $P < 0.01$; Fig. 4).

305 Several factors including sugar metabolism may lead to the modified biofilm
306 formation (Jin et al. 2004; Chai et al. 2012; Cai et al. 2013). In line with work presented here,
307 *L. rhamnosus* GG and vaginal lactobacilli were not able to form biofilm after 24 or 96 h
308 culture in standard culture media used for lactic acid bacteria where polystyrene microplates
309 were used (Lebeer et al. 2007, Terraf et al. 2012). More likely this ability depends on the
310 different strains and environmental factors involved. Martín et al. (2008) showed that vaginal
311 *L. jensenii* strains adhere strongly to the plastic substrate in standard MRS medium.
312 Variations in availability of fermentable carbon and C/N ratio can also modulate biofilm
313 formation. Lack of carbon sources in MRS medium (e.g. omission of glucose or
314 supplementation of lactose or sucrose) as well as of divalent cations (e.g. omission of
315 $MnSO_4$), which can affect bacterial adhesion, did not stimulate biofilm development by
316 vaginal lactobacilli (Terraf et al. 2012). On the other hand, *L. rhamnosus* GG formed biofilm
317 in modified TSB medium (Trypticase soy broth) with low C/N ratio and when glucose and
318 $MnSO_4$ were absent in MRS broth. However, no biofilm formation was evidenced in glucose-
319 depleted MRS by *L. rhamnosus* and *L. casei* strains (Lebeer et al. 2007). In *L. johnsonii*
320 FI9785 the omission of glucose and low relative ratio of C/N in growth medium reduced the
321 biofilm formation. The suppressive effect of glucose may be medium dependent or strain
322 specific. The removal of salts solution containing Mn^{2+} and Mg^{2+} from the MRS medium
323 significantly reduced biofilm formation of *L. johnsonii* FI9785 and its mutants possibly due to
324 the important role of these metal ions in cell metabolism (Dertli et al. 2013).

325 Our next analysis focused on the effect of Tween 80 on biofilm formation (Fig. 3)
326 where Tween 80, a nonionic surfactant and source of fatty acids, was absent in the MRS
327 medium. Additionally, the effect of this component was investigated after its addition to PYG

medium in the same concentration as found in MRS medium. The omission of Tween 80 from MRS medium favored the formation of biofilm ($p < 0.001$) by *L. reuteri* strains. Nevertheless, the biofilm formation was less distinct in MRS medium without Tween 80 when compared with standard PYG medium. Biofilm-repressing effect of Tween 80 was confirmed by addition of this component to PYG medium what resulted in significant reduction ($p < 0.001$) of biofilm formation. In agreement with our results Terraf et al. (2012) reported that the omission of Tween 80 surfactant in the standard culture media favored the formation of biofilm by biofilm forming vaginal lactobacilli. Nevertheless, the effect of Tween 80 appears to be medium and strain specific. Its omission does not seem to be sufficient to induce biofilm formation induced by *L. johnsonii* FI9785. In general, salts solution composition appeared to be more effective than Tween 80 on biofilm formation by *L. johnsonii* FI9785 (Dertli 2013). Biofilm-repressing effect of Tween 80 has not been confirmed in mTSB (modified Trypticase soy broth). The addition of Tween 80 to this medium did not reduce the biofilm formation generated by *L. rhamnosus* GG (Lebeer et al. 2007).

Formation of biofilms in all tested strains was affected by GIT environmental factors such as low pH, bile and mucin (Fig. 6). In contrast to the neutral pH low pH (pH3) stimulated biofilm formation ($p < 0.001$) in *L. reuteri* L2/6 and *L. reuteri* B10/1 cultured in MRS-TWEEN medium and in PYG medium. When compared to controls a significant increase ($p < 0.001$) in biofilm formation after addition of bile at the concentrations of 0.05 and 0.3% in MRS-TWEEN medium and 0.05% in PYG medium was observed. However, increase of bile concentration to 1%, in MRS-TWEEN medium, markedly decreased biofilm formation ($p < 0.001$) made by *L. reuteri* L2/6 strain, but did not affect biofilm formation created by *L. reuteri* B10/1. On the contrary a significant decrease ($p < 0.001$) in biofilm formation was detected in both tested strains where bile concentrations in PYG medium were 0.3 or 1 %. The addition of mucin into the MRS-TWEEN medium, at concentration of 2.5 g/l, significantly

induced biofilm formation by *L. reuteri* L2/6 ($p < 0.001$) and *L. reuteri* B10/1 ($p < 0.01$). The effect of mucin seems to be affected by culture medium. Mucin added to the PYG medium did not stimulated biofilm formation.

Based on available literature, bile induced the expression of specific adhesins such as EPS (Hung et al. 2006; Ruas-Madiedo et al. 2009) and fimbria-like appendages (Pumbwe et al. 2007) in some pathogenic and indigenous commensal bacteria. *In vitro* and *in vivo* models revealed a correlation between the EPS production and bile tolerance in bifidobacteria. For instance, in *B. breve* UCC2003, the EPS coat was essential for bile survival and *in vivo* mice gut colonization (Fanning et al., 2012). Nevertheless, the effect of bile on EPS production in lactobacilli is not well defined yet. Transcriptomic and proteomic data in *L. rhamnosus* GG point to a reduced production of enzymes involved in EPS biosynthesis in bile-containing environments (Koskenniemi et al. 2011), whereas in *L. delbrueckii* no variations were found following bile exposure where acquisition of stable bile-resistance correlated with significant overproduction of enzymes involved in EPS biosynthesis (Burns et al. 2010). It still remains to be determined whether bile exposure affects the composition and properties of the EPS layers. Other cell wall structures and characteristics may be affected in response to bile as well. In the case of *L. acidophilus* increased S-layer protein production was detected when cultured in the presence of 0.05% bile (Khaleghi et al. 2010). Biofilm formation generated by non-autoaggregating strains such as *L. plantarum* F44, *L. paracasei* F19 and *L. rhamnosus* 18243 grown in MRS broth with 0.5% taurocholic acid, 5% porcine bile or 0.25% mucin, could be correlated with an enhanced cell surface hydrophobicity. These non-autoaggregating strains grown in bile induced the autoaggregating behavior and facilitate biofilm formation (Ambalam et al. 2012). Walter et al. (2008b) investigated the ecological role of a glucosyltransferase (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 and a fructosyltransferase (FtfA) of *L. reuteri* LTH5448. *In vitro* experiments on isogenic mutants

revealed that GtfA was essential for the sucrose-dependent autoaggregation of *L. reuteri* TMW1.106 cells under acidic conditions, while inactivation of Inu slowed the formation of cell aggregates. Enhanced biofilm formation by *Lactobacillus plantarum* and *Lactobacillus rhamnosus* was also noted in a mucin-based medium (Filoche et al. 2004). Therefore the presence of polysaccharides may have a general ability to promote biofilm formation by lactobacilli.

L. reuteri is a gut symbiont colonizing stratified squamous epithelia in the upper intestinal tract of animals and is a stable part of colonic and vaginal microbiota of humans (Reid and Bocking 2003; Walter 2008a; Martín et al. 2008). *L. reuteri* is also a stable member of sourdough microbiota (De Vuyst et al. 2009). As it has already been mentioned the population genetic analysis and experimental approaches using *Lactobacillus*-free and monoassociated mouse models revealed that host-specific subpopulations exist among the members of *L. reuteri* species (Frese et al. 2011, 2013; Su et al. 2012; Schwab et al. 2014).

The extensive study Frese et al. (2013) explored the mechanisms that underlie colonization and biofilm formation in specific strains of the gut symbiont *Lactobacillus reuteri*. The biofilm formation of wild-type strains originated from rodent, human, pig, and chicken were evaluated on monoassociated mouse model. The confocal microscopy analysis confirmed the adherence of rodent strains to the forestomach epithelium and biofilm formation, while non-rodent strains were absent from the stratified squamous epithelium. In contrast to the previous findings on *Lactobacillus*-free mice (Frese et al. 2011) the non-rodent strains, in the absence of competitive microbiota, were able to colonize the gut lumen of germ-free mice in high numbers. Despite gut colonization these non rodent strains were not able to adhere to the forestomach epithelium or form biofilms. The complete absence of biofilm formation in the Lr70902 mutant strain of rodent *L. reuteri* 100-23 suggests that this is the adhesion step that confers host specificity. Homologues of Lr70902 can only be found in

rodent and pig isolates of *L. reuteri*, and these proteins may play a key role in exclusive binding to the epithelium of their respective hosts. The authors in theory suggest that the low sequence similarity between the proteins of rodent and pig strains might be accounted for the observed host specificity.

Su et al. (2012) indicated that sourdough isolates of *L. reuteri* emerge from the same phylogenetic line as rodent strains. It was reported that rodent *L. reuteri* isolates are capable of long-term persistence in food fermentation and that sourdough isolate LTH5448 can colonize the murine gastrointestinal tract of *Lactobacillus*-free mice and form population sizes comparable to the rodent strain *L. reuteri* 100-23 (Walter et al. 2008b). Since *Lactobacillus*-free mice are colonized efficiently only by *L. reuteri* of rodent origin, the colonization phenotype provides a strong evidence for the intestinal (rodent) origin of this sourdough isolate. Thus, the assignment of sourdough isolates to the intestinal origins does not represent recurrent contamination with raw material by rodent feces. As a consequence and due to the similarities between those two habitats (i.e., availability of carbon source) *L. reuteri* can stably colonize two environmental niches, the upper intestine of mammals that consume cereal-based foods and sourdough (Su et al. 2012).

We investigated the spatial organization of exogenously applied non-rodent *L. reuteri* L2/6 (primarily isolated from the pig gut contents) in stomach, duodenum and caecum of monoassociated mice. Hybridization with Lab158 probe suggested that *L. reuteri* L2/6 formed biofilm-like structures in mice forestomach (Fig 7). In the duodenum and caecum (Fig 8), mucus ceiling dispersed cells (bacteria in the outer portions of the mucus), occasionally found in small microcolonies appeared to be the colonization form of this strain. Based on the above data we can hypothetically suggest that the examined *L. reuteri* L2/6 strain isolated from pig's intestine could be originated from cereal-based food and have traits of rodent strains.

However, to confirm this hypothesis, a further investigation utilizing the genomic basis for the strain host specificity is required.

The study of factors associated with the production of biofilm will lead to the better understanding of mechanisms by which beneficial bacteria adapt to the stress and colonize natural environment to establish stabile and balanced microbiocenoses. This study provides a preliminary *in vitro* analysis of biofilm formation by potential probiotic strains of *L. reuteri*. Biofilm formation appears to be strain specific and modulated to the various extents by environmental factors. Further studies will be performed to understand in better detail the physicochemical and matrix composition properties of *L. reuteri*, and to identify the mechanisms involved in biofilm formation.

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Figure captions

Figure1 Electrophoresis of PCR products obtained with PCR primers L-reu-1f and L-reu-1r.

1, DNA Ladder; 2, positive control *Lactobacillus reuteri* DSM 17938; 3, negative control (without template); 4, strain L2/6; 5, strain B2/1; 6, strain B10/1; 7, strain B1/1; 8, strain L26

Figure 2 Production of biofilm by *Lactobacillus reuteri* strains (intestinal isolates) and *Lactobacillus reuteri* DSM 17938 in MRS and PYG medium.

The data are expressed as mean OD_{570 nm} with $\pm$ SEM values. The error bars represent standard deviations of eight biological repeats. The data shown are representative of three independent experiments.

Figure 3 Biofilm formation by of *Lactobacillus reuteri* L2/6 and B10/1 strains incubated with assorted sugars at 1% concentration.

The data express the mean OD_{570 nm} values $\pm$ SEM. The error bars represent standard deviations of eight biological repeats. The data shown are representative of three independent experiments. Abbreviations used: PYG - glucose; PYS - sucrose; PYL - lactose; PYF - fructose; PYM - mannose; PYGAL - galactose.

Figure 4 Biofilm formation by of *Lactobacillus reuteri* L2/6 strain incubated with different sugars concentration.

The data express the mean OD_{570 nm} values $\pm$ SEM. The error bars represent standard deviations of eight biological repeats. The data shown are representative of three independent experiments.

Figure 5 Production of biofilm by strains *Lactobacillus reuteri* L2/6 and B10/1 in MRS and PYG medium in the absence or presence of Tween 80.

The data express the mean OD_{570 nm} values $\pm$ SEM. The error bars represent standard deviations of eight biological repeats. The data shown are representative of three independent experiments.

Figure 6 The effect of simulated GIT conditions on biofilm formation by *Lactobacillus reuteri* L2/6 and *Lactobacillus reuteri* B10/1.

The data express the mean OD_{570 nm} values $\pm$ SEM; MRS_{TWEEN} - MRS medium without Tween; PYG – standard PYG medium

Figure 7 Spatial organization of *L. reuteri* L2/6 in the murine forestomach.

The samples from female BALB/c monoassociated mice were analyzed by FISH after administration of *L. reuteri* L2/6. Lab158 probe was TxRd labelled (red color) and DAPI was used as a counterstain (blue color). Panel A-show bacteria enclosed in an exopolymeric matrix, resembling biofilm-like structures (group with *L. reuteri* L2/6). Panel B - control group without *L. reuteri* L2/6. Green color shows autofluorescence of food particles. (100 x)

Figure 8 Spatial organization of *L. reuteri* L2/6 in the murine duodenum and caecum.

The samples from female BALB/c monoassociated mice were analyzed by FISH after administration of *L. reuteri* L2/6. Samples of duodenum (A) and caecum (B) were hybridized with the Lab158 probe TxRd labelled (red color). DAPI was used as a counterstain (blue color). Mucus ceiling dispersed cells or microcolony could be observed in the duodenum and caecum. The mucus layer is visible through autofluorescence.

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