

# Exogenous transglutaminase improves multiple-stress tolerance in *Lactococcus lactis* and other lactic acid bacteria with glutamine and lysine in the cell wall

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## Abstract

**Objective** To increase the resistance of ingested bacteria to multiple environmental stresses, the role of transglutaminase in *Lactococcus lactis* and possible mechanisms of action were explored.

**Results** *L. lactis* grown with transglutaminase exhibited significantly higher resistance to bile salts, stimulated gastric juice, antibiotics, NaCl, and cold stress compared to the control (cultured without transglutaminase), with no negative influence on cell growth. Transmission electron microscopy revealed that the cell walls of *L. lactis* cultured with 9 U transglutaminase/ml were approx. 1.9-times thicker than the control. Further analysis demonstrated that the multi-resistant phenotype was strain-specific; that is, it occurred in bacteria with the presence of glutamine and lysine in the peptidoglycan.

**Conclusion** Supplementation of culture media with transglutaminase is an effective, simple, and inexpensive strategy to protect specific ingested bacteria against multiple environmental challenges.

**Keywords** Transglutaminase · Cell wall · Lactic acid bacteria · *Lactococcus lactis* · Multiple-stress tolerance

## Introduction

*Lactococcus lactis* is extensively used in the manufacture of cheeses. It is also used as a live vector for the mucosal delivery of therapeutic proteins (Bahey-El-Din et al. 2010). Due to its economic importance, *L. lactis* is the best characterized member of lactic acid bacteria (LAB) and is considered the model organism of this group (Pontes et al. 2011). It is widely acknowledged that ingested microbes, such as live vectors and probiotics, must be present in a sufficient concentration at mucosal sites to achieve beneficial effects. However, during food processing and passage through the digestive system, ingested bacteria are exposed to a variety of environmental stresses, such as osmotic stress, low temperatures, acids, bile salts and antibiotics. These stresses can influence the physiological activities of cells and reduce cell viability and can be bottlenecks for guaranteeing final product efficacy. It is therefore essential for ingested bacteria to have mechanisms which protect against these stresses.

Our previous studies have shown that the recombinant transglutaminase-producing *L. lactis* exhibited a thickened cell wall relative to the control strain *L. lactis* NZ9000 (Fu et al. 2005). Some studies have demonstrated that cell wall thickness may be responsible for the resistant phenotype of some microbes. For example, Kawai et al. (2009) reported that cell wall thickness may be responsible for acriflavin resistance

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in the methicillin-resistant *Staphylococcus aureus* isolate KT24. Although transglutaminase has been widely used in the food industry (Gaspar and de Goes-Favoni 2015), information regarding the beneficial effects of transglutaminase on microbial stress tolerance is not available. Thus, we set out to explore transglutaminase's role in *L. lactis* NZ9000 environmental resistance and possible mechanisms of action. In this study, we compared the tolerance of *L. lactis* NZ9000 (cultured with and without transglutaminase) for bile salts (0.04 %, w/v), stimulated gastric juice (pH 3.0, 1.5 g pepsin/l), NaCl (range 1–4 %, w/v), antibiotic stress (three different antibiotics), and low temperature stress (4 and  $-20$  °C). The mechanism, which explains why transglutaminase-producing cells exhibit improved multiple stress tolerance, was also investigated.

## Materials and methods

### Bacterial strains and culture conditions

*Lactococcus lactis* subsp. *cremoris* NZ9000(pNZ8148) was a gift from Dr Jeroen Hugenholtz, Wageningen Centre for Food Sciences and NIZO Food Research, Netherlands. *Lactococcus lactis* subsp. *lactis* NFL and *Lactobacillus fermentum* 415 were used to verify the possible mechanisms involved in the multi-resistant phenotype of transglutaminase-treated cells and preserved in the Food Microbiology Laboratory at Anhui Agricultural University. *L. lactis* NZ9000 was grown statically at 30 °C in M17 broth (Difco) containing 0.5 % (w/v) glucose (refer to as GM17 broth). Chloramphenicol (10 µg/ml) was added to the media as a selection marker for the plasmid pNZ8148. *L. lactis* NFL was grown statically at 30 °C in MRS broth (Difco) and *L. fermentum* 415 was grown aerobically (150 rpm) at 37 °C in MRS broth. Inocula of these strains were transferred from  $-70$  °C stock cultures to the appropriate culture medium and incubated statically overnight as a preculture.

### Stress tolerance assays

For the bile salts stress assay, *L. lactis* NZ9000 was inoculated at 5 % (v/v) preculture and grown in GM17 broth containing 0, 3, 6, 9, and 12 U transglutaminase/ml (Ruibio), respectively, and incubated to stationary

phase (8 h). The cultures were then harvested by centrifugation (3 min at  $10,000\times g$ ), washed twice with saline, and resuspended in saline, with or without addition of sodium taurocholate and incubated at 37 °C. After 2 h, cells were pelleted by centrifugation (3 min at  $10,000\times g$ ) and washed again with saline to remove the residual bile salts. Cells were resuspended in the same volume of saline. Cells not subjected to bile stress were used as controls. 100 µl of resultant cell suspensions was used for serial dilution and cells were spotted onto the surface of GM17 agar plates. Plates were incubated at 30 °C for 48 h, and CFU were counted. The survival rate was calculated as: survival rate (%) =  $(N_s/N_c) \times 100$  %, where  $N_s$  is the total viable count of strains under stress conditions and  $N_c$  the total viable count under control conditions.

Assays to measure the resistance to simulated gastric juice and antibiotics were performed similarly, replacing bile salts with simulated gastric juice, 500 µg erythromycin/ml, 800 µg cefazolin/ml or 500 µg tetracycline hydrochloride/ml. Simulated gastric juice was prepared by dissolving pepsin from porcine gastric mucosa (Amresco) in 0.5 % (w/v) sterile saline to 1.5 g/l and adjusting the pH to 3. The effects of NaCl were examined in GM17 medium with NaCl at 1, 2, 3, 4 % (w/v). Precultures were inoculated and growth for 9 h statically; the culture was centrifuged at  $10,000\times g$  for 3 min, and the cells were resuspended in the same volume of saline to determine the survival ratio.

For the cold stress assay, cells were grown to stationary phase, collected by centrifugation (3 min at  $10000\times g$ ), washed with saline to remove the residual medium, pelleted again, and resuspended in 10 ml fresh GM17 medium. The suspension was evenly divided into 1 ml aliquots, and placed at different temperatures (4 °C,  $-20$  °C) for cold treatment. Samples were collected at day 3, 6, 9, 12 and 15 for survival rate measurement, as described above. Tolerance of *L. lactis* NFL and *L. fermentum* 415 to various stress conditions were carried out similarly as described above (detailed in the individual figure legends).

### Transmission electron microscopy

*L. lactis* N9000, grown in the presence or absence of 9 U/ml transglutaminase to the stationary-phase (8 h), was fixed in glutaraldehyde (2.5 %, v/v) in 10 mM

sodium phosphate buffer, pH 7.2) for 1 h at 4 °C and then rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.2). The specimens were prepared on copper grids coated with a thin carbon film and post-stained with 1 % phosphotungstic acid. All preparations were observed with an H-7000 electron microscope (Hitachi, Japan).

#### Determination of the amino acid composition of cell wall peptidoglycan

Isolation of *L. lactis* NZ9000, *L. lactis* NFL and *L. fermentum* 415 peptidoglycan was performed according to the method outlined in Wu et al. (2013). Peptidoglycan was hydrolyzed by 6 M HCl at 110 °C for 24 h. Quantitative analysis of amino acids was performed using an amino acid analyzer (Hitachi, Japan). The glutamine content of the peptidoglycan was assayed according to the method outlined in Kuhn et al. (1996).

#### Statistical analysis

Results are expressed as mean of triplicate determinations  $\pm$  standard deviation. Statistical significance was calculated by analysis of variance supported by Student's t-test. Differences were considered significant when  $P < 0.05$ .

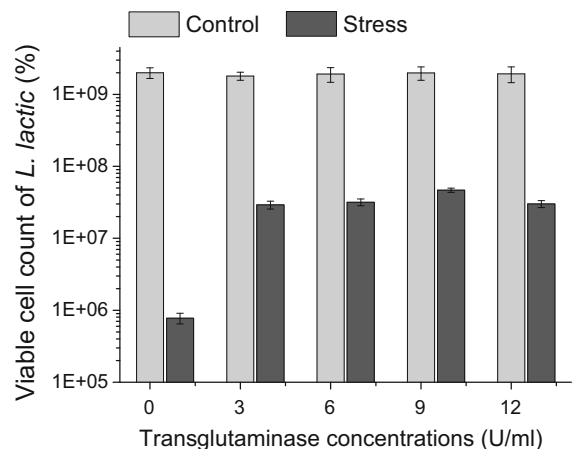
## Results and discussion

Exogenous transglutaminase thickens the cell wall and confers *Lactococcus lactis* NZ9000 tolerance to multiple environmental stresses

Maintaining the viability of ingested cells throughout gastro-intestinal transit is of paramount importance for the microorganisms to reach their intended site of action in sufficient numbers ( $10^8$  CFU/g) of intestinal contents and have a positive effect on the host, so it is important to increase the cells' ability to withstand the high acid in the stomach and bile of the small intestine. *L. lactis* is generally considered a non-intestinal bacterium, and is more sensitive to bile compared to the natural gastrointestinal tract microflora. Therefore, a relatively low concentration (0.04 %, w/v) was chosen to evaluate the tolerance of *L. lactis* to bile salts. *L. lactis* was incubated in GM17 broth

supplemented with or without transglutaminase in preparation of the  $tg^+$  cells and  $tg^-$  cells. Figure 1 shows that the addition of transglutaminase did not affect its growth as the CFU/ml value of  $tg^-$  cells under control conditions was similar to the  $tg^+$  cells treated with transglutaminase. However, the  $tg^+$  cells displayed improved resistance to the 0.04 % bile salts compared with the  $tg^-$  cells and this difference increased with the concentration of transglutaminase. Nevertheless, the improvement was lower when transglutaminase was 12 U/ml, therefore, 9 U transglutaminase/ml was chosen to use in subsequent stress tests. When exposed to stimulated gastric juice (pH 3, pepsin 1.5 g/l), the survival superiority of *L. lactis* NZ9000 cells treated with 9 U/ml transglutaminase over  $tg^-$  cells was also significant ( $P < 0.05$ ), with a 36-fold survivability difference between  $tg^+$  and  $tg^-$  cells incubated in stimulated gastric juice for 2 h.

Antibiotic-induced alterations in the composition of gastrointestinal microbiota increase the risk of antibiotic-associated gastrointestinal disorders in susceptible individuals, therefore antibiotic tolerance is an essential characteristic for ingested bacteria. In most related studies, antibiotic discs are used to estimate growing-cells' sensitivity to a particular antibiotic by using antibiotic-impregnated wafers to test whether bacteria's growth are affected by this antibiotic. In our study, erythromycin tolerance was determined by measuring the viability of cells after



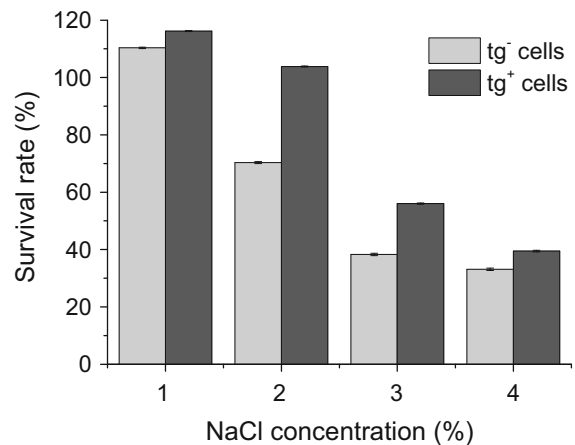
**Fig. 1** Bile survival rate of *L. lactis* NZ9000. The strain was grown statically in GM17 medium with 0, 3, 6, 9, 12 U transglutaminase/ml until THE stationary phase was reached (8 h), then cells were obtained by centrifugation and resuspended in 0.04 % (w/v) bile salt solutions at 37 °C for 2 h

exposure to 500 µg erythromycin/ml in saline. This assay method can evaluate the antibiotic resistance of non-growing cells rather than growing cells, since treatment in such nutrient-poor conditions will lead to the arrest of cell growth. Results showed that survivability reached 39 % after exposure to 500 µg erythromycin/ml in saline for 1 h. The difference in assay method and the action mechanism of erythromycin could explain this lower level mortality.

Erythromycin is bacteriostatic and inhibits bacterial protein synthesis, so under nutrient-limited condition there may simply be little additional effect on *L. lactis* cells. Nonetheless, the erythromycin accumulated in the cytoplasm over the incubation period was expected to inhibit cell regrowth. Our results revealed that the  $tg^+$  cells showed 1.6-fold and 5-fold increase in viability after 500 µg erythromycin/ml treatment for 1 and 2 h, respectively, compared to the  $tg^-$  cells. It is likely that transglutaminase played a role in slowing the entry of erythromycin into the cytoplasm. Furthermore, exposure to two other bacteriostatic antibiotics, i.e., cefazolin, and tetracycline, for 2 h resulted in a significant 5.3- and 3.2-fold increase in viability of  $tg^+$  cells compared to  $tg^-$  cells.

Before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions, and high concentrations of substrate and end product exhibits severe inhibition on the performance of cells during the process of high cell density fermentation. Osmotic stress is therefore another inevitable problem for probiotics. In addition, salting has a detrimental effect on the stability of probiotics during storage of cheese (Fortin et al. 2011). Therefore, being able to surmount this stress is also vital. *L. lactis* NZ9000 growth was inhibited when NaCl was higher than 1 %, regardless of the addition of transglutaminase (Fig. 2). The  $tg^+$  cells, however, showed significantly improved growth when challenged with a mild NaCl stress, displaying greater survivability over  $tg^-$  cells when grown in 2 % (w/v) and 3 % (w/v) NaCl than 4 % (w/v) NaCl.

Additionally, ingested microbes also need to survive and retain their functionality at low temperatures. The cold resistance of *L. lactis* NZ9000 is shown in Fig. 3. The  $tg^+$  cells displayed significantly higher viability than  $tg^-$  cells during low temperature treatment ( $P < 0.05$ ), in particular, when exposed to cold temperatures for a longer time. The difference of survivability during frozen conditions ( $-20\text{ }^\circ\text{C}$ )

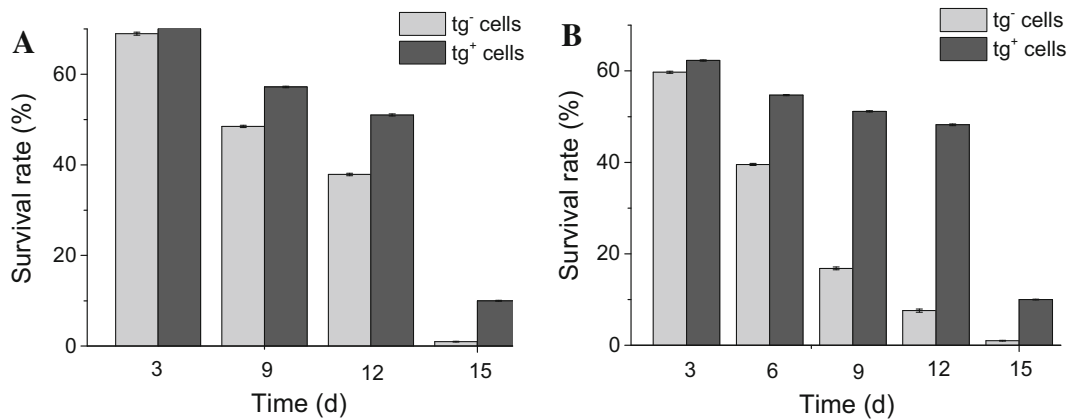


**Fig. 2** Survival rate of *L. lactis* NZ9000 exposed to osmotic stress induced by NaCl. The strain was grown in GM17 broth with 9 U transglutaminase/ml until stationary phase was reached (8 h), then cells were inoculated into fresh GM17 medium containing different concentrations of NaCl and incubated for 9 h

increased from 1-fold for 3 days to 6.4-fold for 15 days.

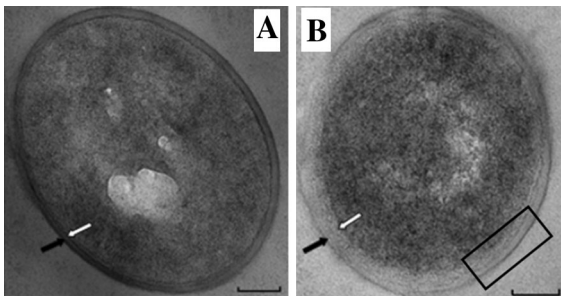
Possible mechanisms involved in the multi-resistant phenotype of transglutaminase-treated cells

The above results demonstrate that the  $tg^+$  cells possess improved resistance against a wide range of physicochemical stresses. Therefore, we hypothesized that there was more than one mechanism involved in the multi-resistant phenotype. First, we suspected that an enhanced permeability barrier in  $tg^+$  cells could be one of these mechanisms, since bile salts stress and antibiotic stress act in vastly different ways—the former at the cell membrane and the latter via intracellular protein synthetic organizations. Bile salts penetrate the cell wall and disaggregate the lipid bilayer structure of cellular membranes, inducing the formation of pores which allow for the release of intracellular content (Begley et al. 2005). If the cell wall acts as a protective barrier to reduce bile salt passage into the cell membrane then the cell would show stronger tolerance. Such a barrier is characterized by physical rather than chemical properties, and therefore depends on the molecular size of the toxic substance. Thus, if the toxic substance is larger than the pore size of the peptidoglycan sac, *L. lactis*  $tg^+$



**Fig. 3** Survival rate of *L. lactis* NZ9000 stored at low temperatures. The strain was grown in GM17 broth with 9 U transglutaminase/ml until stationary phase was reached (8 h),

then cells were obtained by centrifugation and resuspended in fresh GM17 medium, and stored at 4 °C (a) and -20 °C (b)

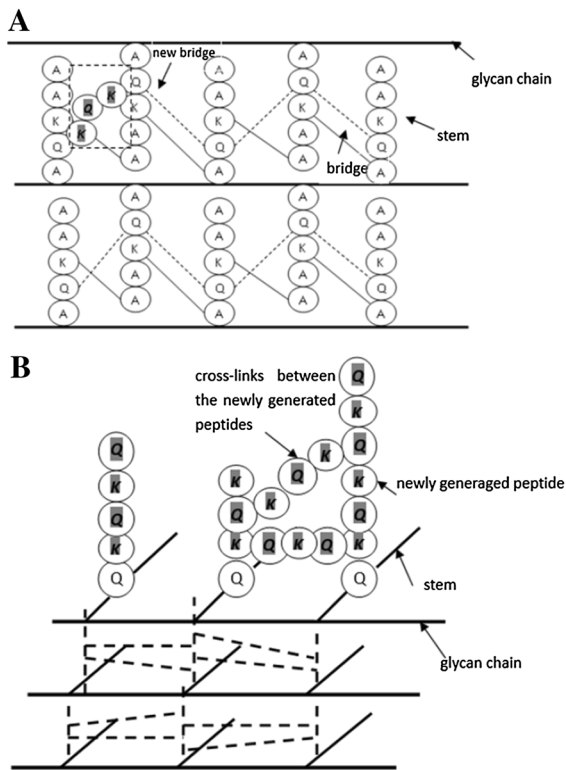


**Fig. 4** Transmission electron micrograph of *L. lactis* NZ9000 grown in GM17 broth with no transglutaminase (a) and with 9 U transglutaminase/ml (b) for 8 h. The cell wall is highlighted with arrows: the white arrow identifies the interior face and the opposing black arrow identifies the exterior face. The transition region of the cell wall is marked by the black box in (b). Scale bars = 200 nm

cells would display an enhanced degree of tolerance against the toxic substance, and vice versa. As predicted, we found that there was no significant difference in the level of resistance to lactic acid between *L. lactis* NZ9000 tg<sup>+</sup> and tg<sup>-</sup> cells (data not shown). This phenomenon could be explained by our hypothesis: the lactic acid molecule is too small to be blocked by the modified cell wall. Additionally, modifications in the cell wall structure should not negatively affect normal nutrient absorption, which was also demonstrated by our present work (Fig. 1). In order to understand these differences, we examined morphological changes in *L. lactis* cells treated with 9 U transglutaminase/ml. Figure 4 shows transmission

electron micrographs of representative cells. The cell wall thickness of tg<sup>+</sup> cells was approx. 1.9-fold that of tg<sup>-</sup> cell walls, suggesting a possible correlation between the action of transglutaminase and thickened cell walls. Interestingly, the cell wall of tg<sup>+</sup> cells also appeared more opaque than that of tg<sup>-</sup> cells. Moreover, the alteration in the cell wall density seemed to start from the outer surface of the cytoplasmic membrane, as observed from a transition region of the cell wall, where the portion adjacent to the cell membrane was more opaque than the outer portion (Fig. 4b).

Peptidoglycan is the major component of the Gram-positive bacterial cell wall. It is a large polymer composed of many identical subunits which contain two sugar derivatives and a stem peptide chain connected to the carboxyl group of *N*-acetylmuramic acid. For Gram-positive bacteria, the carboxyl group of the terminal D-alanine is often connected to the amino group of the third L-lysine through a peptide interbridge and this produces a molecular sieving effects. We suspect that such cross-linkings are strengthened by transglutaminase, which catalyses crosslinking between proteins via intra- or interchain glutamine (acyl donor) and lysine (acyl acceptor) peptide residues (Gaspar and de Goes-Favoni 2015). *L. lactis* NZ9000, a derivative of *L. lactis* MG1363, has a peptidoglycan with a monomer primary structure consisting of GlcNAc-MurNAc-Ala-Gln-Lys-Ala-Ala (Courtin et al. 2006). We suspect that, as shown in Fig. 5a, transglutaminase catalyzes the crosslinking of

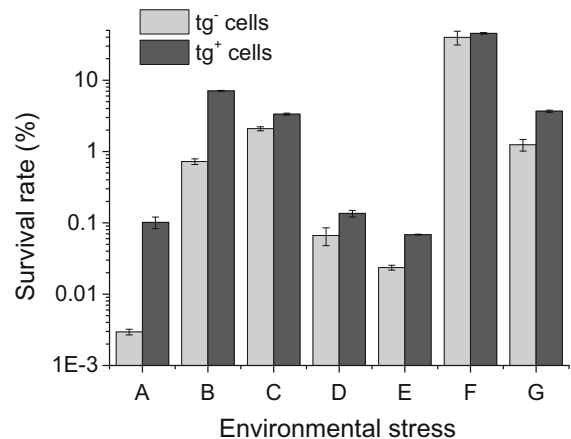


**Fig. 5** **a** Two-dimensional schematic diagram of one layer of the peptidoglycan of *L. lactis* NZ9000 cells grown in GM17 broth with transglutaminase. A newly generated interpeptide bridge is shown by the dotted box and the dotted lines. **b** Three dimensional schematic diagram of the top layer of the peptidoglycan. (A) (Q) (K): alanine, glutamine and lysine located in the stem peptide; (Q) (K): glutamine and lysine coming from the extracellular environment

glutamine on the stem peptide with unbonded lysine and glutamine molecules derived from the culture. The resulting new interpeptide bridges may be the cause of the observed increase in cell wall density. The thickened cell walls, may be due to some newly-generated peptides on the top layer of the peptidoglycan of  $tg^+$  cells (Fig. 5b), that is, the newly generated peptide could be connected to the glutamine in the stem peptide, and extended by alternately linking lysines and glutamines. At the same time, transglutaminase may be cross-linking these newly generated peptides together, and increasing the cell wall thickness. Thus, cell wall modification induced by transglutaminase makes it more difficult for bile salts and antibiotics to pass through cell wall. In order to test

this hypothesis, the peptidoglycan of *L. lactis* NZ9000 was isolated and analyzed. Results revealed that there was  $6.02 \pm 0.42 \mu\text{g}$  glutamine and  $51.6 \pm 3.05 \mu\text{g}$  lysine per mg of peptidoglycan in the  $tg^-$  cells of *L. lactis* NZ9000.

The role of transglutaminase in cell wall thickening may vary between different species of LAB, as the composition of the stem peptide in Gram-positive bacteria is species-specific. In some Gram-positive bacteria, such as *L. lactis* ssp. *cremoris* (Courtin et al. 2006), *Staphylococcus aureus* (Kim et al. 2015) and *Streptococcus pneumoniae* (Bui et al. 2012), the stem peptide is primarily composed of L-Ala-D-Gln-L-Lys-D-Ala-D-Ala. In other Gram-positive bacteria, the second pentapeptide amino acid is glutamic acid instead of glutamine (Wu et al. 2013). In order to investigate this hypothesis, *L. lactis* ssp. *lactis* NFL and *L. fermentum* 415 were assayed for peptidoglycan amino acid composition and environmental resistances. Results indicated that *L. fermentum* 415 contained  $4.45 \pm 0.29 \mu\text{g}$  glutamine and  $47.33 \pm 2.05 \mu\text{g}$  lysine per mg cell wall peptidoglycan. The other strain, *L. lactis* NFL, contained  $64.62 \pm 2.65 \mu\text{g}$  lysine per mg peptidoglycan, but no glutamine, suggesting that the amino acid composition of peptidoglycan is strain-specific. As expected,



**Fig. 6** Survival rates of *L. fermentum* 415. The strain was grown aerobically in MRS broth with 9 U transglutaminase/ml until stationary phase was reached (10 h), then cells were obtained by centrifugation and exposed to 0.04 % bile salts (a), simulated gastric juice (pH 3.0, pepsin 1.5 g/l) (b), 800  $\mu\text{g}/\text{ml}$  erythromycin (c), 800  $\mu\text{g}$  cefazolin/ml (d), 400  $\mu\text{g}$  tetracycline/ml (e), 4 % NaCl (f) and  $-20^\circ\text{C}$  (12 days) (g) stresses, and the cell viability was determined

*L. fermentum* 415 tg<sup>+</sup> cells exhibited more tolerance to different adverse conditions compared to tg<sup>-</sup> cells (Fig. 6). Nevertheless, variations in transglutaminase levels did not influence the persistence of *L. lactis* NFL exposed to the same stresses (data not shown). These results further support the hypothesis of the mechanism of transglutaminase in stress tolerance. It is worth noting that the glutamine and lysine content in *L. lactis* NZ9000 cells treated with 9 U transglutaminase/ml was  $23.78 \pm 2.05$  and  $135.63 \pm 9.92$  µg per mg peptidoglycan, respectively, which was significantly higher than in tg<sup>-</sup> cells.

Second, we thought the increase in cell wall density and thickness may contribute to its sturdiness thus helping it to resist cold temperatures. Corcoles-saez et al. (2012) showed that the cell wall integrity pathway plays an important role in the growth of *Sacchromyces cerevisiae* at low temperatures. In addition, our observation of higher tg<sup>+</sup> cell resistance to osmotic stress induced by high levels of NaCl was comparable to results reported in Xie et al. (2004), in which the genes involved in peptidoglycan biosynthesis were induced during osmotic stress with 4 % NaCl.

Third, we considered that the thickened cell wall acts not only as a physical protective barrier, but also as a chemical barrier contributing to the buffering capacities of the cell wall in the presence of acidic compounds, which was comparable to the report of Heidebach et al. (2009a), in which the improved survival of encapsulated cells can be attributed to the higher local pH value within the protein matrix of the capsules caused by the protein buffering capacity.

Microencapsulation is a potent strategy for maintaining high viability and stability of probiotic bacteria during multiple stresses. It is worth noting that survival rates of the encapsulated cells by transglutaminase-induced gelation were obvious better than that of free cells in stimulated gastric juice and refrigerated storage (Heidebach et al. 2009b; Zou et al. 2012). That process is based on a transglutaminase-catalysed gelation of casein or whey protein suspensions containing probiotic cells, which is obviously much more complex than the method described here. Take, for example, the microencapsulation method used by Heidebach et al. (2009b). 10 U transglutaminase/g casein was added to the protein-cell mixture at 40 °C, immediately dispersed in five volumes of vegetable oil with continuous stirring until the casein/

probiotic droplets were converted to gelatinized beads, separated by centrifugation, washed, and freeze dried. Such complex operations led to the fact that overall manufacturing price might not be accepted. Additionally, the size of spherical capsules formed from the method was  $165 \pm 23$  µm, which was much larger than the diameter observed in our study, and the large capsule size will negatively affect the textural and the sensorial properties of the product (Burgain et al. 2011).

Our observation that transglutaminase-induced thickened cell wall protected cells against harsh environments is somewhat similar to a recent study, which demonstrated that heterologous production of exopolysaccharides in *Lactobacillus paracasei* NFBC 338, which can form a capsule around the cell was associated with a significantly increased protection during heat stress, acid stress, simulated gastric juice stress, and bile stress (Stack et al. 2010). The exopolysaccharide also provides a layer around the bacterial cell, thus creating another natural barrier. This exopolysaccharide layer resembles the effect of transglutaminase since transglutaminase can also elevate the cell's resistance against hostile environment by thickening the cell wall. However, probiotics LABs that have been enhanced in this way are genetically-modified organisms and, with the exception of the USA and Canada, there is still uncertainty in the public arena towards the use of genetic manipulation. For this reason, studies which evaluate the safety of engineered probiotics are crucial if the technology is to gain acceptance (Mills et al. 2011).

## Conclusion

Here we investigate the transglutaminase-induced protected viability of two different LAB strains cultures against several environmental stresses typical of product manufacture and gastrointestinal transit (high osmotic, cold, stimulated gastric juice, bile salts and antibiotics stresses). We speculate that this behavior can potentially occur in other bacteria with glutamine and lysine-rich peptidoglycan. Therefore, the persistence of commercially available ingested bacteria during manufacturing and passage through the gastrointestinal tract can be improved by selecting strains whose peptidoglycans contain glutamine and lysine and incubating those strains in the presence of

transglutaminase. To our knowledge, this is the first study to observe the role of the addition of transglutaminase in enhancing the robustness of the cell in the presence of lethal external environments. This approach may be a promising commercial application due to its effectiveness, simpleness and low-cost.

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