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Adhesive Properties, Extracellular Protein Production, and Metabolism in the *Lactobacillus rhamnosus* GG Strain when Grown in the Presence of Mucin

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This paper examines the probiotic bacterium *Lactobacillus rhamnosus* GG, and how it reacts to the presence of mucin in its extracellular milieu. Parameters studied included cell clustering, adhesion to mucin, extracellular protein production, and formation of final metabolites. *L. rhamnosus* GG was found to grow efficiently in the presence of glucose, *N*-acetylglucosamine, or mucin (partially purified or purified) as sole carbon sources. However, it was unable to grow using other mucin constituents, such as fucose or glucuronic acid. Mucin induced noticeable changes in all the parameters studied when compared with growth using glucose, including in the formation of cell clusters, which were easily disorganized with trypsin. Mucin increased adhesion of the bacterium, and modulated the production of extracellular proteins. SDS–PAGE revealed that mucin was not degraded during *L. rhamnosus* GG growth, suggesting that this bacterium is able to partially use the glucidic moiety of glycoprotein. This study goes some way towards developing an understanding of the metabolic and physiological changes that *L. rhamnosus* GG undergoes within the human gastrointestinal tract.

Keywords: *Lactobacillus rhamnosus* GG, mucin, adhesion, extracellular proteins, glyceraldehyde-3-phosphate dehydrogenase

Lactobacillus rhamnosus GG (ATCC 53103) is one of the most studied probiotic strains, with sound safety assessment records [17], and is commonly included in dairy products. *L. rhamnosus* GG is characterized by a high tolerance to the acidic conditions in the stomach, and by a high capability of binding to human mucus [6]. These traits, among others, make *L. rhamnosus* GG an effective colonizer of the human gastrointestinal tract. In humans, *L. rhamnosus* GG has been used in the treatment of acute rotavirus, in antibiotic-associated diarrhea [2], in the treatment of inflammatory bowel disease [7], and in certain autoimmune diseases [10].

It has been speculated that some of the probiotic effects exerted by probiotic lactobacilli are mediated by its interaction with the intestinal mucosa, its mucus cover being considered an important site for bacterial adhesion and colonization [25]. This layer is composed of a protein backbone of mucin, strongly *O*-glycosylated at the Ser and Thr positions [4]. Once in the gastrointestinal tract, *L. rhamnosus* GG has been shown to favorably balance the microbiota, promoting gut-barrier functions, diminishing the production of carcinogenic compounds, activating the innate immune system, and enhancing adaptive immunity, especially during infections [22].

During its passage through the gastrointestinal tract, *L. rhamnosus* GG growth is affected by the presence of fermentable carbon sources, such as human mucins. Recently, it has been reported that an addition of mucin to the culture medium, at an estimated concentration level of that of the transverse colon (2.5 g/l), increased the biofilm formation of *L. rhamnosus* GG, which might enhance the implantation of the bacterium on the intestinal mucosa [12]. Similarly, the addition of mucin to the growth medium induces a strong mucus binding activity in *Lactobacillus reuteri* [9].

In the present paper, we report on the capability of *L. rhamnosus* GG to ferment different sugars present in the human gastrointestinal tract, and the effects of carbon

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sources on the binding capacity of *L. rhamnosus* GG to mucin, production of organic acids, and production of extracellular proteins.

Material and Methods

Mucin Purification

Partially purified porcine gastric mucin (Type III; Sigma-Aldrich, St. Louis, MO, U.S.A.) was further purified in order to eliminate bound sialic acid. Briefly, 10 g was stirred for 24 h at room temperature in 500 ml of 0.1 M NaCl in 0.02 M phosphate buffer (pH 7.8), with a few drops of toluene. Mucin was further purified after three precipitations with 60% (v/v) prechilled ethanol, utilizing well-documented methods [27]. The resulting pellet was resuspended and extensively dialyzed against mQ water. Finally, mucin was lyophilized and added to the growth culture when required.

Bacterial Culture and Growth Conditions

The *L. rhamnosus* GG strain (ATCC 53103, FlorVis GG, Novartis, Italy) was grown aerobically at 37°C in MRS broth (Becton Dickinson France SAS, Le Pont-De-Claix, France) in static cultures. Overnight cultures were centrifuged and pellets were washed twice in fermentation broth (MRSF) [10 g/l proteose peptone No. 3, 10 g/l beef extract, 5 g/l yeast extract (Becton Dickinson), 1 g/l ammonium citrate, 5 g/l sodium acetate, 0.1 g/l magnesium sulfate, 0.05 g/l manganese sulfate, and 80 g/l Tween]. In order to perform fermentation experiments, MRSF was supplemented with 10 g/l of one of the following sugars: fucose, glucuronic acid, *N*-acetylglucosamine, or sialic acid, as being the monomeric components of mucin. MRSF was also supplemented with 10 g/l lactose, 2.5 g/l mucin (Sigma-Aldrich), or 2.5 g/l purified mucin. MRSF supplemented with 10 g/l glucose was used as the positive control. Growth curves of *L. rhamnosus* GG in MRSF supplemented with 10 g/l glucose, 10 g/l *N*-acetylglucosamine, or 2.5 g/l mucin were performed by monitoring A_{600}. Agarose was incorporated into the medium [1.5% (v/v)] when required.

For the calculation of colony forming units per milliliter (CFU/ml), cultures were first decimally diluted in 1× Trypsin–EDTA solution (Invitrogen/Gibco, Karlsruhe, Germany), and incubated at 37°C for 3 min. Further dilutions were elaborated using PBS.

HPLC Analysis of Organic Acids

For organic acid determination, 20 ml of *L. rhamnosus* GG cultures, grown overnight in the presence of 10 g/l glucose, 10 g/l *N*-acetylglucosamine, or 2.5 g/l mucin, were collected by centrifugation (9,500 ×*g*, 15 min) and washed twice with 100 mM Tris-HCl, pH 5.6. The A_{600} was then adjusted to 0.6, and the cell suspensions were incubated in 100 mM Tris-HCl, pH 5.6, containing 25 mM glucose, for 4 h at 37°C with constant stirring. After incubation, cells were removed by filtration (0.45 µm) and the cell-free supernatants were diluted in 5 mM H_2SO_4. Twenty µl of the resulting solution was injected in an Aminex HPX-87H column (300×7.8 mm; BioRad, Hercules, CA, U.S.A.) and protected with a Micro-Guard precolumn (BioRad). Elutions were done isocratically in 5 mM H_2SO_4 at a constant flow rate of 0.6 ml/min at 35°C. Organic acids and residual glucose were detected in an UV detector (model 486) set to 215 nm, and in a differential refractometer RI detector 486 (Kontron Instruments Ltd, Bletchley, U.K.), respectively. Data acquisition was carried out using the Millenium 32 station software (Waters), and quantitative analyses were performed using the corresponding standard curves.

Enzymatic and Biochemical Pretreatments of Bacteria

Fifty-ml aliquots of cultures at the early stationary phase (usually after 11 h), which corresponds approximately to 5×10^{10} CFUs, as determined by plate counting, were harvested by centrifugation (10 min, 3,500 ×*g*, 4°C). Cells were washed twice with sterile citrate buffer, pH 5 (8.3 g/l NaCl, 0.26 g/l KCl, and 1.5 g/l Na_2HPO_4, adjusted to pH 5 with 1 M citrate), and resuspended in 5 ml of 2 mg/ml trypsin in PBS, or in 5 M lithium chloride (LiCl) in PBS (all reagents purchased from Sigma-Aldrich). Suspensions were incubated for 30 min at 37°C under gentle agitation, and cells were then harvested by centrifugation. Suspensions were finally plated for quantifying changes in cell viability.

Adhesion to Mucin

Adhesion assays were performed in 96-well polystyrene microtiter plates (F96 Maxisorp Immunoplate; Nunc, Roskilde, Denmark) using mucin as the matrix. One hundred µl of a mucin solution in PBS (10 mg/ml) was immobilized on the plate wells for one hour at 37°C, followed by overnight incubation at 4°C. Wells were washed twice with 200 µl of PBS and incubated with 20 g/l bovine serum albumine (BSA) (Sigma-Aldrich), for 2 h at 4°C. Wells were washed twice with 200 µl of PBS in order to eliminate non-bound BSA, and 100 µl of treated or non-treated bacterial cell suspensions, adjusted to cell counts of approximately 10^9 CFU/ml, was added to the wells and incubated at 37°C for 1 h. The amount of initial bacteria added was determined in all cases by plating out. After incubation, wells were washed five times with 200 µl of sterile citrate buffer to remove unbound bacteria. Two hundred µl of 0.5% (v/v) Triton X-100 was added to eliminate attached bacteria. The content of each well was thoroughly mixed with a micropipette, and 100 µl of the resulting suspensions was sampled and plated to obtain the CFU/well. Experiments were performed in quadruplicate and results were expressed as mean ± SD.

Protein Extraction, Manipulation, and Identification

Secreted proteins were obtained and resolved as previously described [20]. Briefly, the protein amount present in 5 ml of culture supernatant, collected in the stationary phase of growth, was precipitated with TCA, washed with acetone, and resolved by SDS–PAGE. This resulted in a protein yield ranging from 20 to 50 µg. Cytoplasmic extracts, used as controls, were obtained by sonication in ice using a Vibracell 75021 Ultrasonic Processor equipped with a 3 mm microtip (Fisher Scientific Bioblock, Illkirch, France) for 3–7 cycles of 3 min (amplitude 12); samples were then centrifuged (10 min, 10,000 ×*g*, 4°C) and the cell-free supernatant was kept. Gels were repeated three times from independent cultures. Selected bands were excised from the gels and digested with trypsin using standard protocols, the resulting peptide mixture being analyzed by tandem mass spectrometry (MS/MS). Data were acquired using a MALDI Q-Tof Premier mass spectrometer (Waters, Manchester, U.K.), with α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich) used as a matrix [3.6 mg/ml solution in acetonitrile:trifluoroacetic acid:water (50:0.1:49.9)]. Monoisotopic masses were corrected by using the pseudomolecular ion of Glu-fibrinopeptide as a lock mass (1570.6774 Da).

Proteins were identified using the MS/MS search module from MASCOT Software (http://www.matrixscience.com) against the

nonredundant protein NCBI database, using the monoisotopic masses derived from trypsinolysis. The following parameters were used: peptide charge +1, peptide tolerance ±0.1 Da, MS/MS tolerance ±0.1 Da, and one missed cleavage allowed for trypsin.

Mucin Degradation Assay in Solid Medium

Mucin and purified mucin were incorporated into solid MRSF at a concentration of 2.5 g/l, with or without 10 g/l glucose. Ten µl of overnight cultures of *L. rhamnosus* GG grown in MRSF supplemented with 2.5 g/l mucin, 2.5 g/l purified mucin, or 10 g/l glucose were inoculated in a spot on the surface of the agar plate in a Petri dish. Plates were incubated at 37°C aerobically for 72 h. The presence of mucin degradation (discolored halo) was revealed by Amido black staining (2× Amido black staining solution; Sigma).

Mucin Degradation Assay in Liquid Medium

Overnight precultures of *L. rhamnosus* GG were grown first in MRSF supplemented with 2.5 g/l mucin, 2.5 g/l purified mucin, or 10 g/l glucose. These cultures were used to inoculate [1% (v/v)] 10 ml of fresh MRSF supplemented with the same sugars. In addition, tubes containing mucin or purified mucin were in some cases supplemented with 10 g/l glucose. Cultures were grown at 37°C for 48 h, and mucin was precipitated with two volumes of cold ethanol at 4°C overnight. Mucin was resolved by SDS−PAGE and visualized using a specific glycoprotein staining kit (Thermo Fisher Scientific, Cergy Pontoise, France).

Extracellular Glyceraldehyde-3-Phosphate Dehydrogenase Activity Assay

GAPDH enzymatic activity was determined spectrophotometrically at room temperature by monitoring NADH apparition at 340 nm. Fifty µl of *L. rhamnosus* GG supernatants was incubated with 2 mM glyceraldehyde-3-phosphate, 1 mM NAD^+ in 1 ml of assay buffer (40 mM triethanolamine, 50 mM Na_2HPO_4, 5 mM EDTA, 0.1 mM DTT, adjusted to pH 8.6) (all reagents purchased from Sigma-Aldrich). One unit of GAPDH activity was defined as the amount of protein capable of generating 1 µmole of NADH per minute.

RESULTS

Growth of *L. rhamnosus* GG in the Presence of Different Carbon Sources

The growth of *L. rhamnosus* GG in MRSF supplemented with different sugars, measured as the CFU/ml, was monitored. The sugars chosen were monomeric components of human mucins, and are listed in the Material and Methods section. In addition, partially purified and purified mucin, glucose, and lactose were also tested. *L. rhamnosus* GG failed to grow, or grew weakly, in the presence of sialic acid, glucuronic acid, lactose, or fucose as the sole carbon source. On the contrary, *N*-acetylglucosamine sustained a growth rate comparable to that achieved with glucose (Fig. 1). Finally, *L. rhamnosus* GG was able to grow, although more slowly, in the presence of both partially purified and purified mucin.

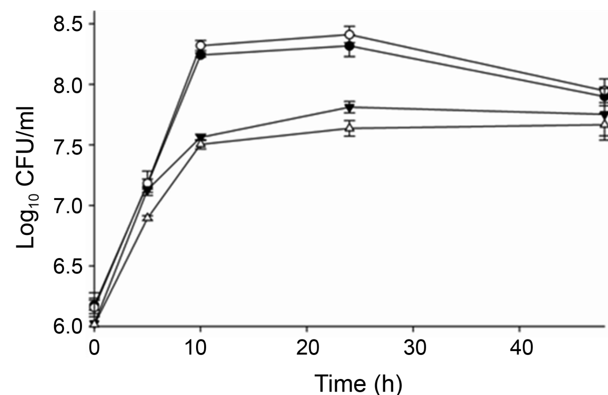

Fig. 1. Growth curves of *L. rhamnosus* GG in MRSF with glucose (○), *N*-acetylglucosamine (●), partially purified mucin (▼), or purified mucin (▽) as sole energy and carbon sources. Bars indicate the standard deviation of triplicate experiments.

In the presence of mucin, *L. rhamnosus* GG growth was characterized by the presence of cell clusters (Fig. 2). These clusters were easily disorganized with trypsin, suggesting a role for surface proteins in this phenotype. Trypsin digestions were systematically performed in all cultures prior to CFU/ml determinations in order to minimize the influence on the adhesion assays.

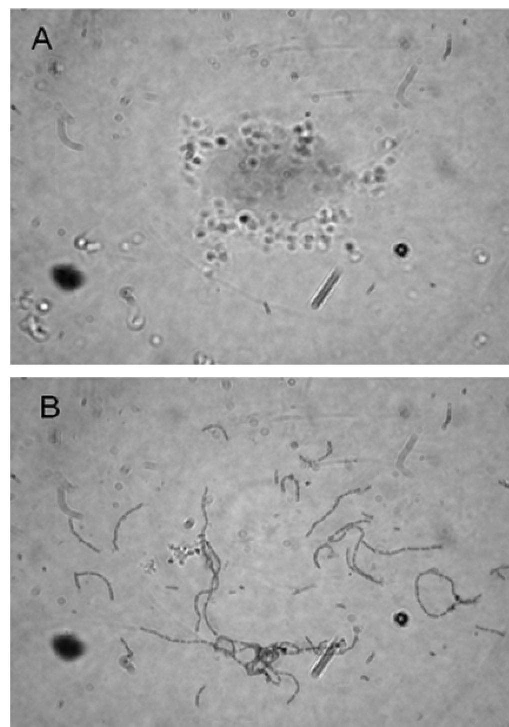

Fig. 2. Effect of trypsin on cell clusters of *L. rhamnosus* GG.
(**A**) Typical cell cluster present in overnight cultures of *L. rhamnosus* GG grown in the presence of mucin. (**B**) The same culture after treatment with 1× trypsin−EDTA solution as described in the Material and Methods section. Cells were visualized by classic Gram staining.

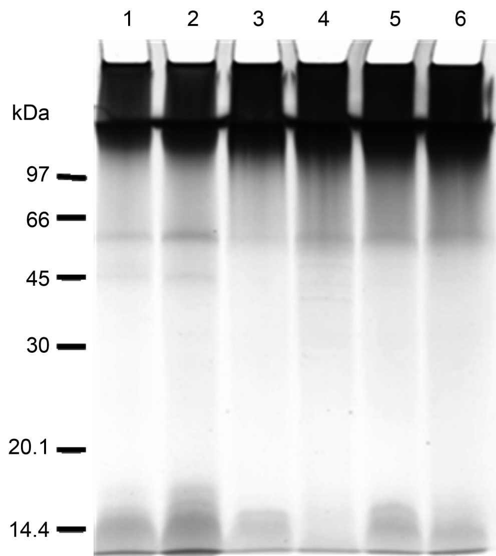

Fig. 3. Representative SDS–PAGE gel showing the absence of mucin degradation after an *in vitro* assay in liquid medium.

Same results were observed in solid medium after Amido black staining (data not shown). In all lanes, the protein precipitated in 10 ml of culture or solution, corresponding to approximately 40 µg of protein, and was further visualized using glycoprotein staining. Lane 1, partially purified mucin solution; lane 2, purified mucin solution; lane 3, culture supernatant corresponding to *L. rhamnosus* GG grown in the presence of partially purified mucin; lane 4, culture supernatant corresponding to *L. rhamnosus* GG grown in the presence of partially purified mucin plus glucose; lane 5, culture supernatant corresponding to *L. rhamnosus* GG grown in the presence of purified mucin; lane 6, culture supernatant corresponding to *L. rhamnosus* GG grown in the presence of purified mucin plus glucose. In all cases, mucin can be visualized as the dark area between stacking and separating gels.

L. rhamnosus GG Does Not Degrade Mucin

In order to establish whether this probiotic strain was able to degrade mucin *in vitro*, degradation assays in both liquid and solid mediums were conducted. Both assays showed that *L. rhamnosus* GG was unable to degrade the glycoprotein (Fig. 3). *L. rhamnosus* GG was not able to grow on an MRSF medium without a fermentable carbon sugar, so growth from any other compound of the fermentation broth could be excluded.

Production of Short-Chain Fatty Acids

The production of acetic and lactic acids from glucose by *L. rhamnosus* GG resting cells, previously grown in the presence of glucose, *N*-acetylglucosamine, or mucin, was measured by high-performance liquid chromatography (HPLC). As listed in Table 1, no acetic acid was produced by *L. rhamnosus* GG resting cells previously grown with glucose or *N*-acetylglucosamine as the sole carbon source. On the contrary, *L. rhamnosus* GG resting cells previously grown in mucin produced acetic acid from glucose. No variations in glucose consumption were observed among the different cell suspensions (around 5 g/l).

Table 1. Formation of acetic and lactic acids from cell suspensions of *L. rhamnosus* GG previously grown in the presence of different sugars as the sole carbon source.

Sugar	Acetic acid production (g/l)	Lactic acid production (g/l)
Glucose	-	7.05±1.49
N-acetylglucosamine	-	8.06±0.40
Mucin	0.64±0.21	7.53±0.24

Results are expressed as the mean ± standard deviations of at least three independent experiments.

Adhesion of *L. rhamnosus* GG to Mucin

L. rhamnosus GG binding was increased 2-fold after growing in the presence of mucin, when compared with glucose (Fig. 4). In addition, both LiCl and trypsin treatments drastically decreased adhesion, regardless of the sugar used. LiCl can extract surface proteins, since, as a chaotropic substance, it alters the electrostatic/hydrophobic interactions between surface proteins and the cell wall [13]. Whole-cell treatment with trypsin digests surface-associated proteins. These results suggest that surface-associated proteins play an important role in the binding of *L. rhamnosus* GG to mucin.

Changes in Extracellular Proteins as Affected by the Carbon Source

Fig. 5 illustrates the extracellular proteins produced by *L. rhamnosus* GG in the presence of the different carbon sources. Remarkably, production of extracellular proteins in the presence of mucin was lower. Extracellular proteins, which changed in the presence of a certain sugar, were identified by tandem mass spectrometry (MS/MS) (Table 2). Bands L2 and L9, identified as glucosamine-6-phosphate isomerase, and an as yet undesignated secreted protein

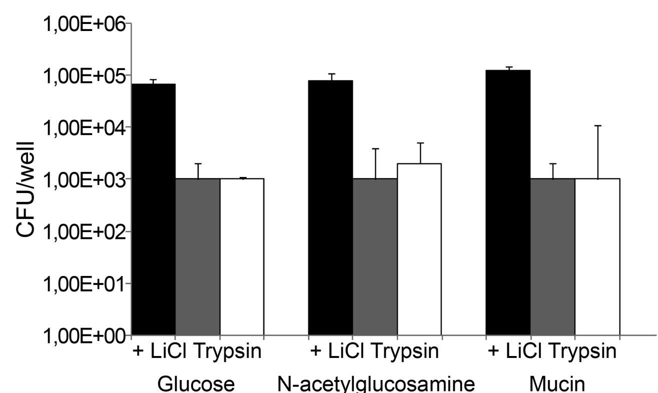

Fig. 4. Adhesion of *L. rhamnosus* GG to mucin after growing in MRSF supplemented with glucose, mucin, or *N*-acetylglucosamine as energy and carbon sources.

Drastic decreases in adhesion were observed after cell treatment with LiCl (grey columns) or trypsin (white columns) with respect to control cultures (black columns). Error bars represent the standard deviations for quadruplicated experiments.

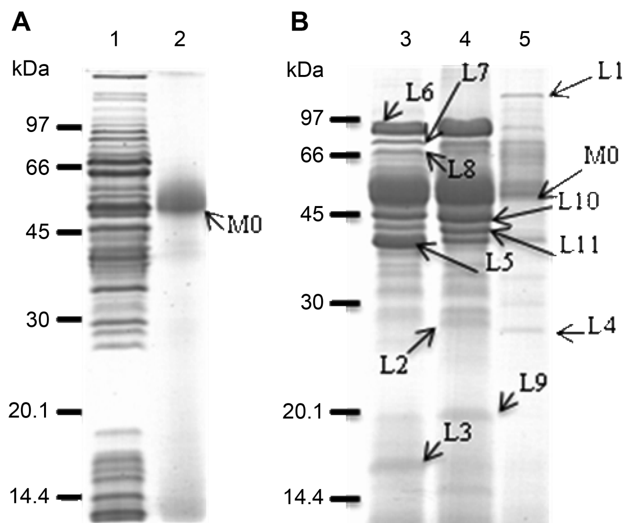

Fig. 5. Representative SDS−PAGE gels showing (**A**) *L. rhamnosus* GG cytoplasmic extract (lane 1) and serpin present in the MRS broth (lane 2); and (**B**) extracellular proteins produced in the presence of glucose (lane 3), *N*-acetylglucosamine (lane 4), or mucin (lane 5). In panel **B**, proteins precipitated from 5 ml of broth were solubilized in 40 µl of Laemmli buffer and separated utilizing the method detailed in the Materials and Methods section.

from *L. rhamnosus* GG, were only present when *N*-acetylglucosamine was the sole carbon source. Bands L5, L6, L7, L8, and L11, overproduced when *L. rhamnosus* GG was grown in the presence of glucose or N-acetylglucosamine, were identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cell wall-associated hydrolase, phosphoenolpyruvate-protein phosphotransferase, and an extracellular transcriptional regulator, respectively. Band L4, overproduced in the presence of mucin, was a hypothetical extracellular protein, which was in contrast with the results obtained for band L1, and also overproduced in the presence of mucin, but was not identified owing to poor peptide profiles yielded after trypsinolysis. Finally, band M0 was identified as a porcine serpin, one of the most abundant proteins in MRS broth.

Interestingly, extracellular GAPDH was shown to be metabolically active, and 0.018 ± 0.002 U/ml was detected in supernatants corresponding to cultures at the stationary phase of growth.

DISCUSSION

In the present study, the influence of the carbon source on adhesion to mucin, organic acid, and exported protein production in *L. rhamnosus* GG is presented. As mentioned above, the sugars used were chosen according to their physiological characteristics. In this way, fucose, glucuronic acid, *N*-acetylglucosamine, and sialic acid were chosen for being the monomeric components of intestinal mucins.

Mucin degradation has traditionally been considered as an undesirable trait for a probiotic bacterium [27]. Our experiments showed that *L. rhamnosus* GG can grow in the presence of purified or partially purified mucin, although it could not degrade such a glycoprotein, as already described [16]. This apparent contradiction could be explained by the fact that *L. rhamnosus* GG might use certain sugars of the glycosidic chains of the mucin, without leading to protein degradation. Recently, two *Bifidobacterium bifidum* strains,

Table 2. Extracellular proteins produced by *L. rhamnosus* GG.

Band[a]	Putative function	Organism	Protein IDs[b]	MM[c]	MS/MS[d]	MWS[e]
M0	Serpin B1	*Sus scrofa*	gi|417185	42.5	2	125
L1	N.I.[f]	-	-	-	-	-
L2	Glucosamine-6-phosphate deaminase	*L. rhamnosus* GG	YP_003172659.1	25.9	2	161
L3	Surface antigen (p40)	*L. rhamnosus* GG	YP_003169777.1	42.5	2	77
L4	Hypothetical protein LGG_00583	*L. rhamnosus* GG	YP_003170329.1	24.7	1	97
L5	Glyceraldehyde-3-phosphate dehydrogenase	*L. rhamnosus* GG	YP_003170679.1	36.6	3	168
L6	Putative cell-wall-associated hydrolase	*L. rhamnosus* GG	YP_003170070.1	49.6	5	173
L7	Phosphoenolpyruvate-protein phosphotransferase	*L. rhamnosus* GG	YP_003171566	63.1	2	134
L8	Putative cell-wall-associated hydrolase	*L. rhamnosus* GG	YP_003170070.1	49.6	4	174
L9	Predicted ORF	*L. rhamnosus* GG	YP_003169755.1	7.8	1	73
L10	Surface antigen (p40)	*L. rhamnosus* GG	YP_003169777.1	42.5	2	80
L11	Transcriptional regulator, LytR family	*L. rhamnosus* GG	YP_003170036.1	40.1	7	315

[a]Labels refer to bands in Fig. 1.
[b]Protein identifier (http://www.ncbi.nlm.nih.gov).
[c]Theoretical molecular mass.
[d]Fragmented MS/MS peptides allowing the identification of the protein.
[e]MOWSE score resulting from the ion MS/MS search against the nonredundant NCBI protein database [14]. All scores are statistically significant (p<0.05).
[f]Not identified, owing to a poor tryptic peptide yield.

among other *Bifidobacterium* species, have been shown to possess the capacity to degrade intestinal mucin through the transcriptional activation of extracellular glycosidases, allowing better growth in the presence of glycoprotein [15]. In agreement with our results, this degradation did not lead to protein degradation [27]. The fact that *L. rhamnosus* GG growth is maintained in the presence of purified mucin, prompted us to hypothesize that *L. rhamnosus* GG may use certain sugars from mucin, although the remote chance of the presence of energy-providing contaminants in mucin preparations cannot be excluded.

L. rhamnosus GG is also known for its ability to bind mucin [24], and can persist in the gastrointestinal tract one week after discontinuation of its oral administration [1]. It is known that strong mucus binding activity is induced in *Lactobacillus reuteri* when grown in the presence of mucin [9]. Similarly, growth of *L. rhamnosus* GG in the presence of mucin increased significantly its adhesion to the same target. Both LiCl and trypsin treatments diminished the adhesion of *L. rhamnosus* GG to mucin, showing a possible involvement of surface-associated proteins, as reported previously in *Lactobacillus plantarum* [23] and in other lactic acid bacteria [24]. The presence of surface proteins mediating bacterial adhesion to intestinal surfaces is well-known [11]. In fact, several mucin binding proteins have been predicted, by bioinformatic means, in the genus *Lactobacillus* [3]. Surface-associated proteins from the *L. rhamnosus* GG cell surface have already been identified, and included cell-wall-associated hydrolase, moonlighting proteins, transporters, and several uncharacterized cell wall and extracellular proteins [18]. Each of these surface proteins need be studied separately in order to identify their individual contribution to the adhesive properties of this bacterium.

Production of organic acids was measured using resting cells, which are cells suspended in a glucose-containing buffer previously grown in different physiological conditions [21]. Use of resting cells avoids interference with growth medium components (such as sodium acetate). Production of organic acids in *L. rhamnosus* GG was similar in resting cells previously grown in the presence of *N*-acetylglucosamine, or glucose. Under these conditions, the metabolism of the bacterium was homofermentative, and glucose was converted exclusively to lactic acid. On the other hand, resting cells of *L. rhamnosus* GG previously grown in mucin produced both acetic and lactic acids from glucose. *L. rhamnosus* is a facultatively heterofermentative species that can produce acetic acid by the action of phosphoketolase or by the activation of pyruvate-formate lyase [5]. Reactions catalyzed by both enzymes renders one molecule of acetyl phosphate, which is converted to acetic acid by acetate kinase, a reaction in which an extra molecule of ATP is generated by substrate-level phosphorylation. In the gastrointestinal environment, these extra ATP equivalents may be used for counteracting the harmful effects of certain substances, such as bile salts, or for improving adhesion and colonization. These results suggest that *L. rhamnosus* GG optimizes its energetic metabolism in the presence of mucin, as already described for *Bifidobacterium animalis* subsp. *lactis* IPLA 4549 [21]. Additional research, using radiolabeled mucin to identify nutrients derived from mucin supporting acetic acid production by LGG cells, is required.

Production of extracellular proteins in the presence of the different sugars was investigated. As pointed out in the introduction, some of these proteins might be key players in the interaction of *L. rhamnosus* GG with the human host [18]. The most abundant extracellular proteins produced in the presence of glucose or *N*-acetylglucosamine, cell-wall-associated hydrolase (CWH) (bands L6 and L8), surface antigen (bands L3 and L10), and GAPDH (band L5) were detected in lower amounts when *L. rhamnosus* GG was grown in the presence of mucin. Extracellular GAPDH mediates plasminogen binding and activation in several bacteria [19]. In *L. rhamnosus* GG, extracellular GAPDH was metabolically active, thus being a moonlighting protein with other functions in addition to its metabolic role in central metabolism [8]. GAPDH from *L. rhamnosus* GG is known to modulate intestinal epithelial cell growth and differentiation [26]. This includes the induction of epithelial cell growth, inhibition of apoptosis mediated by tumor necrosis factor, and reduction of damage caused by hydrogen peroxide in the epithelial barrier [22]. The end result was that band L4 was identified as hypothetical protein LGG_00583, whereas band L9, in the presence of *N*-acetylglucosamine, produced the predicted NCBI protein (ID YP_003169755.1). These novel extracellular proteins, some of them being secretions by *L. rhamnosus* GG, may play important roles in the molecular mediation process between this probiotic bacterium and a human host. A closer examination of the molecular mechanisms of these individual proteins will serve to shed light on the underlying mechanics of probiotic action within this strain.

To summarize up, *L. rhamnosus* GG can grow using mucin as a carbon source, although it is unable to degrade the glycoprotein. It cannot be excluded that other nutrients present in mucin preparations, such as small proteins, support the growth of LGG in mucin. In the presence of this glycoprotein, *L. rhamnosus* GG forms cell clusters, and optimizes its energetic metabolism with the production of acetate, which allows for the synthesis of an extra molecule of ATP. A decrease in protein secretion appears in the presence of mucin, which relates to growth of the bacterium on the intestinal mucosa. GAPDH was shown to be metabolically active, and together with the other uncharacterized extracellular proteins, may make good candidates for further examination of the functions of *L. rhamnosus* GG in the maintenance of gut homeostasis.

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