# Inactivation of Lactobacillus rhamnosus GG by fixation modifies its probiotic properties

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Inactivation of *Lactobacillus rhamnosus* GG by fixation modifies its probiotic properties

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Abstract

Background: Probiotics are microorganisms that have beneficial effects on the host and are safe for oral intake in a suitable dose. However, there are situations, in which the administration of living microorganisms poses a risk for immunocompromised host. The objective of this study was to evaluate the influence of several fixation methods on selected biological properties of *L. rhamnosus* GG, that are relevant to its probiotic action.

Results: Fixation of the bacterial cells with ethanol, 2-propanol, glutaraldehyde, paraformaldehyde, and heat treatment resulted in significant decrease of alkaline phosphatase, peroxidase and β-galactosidase activities. Most of the fixation procedures reduced bacterial cell hydrophobicity and increased adhesion capacity. The fixation procedures resulted in different perception of the bacterial cells by enterocytes, which was shown as changes in gene expression in enterocytes.

Conclusions: The results show that some procedures of inactivation allow to maintain a fraction of enzymatic activity. The adhesion properties of the bacterial cells were enhanced, but the response of enterocytes to fixed cells was different than to live bacteria. Inactivation allows to maintain and modify some of the properties of the bacterial cells.

**Key words**: adhesion, bacteria, enterocyte, gene expression, inactivation
Introduction

*Lactobacillus* sp. are an essential part of the human intestinal microbiota. Many isolates have proven probiotic properties. Probiotics are live microorganisms which, when administered in an appropriate dose, have beneficial effects on the host (Hill et al. 2014). They modulate many metabolic, immunological, and trophic functions of the host organism. Probiotics may be used to maintain the balance of the intestinal microbiota of healthy individuals, as well as to aid its recovery during and after treatment of diseases (Allen et al. 2003). Certain beneficial effects were also observed upon administration of probiotics to patients suffering from diseases not related to the digestive system, e.g. atopic dermatitis (Kalliomaki et al. 2001, Schroeder and Christophers 1989).

The molecular processes in which probiotic microorganisms perform their beneficial action are still not clearly understood. Probiotics can directly interact with the enterocytes by adhesion or influence the host by their enzymatic activity (Lebeer et al. 2008). The process of adhesion is a multistep and complex phenomenon involving both, physical and chemical interactions (Van Loosdrecht et al. 1990, Douillard et al. 2013). Surface proteins produced by bacteria play an important role in these interactions (Detmers et al. 1990). On the surface of *Lactobacillus rhamnosus* GG cells there are adhesive proteins similar to fimbriae (Segers and Leeber 2014). These structures were described as spaCBA heterotrimeric proteinaceous complex by Kankainen et al. in 2009. The SpaCBA protein complex is composed of subunits: SpaA, SpaB and SpaC. SpaA is the main subunit, forming the core of the pilus. SpaB subunit is located at the pilus base, and few SpaB subunits can appear on the pilus fibers. SpaC subunit is an mucus binding component which is located on the tip and throughout the pilus. SpaB is involved in formation of spaCBA pili and has similar mucus-binding capacity as subunit C, although much weaker (Leeber et al. 2011, Reunanen et al. 2012). The role of
SpaCBA as an adhesion molecule was confirmed, but it is also known that it takes part in the formation of biofilm and reduces the production of IL-8 by intestinal epithelial cells, thereby reducing the immune response directed against *L. rhamnosus* GG (Lebeer et al. 2011). The genes encoding SpaCBA fimbriae, as well as sortase participating in its secretion, are located in *L. rhamnosus* GG genome within LGGISL2 island (Sybesma et al. 2013). *L. rhamnosus* GG used in this study has the ability to produce SpaCBA adhesins, which has been confirmed in previous work (Markowicz et. al. 2014).

Cells of the lactic acid bacteria genus present in the gastrointestinal tract can also alleviate the symptoms of lactose intolerance by synthesizing the β-galactosidase enzyme (Li et al. 2012, Palacios et al. 2005). This important metabolic feature that allows lactose metabolism is reduced in the *L. rhamnosus* strain GG because of frameshifts in the antiterminator (*lacT*) and 6-phospho-galactosidase (*lacG*) genes (Kankainen et al. 2009). However, this strain retains minute β-galactosidase activity (Ling et al. 1994).

While probiotics are known for their beneficial influence and effective support of treatment of certain diseases, there are situations in which administration of living microorganisms poses a risk of sepsis to patients. So far, recorded cases concern mainly immunosuppressed individuals, individuals with severe cardiovascular diseases and newborn babies with intrauterine growth restriction (Land et al. 2005, Oggioni et al. 1998, Sadowska-Krawczenko et al. 2014, Spinosa et al. 2000). In such cases, administration of inactivated probiotics or their components might prove a safe alternative, as such cells have lost their ability to proliferate. However, it has been shown that the use of inactivated probiotic microorganisms may reduce the beneficial effects exerted by them on the host (Barrett et al. 2008, Kaila et al. 1996). The inactivation procedures, regardless of the used factors, always cause certain changes in the structure of the bacterial cell components.
The aim of this study was to evaluate the influence of inactivation methods on selected biological properties that are important for the probiotic activity of *L. rhamnosus* GG. Therefore, several fixation techniques (routinely used in experimental studies and in the preparation of antigens for immunization) were tested on *L. rhamnosus* GG cells. Following inactivation, major properties of the bacteria cells were tested: enzymatic activities, adhesion to enterocytes and modulation of selected genes expression in enterocytes.

**Material & Methods**

**Human and bacterial cells.**

The strain used for the study was *L. rhamnosus* GG (ATCC 53103) derived from a dietary supplement (Dicoflor, Vitis Pharma, Warsaw, Poland). Bacterial cultures were carried out in MRS medium (Biocorp, Warsaw, Poland) at 37°C in an anaerobic jar. Probiotic bacteria adapt their metabolism to the environmental conditions. Anaerobic growth was thus used to simulate conditions existing in the lower parts of the human gastrointestinal tract in order to facilitate proper interaction with intestinal epithelial cells. For the adhesion tests, the Caco-2 cell line was used. The cell line was obtained from the European Collection of Cell Cultures (ECEAC) and cultured in Dulbecco's modified Eagle's medium (Lonza, Verviers, Belgium) containing 10% (v/v) heat-inactivated calf serum (Lonza, Verviers, Belgium) and 1% non-essential aminoacids (Lonza, Verviers, Belgium). The cultures of Caco-2 cells were carried out at 37°C, at 10% (v/v) CO₂ in air.

**Fixation method.**

Six different fixation agents were used in various concentrations and treatment times. Solutions of chemical agents were prepared in phosphate buffered saline (PBS) with calcium and magnesium ions, pH 7.5 (Lonza, Verviers, Belgium), composed of (g/L): 0.130 CaCl₂ ·
2H₂O, 0.100 MgCl₂ · 6H₂O, 0.200 KCl, 0.200 KH₂PO₄, 8.000 NaCl, 2.160 Na₂HPO₄ · 7 H₂O.

Table 1. Fixatives and fixation conditions used in this study.

Before fixation, bacterial cells were washed twice with PBS and resuspended in the same buffer. 10 ml of bacterial suspension (optical density of 0.5 ± 0.05 at 600 nm) was centrifuged (16000 x g) and resuspended in equal volume of fixing solution followed by incubation at RT or 4°C, with agitation, for a suitable period of time (Table 1). Alcohol based fixatives were used ice-cold. High temperature fixation was carried out after resuspension of bacterial cell pellet in PBS. After completing the fixation procedure, bacterial cells were washed three times with PBS, and resuspended in the same buffer. PBS with calcium and magnesium ions was used in order to provide optimal conditions for ionic-interactions that may influence structure of bacterial cells. To verify that fixation method kills bacteria, 50 µl of fixed bacteria suspension at optical density of 0.5 ± 0.05 at 600 nm (which corresponds to 10⁸ cfu) was plated on MRS agar followed by incubation for 48 hours at 37°C under anaerobic conditions.

**Enzymatic activity of fixed bacteria.**

The evaluation of enzymatic activity of fixed bacteria was performed using colorimetric assays. Chromogenic substrates were used to detect alkaline phosphatase, peroxidase, and β-galactosidase activity of cells. Before the analysis, bacterial cells resuspended in phosphate buffer with calcium and magnesium ions, were normalized to OD₆₀₀ = 0.05 ± 0.005. Assays were performed in triplicates.

Alkaline phosphatase activity detection was carried out with p-nitrophenyl phosphate (pNPP; Calbiochem, Warsaw, Poland). 200 µl of pNPP solution (1 mg/ml) was added to 200 µl of fixed bacterial cells in suspension and incubated for 2 h at room temperature. The reaction was stopped by the addition of 100 µl of 3 M NaOH per reaction mixture. Bacteria were
centrifuged at 16000 x g for 1 min, 100 µl of supernatant was placed in 96-well plate for colorimetric measurement at 405 nm.

Peroxidase activity detection was done with 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich, Warsaw, Poland). The 200 µl of TMB solution (1 mg/ml) was added to 200 µl of fixed bacterial cells in suspension and incubated for 2 h at room temperature. The reaction was stopped by the addition of 100 µl of 10% (v/v) phosphoric acid per reaction mixture. Bacteria were centrifuged at 16000 x g for 1 min, 100 µl of supernatant was placed in 96-well plate for colorimetric measurement at 450 nm.

The β-galactosidase activity was measured after the incubation of cell suspension in PBS with 5 mg/ml of lysozyme, 25U/ml of mutanolysin and 1mg/ml of 2-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich, Warsaw, Poland). After 2 h of incubation at 37°C, bacteria were centrifuged at 16000 x g for 1 min, and 100 µl of supernatant was placed in 96-well plate for colorimetric measurement at 420 nm.

**MATH assay.**

Cell surface hydrophobicity of the bacteria was determined by measuring their adsorption to n-octane in a two-phase partitioning system based on the technique of Rosenberg (Rosenberg 2006). Briefly, cells were centrifuged at 1000g for 5 min and resuspended in PUM buffer (per litre, 22.2 g K₂HPO₄•3H₂O, 7.26 g KH₂PO₄, 1.8 g urea, and 0.2 g MgSO₄•7H₂O, pH 7.1) to an OD₆₀₀ of 0.5±0.05. A fraction of the cell suspension was added to a test tube containing n-octane at 4:3 ratio. Following vigorous vortexing for 5 min at room temperature the sample was allowed to settle for 30 min to permit separation of the layers. The aqueous phase was carefully extracted and examined spectrophotometrically at 600 nm. The decrease in optical density was used as a measure of cell surface hydrophobicity.

**Adhesion assay.**
Adhesion assay was performed as described previously (Schmidt et al. 2010), briefly: bacterial cells were grown in MRS broth (Biocorp, Warsaw, Poland) at 37°C in anaerobic conditions with methyl-[\(^{3}\)H]-thymidyne (5 µl/ml of broth, 60-90 Ci/mmol, 1mCi/ml; Hartmann Analytic GmbH, Braunschweig, Germany). After 18-20 hours of growth, the bacteria were washed twice with sterile Hank's buffered salts solution (HBSS) and resuspended in the same buffer. Caco-2 cells (passage no. 49-52) used in the adhesion assay were prepared in PTFE filter (0.4 µm pore size) inserts for 6-well tissue-culture dishes (Merck-Millipore, Warsaw, Poland) by inoculating 4x10\(^{5}\) cells/cm\(^{2}\). Twenty one-days-post-confluent Caco-2 cell monolayers were washed with HBSS, briefly, the growth medium was removed and the cell monolayer was overlayed with 1 ml of HBSS, which was removed after 1 min agitation. In the next step, bacterial cells suspension at concentration of approximately 5x10\(^{8}\) cfu/ml were added to each well in 2.0 ml (total volume) of HBSS and incubated at 37°C in an atmosphere of 10 % (v/v) CO\(_2\) in air. After 60 min incubation, the monolayers were washed three times with sterile, HBSS to remove free bacterial cells. The amount of adhered bacterial cells was estimated from the radioactivity remaining in the Caco-2 monolayer. Each assay was performed in triplicate. Radiolabeled bacteria in amount initially added for adhesion and washed Caco-2 monolayer with adhered radiolabeled bacteria were lysed in 0.9 ml of 1 % SDS, then 0.1 ml of 1 M NaOH was added and the lysate was incubated overnight at 60°C to complete lysis. The radioactivity of the lysed suspension was measured by liquid scintillation in Beckmann LS6500 after addition of Hionic-Fluor scintillation cocktail (Perkin-Elmer, Warsaw, Poland).

**RT-qPCR (Reverse Transcription quantitative PCR).**

Total RNA was extracted from the Caco-2 cells using TRI Reagent (Sigma-Aldrich, Warsaw, Poland) as recommended in the instruction manual and resuspended in DEPC-treated water. The RNA solution was DNase treated with TURBO DNA-free Kit (Life Technologies,
Warsaw, Poland) and used for cDNA synthesis with High-Capacity RNA-to-cDNA Kit (Life Technologies, Warsaw, Poland) as recommended by kits manufacturer. RT-qPCR reaction tubes contained 0.2 ng cDNA, 3µM each primer, and GoTaq qPCR master mix (Promega, Warsaw, Poland) in a final volume of 20 µl. Initial DNA denaturation and enzyme activation steps were performed at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 60 °C for 30 s, and elongation at 72°C for 30 s in a 7500 SDS Real-Time PCR thermocycler (Applied Biosystems, Warsaw, Poland). A melt curve analysis was performed to verify the specificity of the amplicons. Five gene targets were chosen to analyse selected gene expression in the enterocytes incubated with inactive *L. rhamnosus* GG cells:

Chemokine (C-C motif) receptor 6 (*CCR*),

Claudin 1 (*CLDN1*),

Occludin (*OCLN*),

Beta-1-catenin (*CTNNB1*),

Interleukin 8 (*IL8*).

Oligonucleotides used in this study for human gene transcripts were as follows:

CCR6 (GGGATCAATGAATTTCAGC, CAATCGGTACAAATAGCCTG),

CLDN1 (TTGGCATGAAGTGTATGAAG, ACCTGCAAGAAGAAATATCG),

OCLN (GGACTGGATCAGGGAATATC, ATTCTTTATCCAAACGGGAG),

CTNNB1 (CTTAAGGATGACTGCTG, AGAGTGAAAAGAACGATAGC),

IL8 (GTTTTTGGAAGGCTGAG, TTTGCTTGAAGTTTCACTGG),

Primers were designed using Primer 3 online tool (Untergrasser et al. 2012).

**Statistical analysis.**
Each assay was performed in triplicate. Differences between measurements were examined for significance by Student’s t-test after analysis of variance with Statistica software (v6; StatSoft). P > 0.05 was not statistically significant. RT-qPCR data was analysed using comparative Ct (ΔΔCT) method (Schmittgen and Livak 2008, Vandesompele et al. 2002) with global normalization algorithm (Vandesompele et al. 2002) in DataAssist Software ver. 3.01 (Applied Biosystems, Warsaw, Poland).

Results

**Varoius fixation treatments have dissimilar effect on *L. rhamnosus GG* viability.**

The efficacy of bacteria fixation was evaluated based on the results obtained after incubation of MRS agar plates for 48 h incubation, results are presented in Table 2. Complete fixation (no growth) was obtained after treatment of bacteria with formaldehyde (4 %, 24 h, RT), ethanol (65 %, 70 %, 75 %, all fixation times, 4°C), 2-propanol (65 %, 70 %, 75 %, all fixation times, 4°C), glutaraldehyde (1 %, 2 %, 4 %, all fixation times, RT), paraformaldehyde (1 %, 2 %, 4 %, all fixation times, RT), and after thermal inactivation (80°C). Bacterial cells recovery in form of rare colonies on agar plates was observed after fixation with formaldehyde for 10 min and 1 hour, and after application of a formaldehyde at concentration of 1% and 2% for 24 hours. For subsequent studies following fixation conditions were chosen: 4 % formaldehyde (24 hours), 70 % ethanol (20 min), 70 % 2-propanol (20 min), 1 % glutaraldehyde (10 min), 1 % paraformaldehyde (10 min), and thermal inactivation (80°C). These treatment methods ensured total microbial inactivation. Fixation conditions with aldehyde-based agents were chosen on basis of their best activity and shortest time of action. Alcohol-based fixatives were used at 70% since it is the concentration commonly used in disinfectants.

Table 2. Effectiveness of fixation procedures.
Fixation of bacterial cells partially retains their enzymatic potential.

To assess the influence of fixation on structure and function of bacterial proteins, losses of enzymatic activity of alkaline phosphatase, peroxidase and β-galactosidase were determined. Statistically significant (p < 0.05) decrease of all the enzymatic activities was observed following fixation of bacteria with every fixative used (Figure 1).

Alkaline phosphatase was the most fixation-resistant enzyme. After fixation with ethanol and formaldehyde, the activity of this enzyme in *L. rhamnosus* GG cells decreased 1.2 and 1.4-fold, respectively, compared to the initial activity. Paraformaldehyde and 2-propanol treatment reduced its activity by a factor of 2. Glutaraldehyde fixed cells showed almost 40-fold lower alkaline phosphatase activity than live cells, whereas heat treated cells – 90-fold lower.

Activity of peroxidase was also measured in this study to analyze the influence of fixation on the activity of bacterial proteins. Peroxidase activity of *L. rhamnosus* GG cells decreased 1.3-fold after treatment with formaldehyde. Ethanol and 2-propanol fixation reduced the enzymatic activity of this enzyme by a factor of 2, whereas glutaraldehyde reduced the activity almost 9-fold. Both paraformaldehyde and heat treatment of *L. rhamnosus* GG cells reduced peroxidase activity 10-fold.

β-galactosidase was the most vulnerable enzyme to fixative agents amongst the studied proteins of *L. rhamnosus* GG. The highest activity was retained in the case of glutaraldehyde fixation (2-fold reduction). Formaldehyde and ethanol fixation of the cells reduced the initial enzymatic activity by a factor of 5, whereas heat inactivation and 2-propanol, by a factor of 6. Paraformaldehyde fixed cells showed 10-fold reduction in β-galactosidase activity in comparison to live cells.
Figure 1.

Fig. 1. Relative peroxidase (PER), alkaline phosphatase (AP), and β-galactosidase (BG) activity of live *L. rhamnosus* GG cells (L), and after fixation with 4 % formaldehyde for 24h (F), 1 % glutaraldehyde for 10 min (G), 1 % paraformaldehyde for 10 min (P), 70 % ethanol for 20 min (E), 70 % 2-propanol for 20 min (I), and heat treatment for 20 min (T). Enzymatic activities were measured with chromogenic substrates with normalized amount of bacterial cells and absorbance measured at specific wavelengths. Graph shows average measurements and whiskers ±2·standard deviation.

**Fixation changes surface properties of *L. rhamnosus* GG cells.**

The change of cell surface hydrophobicity of *L. rhamnosus* GG was measured by adhesion to hydrocarbon after fixation treatments. Viable cells exhibited high affinity to n-octane, 91% of suspended bacteria partitioned to this organic phase. The highest reduction in hydrophobicity of the cell surface (61%) was observed after heat treatment. Paraformaldehyde fixation reduced cells surface hydrophobicity to 69%, whereas glutaraldehyde to 78%. Formaldehyde and ethanol fixation changed the property to ~85%. Treatment with 2-propanol did not decrease the cells surface hydrophobicity significantly.

**Fixation of *L. rhamnosus* GG cells increases their ability to adhere to intestinal epithelial cells.**
The measurement of enzymatic activities indicated that proteinaceous molecules possess varying susceptibility to fixation agents. A significant ($p < 0.05$) 2-fold increase in effectiveness of adhesion was observed after use of glutaraldehyde, paraformaldehyde, ethanol, and 2-propanol as fixation agents. Heat treatment and formaldehyde had no statistically significant effects on adhesion (Figure 2).

Figure 2.

Fig. 2. Adhesion efficiency of *L. rhamnosus* GG cells to differentiated enterocytes performed with live (L) bacterial cells, or cells fixed with 4 % formaldehyde for 24h (F), 1 % glutaraldehyde for 10 min (G), 1 % paraformaldehyde for 10 min (P), 70 % ethanol for 20 min (E), 70 % 2-propanol for 20 min (I), and heat treatment for 20 min (T). The adhesion values represent a percentage of bacterial cells that attached to differentiated Caco-2 human intestinal cells. Graph shows average of assays performed in triplicates, whiskers denote average±2·standard deviation, statistically significant changes ($p < 0.05$) are marked with asterisks (*).

**Fixation of bacterial cells changes their perception by intestinal epithelial cells.**

For the analysis of gene expression modulation, two fixation methods were chosen. Fixation of bacterial cells with 70 % ethanol for 20 min and 4% formaldehyde for 24 h. These treatment methods resulted in retention of the highest overall enzymatic activity, and therefore had presumably relatively small impact on protein conformation. Fixation with ethanol has shown to enhance adhesion ability. Formaldehyde is a commonly used inactivating agent in the preparation of vaccines. Moreover, ethanol fixation complies with ‘food grade’ requirements.

Introduction of *L. rhamnosus* GG, despite treatment, resulted in changes in expression of
genes associated with inflammatory response (CCR6, IL8) and sealing the intestinal epithelium barrier (CLDN1, CTNNB1, OCLN). Adhesion of both live and inactivated \textit{L. rhamnosus} GG cells stimulated the expression of CLDN1. Expression of CTNNB1 was also upregulated. However, formaldehyde-treated cells stimulated the expression of this gene more than living cells, while ethanol treatment decreased the extent of modulation. Interestingly, expression of CCR6 and OCLN was downregulated by both live and ethanol-fixed cells, whereas formalin-fixed cells caused stimulation of expression of these genes. In the case of IL8, live cells upregulated expression, while fixed cells downregulated it (Figure 3).

Figure 3.

Fig. 3. Expression changes of selected genes (IL8 – interleukin 8, CCR6 - chemokine receptor 6, CLDN1 – claudin 1, OCLN – ocludin, CTNNB1 – beta-1-catenin) in Caco-2 cells as measured by RT-qPCR after incubation with \textit{L. rhamnosus} GG cells fixed with 70% ethanol for 20 min (E), 4% formaldehyde for 24 h (F) in relation to Caco-2 not treated with bacteria (L). Statistically significant changes (p < 0.05) are marked with asterisks (*).

Discussion

Probiotic bacteria affect many processes in the human body through a number of interactions with intestinal epithelium. They are usually mediated by changes in gene expression in epithelial cells. Changed levels of some gene products may lead to the development of inflammation in the intestines (Daig et al. 1996, Mahida et al. 1992, Mazzucchelli et al. 1994) or affect the continuity and proper function of the intestinal barrier (Ivanov et al. 2004).
Therefore, it is advantageous to administer probiotic bacteria in cases of intestinal epithelial dysfunctions (especially caused by inflammatory processes) and imbalance of intestinal microflora. However, administration of live probiotic bacteria may threaten immunosuppressed individuals (Land et al. 2005). On the other hand, inactivation of bacterial cells can cause structural changes and denaturation of proteins. Thereby the impact of fixed bacteria on the intestinal epithelium may be limited.

Activities of alkaline phosphatase, peroxidase and β-galactosidase in *L. rhamnosus* GG cells were measured to evaluate the maintenance of protein functions within the fixed cells. Alkaline phosphatase and peroxidase are enzymes involved in metabolism of bacteria. β-galactosidase is important for the probiotic activity of *Lactobacillus* sp., its ability to break down lactose contained in dairy products results in reduction of lactose intolerance symptoms (Li et al. 2012, Palacios et al. 2005). Analyzes indicated that β-galactosidase activity, the advantageous feature of probiotic bacteria, was retained to some degree. It has been shown, that the ability to metabolize lactose is lost in strain GG of *L. rhamnosus* because of frameshifts in the antiterminator (*lacT*) and 6-phospho-galactosidase (*lacG*) genes (Douillard et al. 2013, Kankainen et al. 2009). However, the strain retains minute β-galactosidase activity, as it was shown by Ling et al. (1994) and in this study, that might be a result of the presence of β-glucosidase (BglB) which exhibits phospho-β-galactosidase activity. *L. rhamnosus* GG genome contains four *bglB* genes (Morita et al. 2009) that are homologous to beta-glucosidase A (BglS) from *Lactococcus lactis* subsp. *lactis* IL1403. Beta-glucosidase is the major enzyme in *L. lactis* IL1403 involved in lactose hydrolysis (Aleksandrzak-Piekarczyk et al. 2005). Therefore, 6-phospho-beta-glucosidase might have been responsible for the enzymatic activity determined in our experiment (that involved lysosyme treatement for cell permeation) with o-nitrophenyl β-D-galactopyranoside. Even if *L. rhamnosus* GG is not potent enough to alleviate lactose intolerance, fixation treatment of other strains with
methods used hereby could possibly have a similar effect. While, the use of 4% formaldehyde yielded lower losses in enzymatic activity than treatment with 70% ethanol, the latter method may be preferable as the alcohol is considered a ‘food grade’ reagent.

The results confirm that the external structural elements of *L. rhamnosus* GG include factors responsible for adhesion to the enterocytes. These elements are modified by the action of fixative agents, which alters the ability of adhesion. Based on the mechanisms of action of the fixating factors used, it should be expected that proteins were the molecules most influenced by them, whereas polysaccharides were not as prone to changes in conformation. Factors such as high temperature and alcohols are mainly used for disinfection. Temperature in the range of 60°C to 90°C causes denaturation of proteins, nucleic acid degradation and release of low molecular weight substances from cells (Russel 1991).

Increased efficiency of adhesion after application of denaturing agents (ethanol and 2-propanol) and potent cross-linking agents (paraformaldehyde, glutaraldehyde) is likely to be caused by large conformational changes within the proteins present in the outer structures of the bacteria. Alcohols work as coagulants and precipitate proteins. They also affect the cell membrane lipids, making it more rigid and impermeable (Ballesteros et al. 1998).

Aldehydes, such as glutaraldehyde, formaldehyde and paraformaldehyde are most often used to stabilize the tissue or cell cultures (Fox et al. 1985). Formaldehyde and paraformaldehyde reacts mainly with primary amines of proteins, forming a methylene bridge causing cross-linking of molecules that is partially reversible. At higher concentrations, formaldehyde also coagulates proteins. Glutaraldehyde is much more potent than formaldehyde, as it contains two aldehyde groups separated by flexible chain. Substances such as carbohydrates, lipids and nucleic acids are not chemically changed by these aldehydes (Kiernan 2000). However, the substances are thought to be trapped in a matrix of cross-linked proteins by both formaldehyde and glutaraldehyde. Formaldehyde cross-linking effect is
weaker than paraformaldehyde and glutaraldehyde, which probably translates into the least changed adhesion performance obtained in this study. The fact that cell structure has been least affected by the use formaldehyde may correspond to the highest activity of enzymes such as peroxidase or alkaline phosphatase. The fixation treatment of *L. rhamnosus* GG with 4% formaldehyde for 24 hours has probably the weakest effect on the structure of proteinaceous bacterial components.

Fixation of bacteria with formaldehyde or ethanol has impact on the level of expression of certain genes associated with inflammation and the formation of connections between enterocytes. Incubation of formaldehyde-fixed and ethanol-fixed bacteria with Caco-2 cells triggered a different effect on the expression of *CCR6* and *IL8* genes (involved in inflammatory process). The CCR6 chemokine receptor is overexpressed in bowel epithelial cells during chronic inflammatory bowel diseases, especially Crohn’s disease (Annunziato et al. 2007). In vivo tests showed that an increased level of CCR6 leads to less extensive inflammation in the intestine (Wang et al. 2009). Therefore admission of ethanol-fixed *L. rhamnosus* GG (which were shown to downregulate CCR6 expression in this study) may provide a protective function against inflammatory bowel disease. The molecular mechanism behind this phenomenon needs to be better understood.

Elevated levels of IL-8 have been widely observed in cases of chronic inflammatory bowel diseases (Daig et al. 1996, Mahida et al. 1992, Mazzucchelli et al. 1994). So far, numerous studies on the influence of pathogenic bacteria on IL-8 production by epithelial cells have been performed (Jung et al. 1995, Larsson et al. 1999, Schutle and Wattiau 1996). Data on the activation of cytokines production by probiotics are ambiguous and vary depending on the tested strain of probiotic bacteria. (Haller et al. 2000). It was proven that *L. rhamnosus* GG can reduce the high levels of IL-8 expression in Caco-2 induced with TNFα, flagellin or IL-1beta (Choi et al. 2008, Lopez et al. 2008, Zhang et al. 2006). However, in the
IL-8-uninduced Caco-2, live probiotic bacteria were shown to increase the expression of IL-8. Research of other groups indicated that the effect of inactivated *L. rhamnosus* GG depends on the inactivation procedure and may be either positive or negative (Wong and Ustunol 2006, Zhang et al. 2006). A preferred situation is when inactivated *L. rhamnosus* GG maintains the ability to reduce the level of IL-8 and, at the same time, causes decreased IL-8 expression (Zhang et al. 2006). Dehlink et al. (2007) described in their work, that live, formalin-inactivated and heat-inactivated probiotic strains can induce strain-specific production of IL-8 in Caco-2-leucocyte cocultures. In our study both, ethanol- and formaldehyde-fixed *L. rhamnosus* GG cells caused decrease in IL-8 expression in Caco-2 cells when compared to active *L. rhamnosus* GG cells. Differences in these results may be attributed to the fact, that the reaction of intestinal epithelial cells to bacterial signals may depend on interactions with immunocompetent cells (Haller et al. 2000). This result is interesting and requires detailed studies regarding to the anti-inflammatory properties of the probiotic.

CLDN1 (claudine 1), OCL1 (occludine-1) and CTNNB1 (β-catelin) are important proteins involved in the formation of tight junctions, cell-to-cell adhesion in epithelial and endothelial cells. Furthermore, β-catelin acts mainly as an intracellular and intercellular signal transducer. These proteins are responsible for maintaining normal function of intestinal epithelium (MacDonald et al. 2009, Morita et al. 1994, Morita et al. 1999, Niessen, 2007). Claudine-1 and ocludine-1 expression is known to be reduced in intestinal bowel disease (IBD) and its decreased expression is correlated with the intensity of the IBD symptoms (Ivanov et al. 2004). Increased expression of β-catelin in IBD promotes reduction of inflammation (Leavy 2012). The ability of probiotics to increase CLDN1, OCL1 and CTNNB1 levels may be beneficial to patients with intestinal barrier dysfunctions in inflammatory diseases (Henderson et al. 2011). Effect of *L. rhamnosus* GG cells incubation with Caco-2 on the expression of CLDN1 and CTNNB1 genes by Caco-2 cells was positive.
Both, live and ethanol- or formaldehyde-fixed *L. rhamnosus* GG cells caused a significant increase in the level of expression of these genes. The positive influence of formalin-fixed cells was more significant than the influence of live bacterial cells. However, live and ethanol-fixed *L. rhamnosus* GG cells caused the OCLN1 expression to decrease, whereas formaldehyde fixed *L. rhamnosus* GG cells caused a reverse effect. The differences may be related to the fact that the modulation of gene expression involves also secreted proteins and metabolites, which react differently with the bacterial cell surface under the influence of the fixing agents. The differences may be attributed to the fact that secreted proteins and metabolites are also known to modulate gene expression. These factors may interact differently with the bacterial cell surface under the influence of fixing agents.

There are a few studies on the use of inactivated probiotics in humans that relate almost exclusively to heat inactivated bacteria. Rayes et al. (2002) conducted a study on patients awaiting abdominal surgery who were supplemented with thermally inactivated probiotics. Post-operative infections occurrence decreased by a factor of three in the group receiving the probiotic as compared to the placebo group. There are a few clinical studies which suggest that inactivated probiotics are able to modulate human immune system by changing gene expression or by modulating host T-cell responses (Hirose et al. 2006, Van Baarlen et al. 2009). They can also alleviate the symptoms of asthma (Wheeler et al. 1997). Two studies showed that live lactobacilli are more effective than heat-inactivated probiotics in alleviating the symptoms of diarrhoea (Hirose et al. 2006), irritating bowel syndrome (Van Baarlen et al. 2009) and atopic disease (Kirjavainen et al. 2003). The beneficial effect of inactivated probiotics was maintained, however weakened, than in case of live microorganisms. Lactobacilli inactivated by ultraviolet radiation largely retained β-galactosidase activity and the ability to degrade lactose in the gastrointestinal tract. Thermally inactivated bacteria did not show this activity. It was suggested that probiotics do not have to
be alive to alleviate lactose intolerance. Inactivation method, however, must maintain the
impermeability of the bacterial cell wall (de Vrese et al. 2001). There are no clinical studies in
humans on the impact of probiotics inactivated by other methods than the high-temperature
inactivation. There are some studies showing negative effects resulting from consumption of
probiotics killed by high temperatures. Kirjavainen et al. (2003) found that the treatment of
atopic dermatitis with the inactivated *L. rhamnosus* GG was associated with diarrhea.

The majority of research shows that impact of inactivated probiotics on the human
body is weaker than the impact of live probiotics. Therefore, where it is possible, live
probiotics should be used. Microorganisms classified as probiotic are generally considered as
safe. In appropriate dose, they do not pose a risk to immunocompetent people (Hill et al.
2014). Their use, however, is risky for immunosuppressed patients and therefore
administration of inactivated probiotics is an alternative for people with a weakened immune
system.

The process of fixation with formaldehyde and ethanol does not cause loss of the
selected biological properties of *L. rhamnosus* GG tested in this study. These features of
bacteria are modulated positively or negatively, but do not disappear. Fixation of probiotic
bacteria cells eliminates the risk of bacteremia associated with providing them to the
organism, and, at the same time, maintains their functional characteristics. This fact is
important in cases, where probiotics are to be used in patients with immunosuppression.
Ethanol is a particularly prospective fixative, as it is commonly used as vehicle for a number
of pharmaceuticals. Moreover, fixation may prove to be a suitable technique for modification
of some of the properties of bacterial cells. Further studies need to be conducted in order to
explain the molecular basis of fixative-induced changes of the features of bacterial cells with
regard to human health.
Author contributions: CM contributed to all experimental procedures, data collection and interpretation, drafting and writing of the manuscript. PK contributed to language revision and substantive correction. WG critically read the manuscript. MS contributed to conception of the idea, primer design, data analysis and interpretation, and writing of the manuscript. All read and approved the manuscript.

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Table 1. Fixatives and fixation conditions.

<table>
<thead>
<tr>
<th>Fixing agent</th>
<th>Concentration</th>
<th>Temperature</th>
<th>Fixation time</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>4%; 2%; 1%</td>
<td>RT (22°C)</td>
<td>24 h; 1 h; 10 min</td>
<td>Rutala et al. (2008), Dehlink et al. (2007), MacDonnel and Russell (1999)</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>4%; 2%; 1%</td>
<td></td>
<td></td>
<td>Rutala et al. (2008), MacDonnel and Russell (1999)</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>2%; 1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>75% 70% 65%</td>
<td>4°C</td>
<td>24 h; 1 h; 15 min</td>
<td>Rutala et al. (2008), MacDonnel and Russell (1999)</td>
</tr>
<tr>
<td>2-propanol</td>
<td>75% 70% 65%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High temperature</td>
<td>-</td>
<td>80°C</td>
<td>20 min</td>
<td>Ouwehand et al. (2000), Dehlink et al. (2007), Ostad et al. (2009)</td>
</tr>
</tbody>
</table>
Table 2. Effectiveness of fixation procedures.

<table>
<thead>
<tr>
<th>Fixing agent</th>
<th>Concentration</th>
<th>Time</th>
<th>[cfu/ml]*</th>
<th>Log reduction of viability**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>1%</td>
<td>10 min</td>
<td>uncountable</td>
<td>N/D***</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>1 h</td>
<td>uncountable</td>
<td>N/D</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>24 h</td>
<td>uncountable</td>
<td>N/D</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>10 min</td>
<td>0,6 x 10^2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>1 h</td>
<td>4,6 x 10^2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>24 h</td>
<td>uncountable</td>
<td>N/D</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>10 min</td>
<td>2x10^2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>1 h</td>
<td>1,8 x 10^2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>24 h</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>1%; 2%;4%</td>
<td>10 min; 1 h; 24 h</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>1%;2%</td>
<td>10 min; 1 h; 24 h</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>65%; 70%; 75%</td>
<td>20 min; 1 h; 24 h</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>2-propanol</td>
<td>65%; 70%; 75%</td>
<td>20 min; 1 h; 24 h</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>80ºC</td>
<td>-</td>
<td>20 min</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

* [cfu/ml] – colony forming units (bacterial cells) per 1ml of bacterial suspension, amount of bacterial cells in the suspension plated on MRS agar was 10^8 cfu/ml
** decrease in the number of living bacterial cells in suspension
*** N/D – not determined, numerous colonies, impossible to count
Fig. 1. Relative peroxidase (PER), alkaline phosphatase (AP), and β-galactosidase (BG) activity of live *L. rhamnosus* GG cells (L), and after fixation with 4 % formaldehyde for 24h (F), 1 % glutaraldehyde for 10 min (G), 1 % paraformaldehyde for 10 min (P), 70 % ethanol for 20 min (E), 70 % 2-propanol for 20 min (I), and heat treatment for 20 min (T). The enzymatic activities were measured with chromogenic substrates with normalized amount of bacterial cells and absorbance measured at specific wavelengths. Graph shows average measurements and whiskers ±2·standard deviation, statistically significant changes (p < 0.05) are marked with asterisks (*).

Fig. 2. Adhesion efficiency of *L. rhamnosus* GG cells to differentiated enterocytes performed with live (L) bacterial cells, or cells fixed with 4 % formaldehyde for 24h (F), 1 % glutaraldehyde for 10 min (G), 1 % paraformaldehyde for 10 min (P), 70 % ethanol for 20 min (E), 70 % 2-propanol for 20 min (I), and heat treatment for 20 min (T). The adhesion values represent a percentage of bacterial cells that attached to differentiated Caco-2 human intestinal cells. Graph shows average of assays performed in triplicates with whiskers denoting average±2·standard deviation, statistically significant changes (p < 0.05) are marked with asterisks (*).

Fig. 3. Expression changes of selected genes (IL8 – interleukin 8, CCR6 - chemokine receptor 6, CLDN1 – claudin 1, OCLN – ocludin, CTNNB1 – beta-1-catenin) in Caco-2 cells as measured by RT-qPCR after incubation with *L. rhamnosus* GG cells not treated with bacteria (L), fixed with 70% ethanol for 20 min (E), 4% formaldehyde for 24 h (F) in relation to Caco-2. Statistically significant changes (p < 0.05) are marked with asterisks (*).