Strain-specific differences in behaviour among Lacticaseibacillus rhamnosus cell wall mutants during direct compression

Strain-specific differences in behaviour among *Lacticaseibacillus rhamnosus* cell wall mutants during direct compression

Eline Byl[a], Katarina Jokicevic[a], Shari Kiekens[a], Sarah Lebeer[b], Filip Kiekens[a,*]

[a]*University of Antwerp, Department of Pharmaceutical, Biomedical and Veterinary Sciences, Laboratory of Pharmaceutical Technology and Biopharmacy. Universiteitsplein 1, B-2610 Wilrijk, Belgium*

[b]*University of Antwerp, Department of Bioscience Engineering, Research Group Environmental Ecology and Applied Microbiology, Groenenborgerlaan 171, B-2020 Antwerp, Belgium*

eline.byl@uantwerpen.be (E. Byl), katarina.jokicevic@uantwerpen.be (K. Jokicevic), shari.kiekens@uantwerpen.be (S. Kiekens), sarah.lebeer@uantwerpen.be (S. Lebeer), filip.kiekens@uantwerpen.be ; +32 3 265 27 13 (F. Kiekens[*])

Strain-specific differences in behaviour among *Lacticaseibacillus rhamnosus* cell wall mutants during direct compression

The human body harbours a large variety of microbial communities. It is already well-known that these communities play an important role in human health. Therefore, microbial imbalances can be responsible for several health disorders by different mechanisms. In recent years, probiotic bacteria have been increasingly applied to restore imbalances and stimulate microbiome functions such as immune modulation. Tablets are the dosage form of choice for oral probiotics. Nevertheless, a probiotic tablet with a sufficient amount of viable cells remains a challenge due to the stress of the compression process. Recent research demonstrated that the applied pressure and tableting properties play an important role in the survival of *Lacticaseibacillus rhamnosus* GG during direct compression. This study focused on the importance of the cell surface molecules in the protection of this prototype probiotic strain during direct compression. Spray-dried powders of *L. rhamnosus* GG and its exopolysaccharide-deficient mutant and lipoteichoic acid mutant were blended with two different filler-binders and compacted at various compression pressures. Under each tableting condition, the survival rate and tableting properties were analysed. The results demonstrated that the cell surface molecules play an important role in the behaviour of *L. rhamnosus* GG during direct compression. Specifically, the long, galactose-rich exopolysaccharides of *L. rhamnosus* served a protective shield during tablet production, promoting the survival rate of this probiotic strain. The D-alanylation of the lipoteichoic acids plays also an important role. When the D-alanyl ester content was completely absent, the survival rate was less affected by the tableting properties. Moreover, this research revealed that the sensitivity to the tableting properties is species and strain dependent.

Keywords: probiotics; *Lactobacillus rhamnosus*; *Lactobacillus casei*; tablet; compression; tableting properties; 3-D model; viability

Introduction

A large variety of microbial communities inhabit several sites in the human body. They colonise different body surfaces that come into contact with the outside world, more specifically the skin and mucosal surfaces of the gastro-intestinal tract,

oronasopharyngeal tract and the urogenital tract (Costello et al., 2009). The composition of these communities play an important role in health of the human host and can be influenced by environmental pressures, as well as microorganism-microorganism and host-microorganism interactions (Man et al., 2017). This can lead to dysbiosis which can result in acute or chronic disease. Probiotics, which are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host", are increasingly applied to restore some functions of the microbiota during dysbiosis, for example to inhibit pathogens, reduce inflammation or strengthen the mucosal barrier (FAO/WHO, 2002).

The required dosage that is needed to confer a health benefit is depending on the biological target and pharmaceutical dosage form (Kosin and Rakshit, 2006; Muller et al., 2014). Tablets are the dosage form of choice for oral probiotics because of the ease of administration, accurate dosage form, good patients acceptance and suitability of large-scale production (Ilić et al., 2013; Roopwani and Buckner, 2011). During the different stages of tablet manufacturing (drying, blending and compacting), the probiotic bacteria are exposed to various stresses, which can negatively affect the probiotic viability. Therefore, the development of a probiotic tablet with an adequate amount of viable cells is a substantial challenge. This research focuses on the compression stage. The mechanical stress of the compression process can cause damage to the cell wall and membrane and other bio-active components of the probiotic bacteria and therefore in a loss of viability (Chan and Zhang, 2002; Muller et al., 2014).

Research has shown that the applied pressure and tableting properties of the probiotic powder blend have an important impact on the survival rate of *Lacticaseibacillus rhamnosus* GG ATCC 53103 (*L. rhamnosus* GG) during direct compression (Byl et al., 2019b, 2019a). More specifically, the fast elastic decompression

of a powder blend lowers the mechanical stress during direct compression, resulting in less damage to the bacterial cell wall and therefore in a higher survival rate.

L. rhamnosus GG is a well-studied probiotic strain and has extensive number of proven health benefits (Capurso, 2019; Doron et al., 2005). The taxonomy has recently changed (Zheng et al., 2020). It is a low-G+C gram-positive, nonsporulating and facultative anaerobic strain. The cell wall of gram-positive bacteria consists of a thick, multi-layered peptidoglycan sacculus decorated with proteins, polysaccharides and teichoic acids which are composed of wall teichoic acids and/or lipoteichoic acids (LTA) (Lebeer et al., 2008). Although the basic architecture of the cell wall is similar in all gram-positive bacteria, strain-specific variations exist in the specific decoration with proteins polysaccharides and LTA (Lebeer et al., 2010). To analyse the functions of these specific decorations, knockout mutants of *L. rhamnosus* GG which are deficient in D-alanylation of lipoteichoic acids (*dltD* mutant) or exopolysaccharides (*welE* mutant) have been previously constructed (Lebeer et al., 2012; Velez et al., 2007). The *dltD* knockout mutant is characterised by an altered cell surface charge, a two-fold increased cell length (1.87-fold based on SEM or 2.4-fold based on TEM) and morphological alterations at the level of the septum (Kiekens et al., 2019; Velez et al., 2007). The *welE* knockout mutant lacks the long, galactose-rich exopolysaccharides, which results in an increased adherence to gastro-intestinal mucus and mucosae and biofilm formation capacity due to the increased exposure of the adhesive SpaCBA fimbriae (Lebeer et al., 2009).

In this study, the effect of exopolysaccharides and lipoteichoic acids on the survival rate of *L. rhamnosus* GG during tableting was examined. This probiotic strain and its cell surface mutants were spray-dried after which the powders were blended with two different filler-binders and compacted at various compression pressures. Subsequently, the effect of the applied pressure and tableting properties on the survival

rate during tableting was explored. The tableting properties were analysed by the 3-D modelling technique, which uses the three main variables to characterise the tableting process, namely time, force and displacement. The time plasticity *d*, the pressure plasticity *e* and the angle of rotation ω are the parameters that are derived from the 3-D modelling. The time plasticity *d* describes the plastic deformation as a function of time, which can be influenced by tableting speed. The pressure plasticity *e* describes the relationship between density and pressure. The angle of torsion ω is a measure of the elasticity of the material. Together, these parameters give a rather complete information about the tableting process. Finally, the survival rates were compared with the survival rate of a closely related other probiotic strain, namely *Lacticaseibacillus casei* AMBR2, to investigate the species and strain dependency. This strain, isolated from the upper respiratory tract (URT) of healthy individuals, contains another type of adhesive fimbriae enabling strong adherence to URT epithelium (De Boeck et al., 2019). Both *L. rhamnosus* GG and *L. casei* AMBR2 belong to the *L. casei* group of lactobacilli, recently reclassified in a new *Lacticaseibacillus* genus (Wuyts et al., 2017).

Materials and methods

2.1. Probiotic strains and growth conditions

During this study, *Lacticaseibacillus rhamnosus* GG wild-type (ATCC53103), its exopolysaccharide (EPS)-deficient mutant *ΔwelE*::Tc^R (CMPG5351), its lipoteichoic acid mutant *ΔdltD*::Tc^R (CMPG5540) and *Lacticaseibacillus casei* AMBR2 were examined (Kankainen et al., 2009; Lebeer et al., 2012, 2009). The probiotic strains were provided by the Laboratory of Applied Microbiology and Biotechnology of the University of Antwerp. The bacterial cells were routinely grown under non-shaking conditions in de Man-Rogosa-Sharpe (MRS) medium (Carl Roth, Germany) overnight at

37 °C till stationary phase, as previously described in Kiekens et al (Kiekens et al., 2019).

2.2. Spray drying

After the probiotic bacteria reached the stationary phase, the bacterial suspensions were centrifuged at 3983 × g for 10 min at 20 °C. The cell pellets were re-suspended to their original volume in a spray drying medium. The examined spray drying media were solutions of lactose monohydrate (Fagron, Waregem, Belgium) and trehalose dihydrate (provided by Nagase GmbH, Dusseldorf, Germany) in purified water (2,5% w/V) which were either used as such or enriched with hydroxypropyl methylcellulose (HPMC; Thermo Fisher, Karlsruhe, Germany) with a concentration of 1% (w/V). The re-suspended bacteria were spray-dried with a laboratory-scale spray dryer (B-290; Büchi, Flawil, Switzerland). A co-current configuration was used and the feed was sprayed into the drying chamber using a two-fluid nozzle (orifice diameter 1.4 mm). The feed flow rate, drying air flow rate and atomisation – spray flow rate were 7.5 mL/min, 32.5 m^3/h and 831 l/h, respectively. The spray drying parameters were held constant during the experiments. The re-suspended bacteria were spray-dried at a constant outlet temperature of approximately 55-58 °C. The feed flow rate and the drying air flow rate were 7.5 mL/min and 831 l/h respectively. The spray-dried bacterial powders were collected from a single cyclone separator, by powder harvesting every 15 minutes, as previously optimised by Jokicevic et al (Jokicevic et al., 2020).

2.3. Compression of probiotic powder

The probiotic tablets were produced by direct compression using a single punch tablet press (MCC Corporation, NJ, US) fitted with a flat-faced bevel-edged punches with diameter of 8 mm. The probiotic powder blends consisted of 10% (w/w) spray-dried bacterial powder, 85% (w/w) filler-binder and 5% (w/w) magnesium stearate (magnesium

stearate; Fagron, Nazareth, Belgium). The used direct compression filler-binders were Tablettose® 100 (lactose; Meggle Pharma, Wasserburg, Germany) and Xylisorb® XTAB 240 (xylitol; Roquette pharma, Zaventem, Belgium). These filler-binders were chosen based on the results of previously investigations (Byl et al., 2019b, 2019a). The high concentration of lubricant was selected to avoid sticking to punches and die. To investigate the effect of the compression pressure on bacterial survival, the bacterial powder blends were compacted at different pressures, namely from 40 until 120 MPa.

2.4. Bacterial viability analysis in the probiotic tablets

The bacterial viability was investigated by the plate counting method. Three batches with each filler-binder were manufactured, three powder samples before and three tablets after production were evaluated. The powder blends and tablets were diluted 10 times in purified water. Afterwards, a six-fold serial dilution of these suspensions was made in triplicate and spread onto MRS agar plates (Carl Roth, Belgium) in duplicate. The plates were incubated aerobically at 37 °C for 72 h. After incubation, the colony forming units (CFU) per plate were enumerated. To obtain the bacterial survival rate, the CFU counts of the powder blends before and the tablets after compression were compared. The average survival rate of the analysed probiotic tablets of three batches of each formulation (n=9) is displayed in the graphs. The error bars denote the standard deviation between the different tablets.

2.5. Tableting properties: porosity-pressure-time-profile (3-D model)

The tableting properties of the probiotic powder blends were evaluated by varying following three important tableting variables: compression force, compression time and displacement of the upper punch. They were recorded and converted to pressure, normalised time and $\ln(1/1-D_{rel})$, respectively. These parameters were presented in a 3-D

data plot, which was generated by Advanced Instrumentation monitor software (MCC Corporation, NJ, US). A twisted plane was fitted to this 3-D data by means of a nonlinear least-squares solver according to the Levenberg-Marquardt algorithm (Levenberg and Arsenal, 1943; Marquardt, 1963; More, 1978) (Matlab, Mathworks Inc, Unterföhrung, Germany) with the following equation:

$$z = \ln {}^{1}/_{1 - Drel} = \{[t - tmax] * [d + \omega * (Pmax - P)]\} + (e * P) + (f + d * tmax) \quad (1)$$

where D_{rel} is the relative density, t is the normalised time, t_{max} is the time at maximum pressure, P is the pressure, P_{max} is the maximum pressure, d is the time plasticity, e is the pressure plasticity, f is the intersection and ω is the angle of rotation.

The tableting properties of the different probiotic formulations (table 1) were analysed at 80 MPa. The resulting parameters d, e and ω of the fitted plane of three compaction cycles were averaged and the standard deviations were calculated and presented in a 3-D parameter plot.

2.6. Statistics

The statistical software GraphPad Prism 7.04 (GraphPad software inc, La Jolla, CA, USA) was used to analyse the data. The two-way analysis of variance (ANOVA) test, Turkey's and Sidak's multiple comparisons test were used to determine the statistical differences in bacterial viability. The statistical tests were performed at a significance level $\alpha = 0.05$.

Results

3.1. Influence of compression pressure on probiotic viability

It is previously reported that the loss of viability of *L. rhamnosus* GG is influenced by the

applied pressure (Byl et al., 2019a; Zheng et al., 2020). Specifically, at higher pressures, the probiotic bacteria are exposed to higher levels of mechanical stress, resulting in more damage to the bacterial cells and therefore in a significant loss of bacterial viability. During this research, the survival rate of *L. rhmanosus* GG wild-type in function of the applied pressure is compared with the survival rate of its *welE* mutant, *dltD* mutant and the closely related strain *L. casei* AMBR2 to investigate the importance of the cell surface molecules and strain and species dependency. The following results confirm that the survival rate is highly dependent on the applied pressure during tablet production. Moreover, the results show that the probiotic strains display a different survival profile within the formulations tested, indicating that cell surface molecules play a crucial role in the survival rate.

3.1.1. Lacticaseibacillus rhamnosus GG wild-type

Fig. 1 shows the survival rate of *L. rhamnosus* GG wild-type in function of applied pressure. The values and standard deviations are shown in table 2. Within the tablets, the viability decreased significantly with increasing pressure, excluding for the tablet formulation consisted of spray-dried lactose in combination with HPMC and the filler-binder Tablettose® 100 and the tablet formulation consisted of spray-dried lactose and the filler-binder Xylisorb® XTAB 240 (two-way ANOVA Turkey's multiple comparisons test; Graphpad software inc, La Jola, CA, USA). The strongest decrease in viability was observed within tablets consisted of spray-dried trehalose in combination with HPMC and the filler-binder Xylisorb® XTAB 240. Within this tablet formulation, the viability of *L. rhamnosus* GG wild-type dropped from 86% to 26%. At each compression pressure, the survival rate within the Tablettose® 100 tablets was significantly better when *L. rhamnosus* GG was spray-dried with trehalose. Within the Xylisorb® XTAB 240 tablets,

the survival rate was significantly lower when *L. rhamnosus* GG was spray-dried with lactose. There were no significant differences in viability between the other spray-dried formulations at 80 and 120 MPa.

3.1.2. Exopolysaccharide (EPS)-deficient ΔwelE mutant

Within the tablets, the viability of the *welE* knockout mutant decreased significantly with increasing pressure, except for the tablet formulation consisting of spray-dried lactose in combination with HPMC and the filler-binder Tablettose® 100 and the tablet formulation consisted of spray-dried lactose and the filler-binder Xylisorb® XTAB 240 (fig. 2 - table 3). Similar as for the wild-type, the strongest decrease in viability was observed within the tablet formulation consisted of spray-dried trehalose in combination with HPMC and the filler-binder Xylisorb® XTAB 240. Within this formulation, the viability of the *welE* mutant decreased from 66% to 23%. At each compression pressure, the survival rate within the Tablettose® 100 tablets was significantly better when the *welE* mutant was spray-dried with trehalose. No significant difference in survival rate was observed between lactose and trehalose in combination with HPMC. At 40 and 80 MPa, the survival rate of the *welE* mutant was significantly lower when it was spray-dried with lactose in combination with HPMC. This significant difference in survival rate was not observed at 120 MPa. Within the Xylisorb® XTAB 240 tablets, the survival rate of the *welE* mutant theat 40 and 80 MPa was significantly better when it was spray-dried with trehalose in combination with HPMC. Between the other spray-dried formulations, no significant differences in survival rate were observed, except at 80 MPa. At this pressure, a significantly better survival rate was noticed when the *welE* mutant was spray-dried with trehalose.

3.1.3. Lipoteichoic acid ΔdltD mutant

The relationship between the survival rate of the *dltD* mutant and applied pressure is displayed in fig. 3. The values and standard deviations are shown in table 4. Within the tablets, the viability of the *dltD* mutant decreased significantly with increasing pressure (two-way ANOVA Turkey's multiple comparisons test; Graphpad software inc, La Jola, CA, USA). Within the Tablettose® 100 tablets, the lowest survival rate was observed when the *dltD* mutant was spray-dried with lactose in combination with HPMC. Additionally, at each compression pressure the survival rate of the *dltD* mutant was significantly better when it was spray-dried with trehalose or trehalose in combination with HPMC. No significant difference was observed between lactose and lactose in combination with HPMC. Within the Xylisorb® XTAB 240 tablets, the survival rate of the *dltD* mutant at each compression pressure was better when it was spray-dried with lactose. The lowest survival rate was noticed when the *dltD* mutant was spray-dried with lactose in combination with HPMC.

3.1.4. Lacticaseibacillus casei AMBR2

Within the *L. casei* AMBR2 tablets, the viability decreased significantly with increasing pressure (fig. 4 – table 5). The strongest decrease in viability was observed within the tablet formulation consisted of spray-dried trehalose in combination with HPMC and the filler-binder Tablettose® 100 and the tablet formulation consisted of spray-dried trehalose in combination with HPMC and the filler-binder Xylisorb® XTAB 240. Within these formulations, the viability went down from 92% to 40% and 60% to 14% respectively. At each compression pressure, the survival rate within the Tablettose® 100 tablets was significantly better when *L. casei* AMBR2 was spray-dried with trehalose in combination with HPMC. The significant lowest survival rate was observed when *L. casei* AMBR2

was spray-dried with lactose. No significant difference was noticed between lactose in combination with HPMC and trehalose, excepting at 120 MPa. At each applied pressure, the survival rates within the Xylisorb® XTAB 240 tablets differed significantly from each other, with exception of tablets consisted of spray-dried trehalose and the filler-binder Xylisorb® XTAB 240 and tablets consisted of spray-dried trehalose in combination with HPMC and the filler-binder Xylisorb® XTAB 240 at 40 MPa. The survival rate of *L. casei* AMBR2 was significantly better when it was spray-dried with lactose. The significantly lowest survival rate was observed when it was spray-dried with lactose in combination with HPMC.

3.2. Tableting properties of the probiotic powder blends

Different survival rates among the tablet formulations are observed. It is already reported that the tableting properties play an important role in the protection of *L. rhmanosus* GG during compression (Byl et al., 2019a, 2019b). Therefore, the tableting properties of the formulations (different spray drying medium and filler-binder) are analysed with the 3-D modelling technique.

Among the different probiotic strains tested, each tablet formulation showed similar tableting properties. The average values and standard deviations of the parameters d, e and ω were calculated and are presented in table 6. When the spray-dried powders were blended with Xylisorb® XTAB 240, the d values were higher in comparison with Tablettose® 100 which indicates that the Xylisorb® XTAB 240 blends deformed faster during tableting. It is also notable that the ω values were higher, meaning that these powder blends showed less elastic decompression than the Tablettose® 100 powder blends. When the probiotic stains were spray-dried with HPMC and blended with Tablettose® 100, the angle of rotation ω increased whereas this value decreased when the

spray-dried powders were blended with Xylisorb® XTAB 240. The lowest averaged value of *d*, *e* and ω was observed when the probiotic strains were spray-dried with trehalose and compacted with Tablettose® 100. This powder blend needed a lot of time and pressure for deformation. Therefore, it did not deform easily and after some deformation it probably fractured under pressure. Because of the low ω value, the particles undergo some fast elastic recovery. The highest averaged values of *d*, *e* and ω were observed when the probiotic strains were spray-dried with lactose and compacted with Xylisorb® XTAB 240. This powder blend deformed more easily by low pressure and the deformation is probably totally plastic.

3.3. Influence of tableting properties on probiotic viability

For each probiotic strain tested, the tableting properties of the formulations are related to the survival rate at an applied pressure of 80 MPa. The results demonstrate that the survival rate is highly dependent on the tableting properties. Moreover, the cell surface molecules play a crucial role in the sensitivity to the tableting properties.

3.3.1. Lacticaseibacillus rhamnosus GG wild-type

The survival rate of *L. rhamnosus* GG wild-type within the different tablet formulations is shown in fig. 5 (A). This probiotic strain survived significantly better when it was spray-dried with trehalose and compacted with Tablettose® 100. This probiotic powder blend exhibited the lowest values of *d, e* and ω. Therefore, it needed time and pressure to deform and after some deformation it probably fractured. During decompression, the fractured particles underwent fast elastic recovery. Remarkably, the survival rate within the Tablettose® 100 tablets strongly decreased when *L. rhamnosus* GG was spray-dried in combination with HPMC. When lactose was spray-dried in combination with HPMC,

the angle of rotation ω increased meaning that the powder blend underwent less fast elastic recovery. When trehalose was spray-dried in combination with HPMC, the time plasticity *d* increased and it deformed faster. Therefore, it can be concluded that *L. rhamnosus* GG wild-type is sensitive to the time plasticity *d* and angle of rotation ω. More specifically, lower values resulted in higher survival rates. Within the Xylisorb® XTAB 240 tablets, no significant differences in survival rates were observed. No lower survival rates were noticed when this strain was spray-dried in combination with HPMC. Therefore, it can be assumed that HPMC itself is not toxic for *L. rhamnosus* GG wild-type.

3.3.2. Exopolysaccharide (EPS)-deficient ΔwelE mutant

Within the Tablettose® 100 tablets, the survival rate decreased significantly when the exopolysaccharide-deficient mutant was spray-dried with lactose or trehalose in combination with HPMC (fig. 5 (B)). The addition of HPMC caused a change in tableting properties, which is explained earlier. The survival rate of this mutant was also significantly lower when it was spray-dried with lactose and lactose in combination with HPMC and compacted with Xylisorb® XTAB 240. These powder blends exhibited the highest values of *d, e* and ω. Therefore, they deformed easily without fast elastic recovery during decompression. These results make it possible to conclude that the *welE* mutant is hypersensitive to *d, e* and ω, and therefore to the tableting properties of the powder blend.

3.3.3. Lipoteichoic acid ΔdltD mutant

The lipoteichoic acid mutant survived significantly better when it was spray-dried with lactose and compacted with Xylisorb® XTAB 240 (fig. 5 (C)). The significant lowest survival rate was observed when it was spray-dried with lactose in combination with

HPMC and compacted with Xylisorb® XTAB 240. There were no significant differences in survival rate between the other formulations.

3.3.4. Lacticaseibacillus casei AMBR2

L. casei AMBR2 survived significantly better when it was spray-dried with lactose and compacted with Xylisorb® XTAB 240 (fig. 5 (D)). As mentioned earlier, this tablet formulation exhibited the highest values of d, e and ω. Therefore, the powder blend deformed easily by low pressure and the deformation was probably totally plastic without elasticity. Remarkably, the survival rate decreased significantly when *L. casei* AMBR2 was spray-dried with trehalose in combination with HPMC and compacted with Xylisorb® XTAB 240. This powder blend exhibited lower values of d, e and ω. Therefore, it can be concluded that the survival rate of *L. casei* AMBR2 is influenced by these parameters. *L. casei* AMBR2 survived also significantly worse when it was spray-dried with lactose and compacted with Tablettose® 100. It should be noticed that the survival rate of *L. casei* AMBR2 within the Tablettose® 100 tablets improved when it was spray-dried in combination with HPMC. Therefore, it can be concluded that *L. casei* AMBR2 survives better the compression process when it is compacted with a powder blend that deforms easily during compression and does not show elasticity during decompression.

Discussion

During this study, the survival of different probiotic strains during direct compression was explored. The probiotic strains were spray-dried with different spray drying media and compacted with the filler-binder Tablettose® 100 or Xylisorb® XTAB 240 at various compression pressures. Within each tablet formulation, the survival rate of the probiotic strains decreased significantly with increasing pressure. An exception to this were the

survival rates of *L. rhamnosus* GG and its isogenic *welE* mutant within tablets consisted of spray-dried lactose in combination with HPMC and the filler-binder Tablettose® 100 and tablets consisted of spray-dried lactose and the filler-binder Xylisorb® XTAB 240. Within the Tablettose® 100 tablets, the survival rates of *L. rhamnosus* GG and its *welE* mutant were significantly better when they were spray-dried with trehalose whereas a better survival rate was achieved with *L. casei* AMBR2 and the *dltD* mutant of *L. rhamnosus* GG when they were spray-dried with trehalose in combination with HPMC. Within the Xylisorb® XTAB 240 tablets, *L. casei* AMBR2 and the *dltD* mutant of *L. rhamnosus* GG survived significantly better when they were spray-dried with lactose and significantly worse when they were spray-dried with lactose in combination with HPMC. This significant decrease did not occur within other HPMC formulations. Hence, it can be assumed that HPMC itself is not responsible for this decrease and not toxic for these probiotic strains. Remarkably, *L. rhamnosus* GG and its *welE* mutant survived significantly worse when they were spray-dried with lactose and compacted with Xylisorb® XTAB 240.

The analysis of the results of the 3-D modelling technique revealed the sensitivity of the probiotic strains to the tableting properties of the powder blends. *L. rhamnosus* GG wild-type survived significantly better when it was spray-dried with trehalose and compacted with Tablettose® 100. This powder blend exhibited the lowest values of d, e and ω. It needed time and pressure to deform and after some deformation it probably fractured. During decompression, the fractured particles underwent fast elastic recovery. Remarkably, when the value of d increased, the survival rate decreased significantly. This result reveals the sensitivity of *L. rhamnosus* GG wild-type to the time plasticity which is not previously reported. The results of the 3-D modelling technique confirm the

importance of pressure plasticity and fast elastic recovery in the survival of *L. rhamnosus* GG wild-type during direct compression (Byl et al., 2019b, 2019a).

The results of this study demonstrate that the exopolysaccharide-deficient mutant is more sensitive to the tableting properties in comparison with *L. rhamnosus* GG wild-type. More specifically, the survival rate of the *welE* mutant was significantly lower within tablet formulations that exhibited higher values of *d*, *e* and/or ω. These results reveal a hypersensitivity of this strain, which is not observed with *L. rhamnosus* GG wild-type. Therefore, it can be concluded that the large galactose-rich cell wall polysaccharides, which are absent in the *welE* mutant, play an important role in the protection of *L. rhamnosus* during direct compression. This is in agreement with previous work these exopolysaccharides play an important role in protecting *L.* rhamnosus GG against various stressors (Lebeer et al., 2011). The lipoteichoic acid mutant showed less variation in survival rates among the different formulations tested. Therefore, it can be assumed that this mutant is less influenced by the tableting properties. The better resistance may be explained by its increased cell length and morphological alteration at the level of the septum (Kiekens et al., 2019; Velez et al., 2007). The higher resistance was also observed with *L. casei* AMBR2. The *dltD* mutant of *L. rhamnosus* GG and *L. casei* AMBR2 survived significantly better when they were spray-dried with lactose and compacted with Xylisorb® XTAB 240. This tablet formulation exhibited the highest values of *d*, *e* and ω. It deformed easily by low pressure and the deformation was probably totally plastic. It should be noticed that the *dltD* mutant of *L. rhamnosus* GG and *L. casei* AMBR2 wild-type behaved differently within the other formulations. The survival rates indicated that *L. casei* AMBR2 is more sensitive to the tableting properties. More specifically, *L. casei* AMBR2 has a higher need to a powder blend which exhibits lower values of *d*, *e* and ω.

Conclusion

This study demonstrates that cell surface molecules play an important role in the behaviour of probiotic bacteria during direct compression, which was not previously reported. The long galactose-rich cell wall polysaccharides of the *L. rhamnosus* GG wild-type protect this strain against the mechanical stress during direct compression. Moreover, the results show that the lipoteichoic acid mutant is less vulnerable to the compression process. Finally, this study demonstrates that the sensitivity to the tableting properties is species and strain dependent.

Acknowledgments

This work was supported by the research foundation – Flanders (FWO) under Grant 1S 448 17N. Work on nasopharyngeal probiotics in the teams of Sarah Lebeer and Filip Kiekens is also supported by the IWT-SBO Grant ProCure IWT150052 (www.procureproject.be).

Disclosure of interest

None

References

Byl, E., Bladt, P., Lebeer, S., Kiekens, F., 2019a. Importance of pressure plasticity during compression of probiotic tablet formulations. Eur. J. Pharm. Biopharm. 145, 7–11. https://doi.org/10.1016/j.ejpb.2019.10.001

Byl, E., Lebeer, S., Kiekens, F., 2019b. Elastic recovery of filler-binders to safeguard viability of Lactobacillus rhamnosus GG during direct compression. Eur. J. Pharm. Biopharm. 135, 36–43. https://doi.org/10.1016/j.ejpb.2018.12.005

Capurso, L., 2019. Thirty Years of Lactobacillus rhamnosus GG. J. Clin. Gastroenterol. 53, S1–S41. https://doi.org/10.1097/MCG.0000000000001170

Chan, E.S., Zhang, Z., 2002. Encapsulation of probiotic bacteria Lactobacillus acidophilus by direct compression. Food Bioprod. Process. Trans. Inst. Chem. Eng. Part C 80, 78–82. https://doi.org/10.1205/09603080252938708

Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I., Knight, R., 2009. Bacterial Community Variation in Human Body Habitats Across Space and Time. Science (80-.). 326, 1694–1697. https://doi.org/10.1126/science.1177486

De Boeck, I., van den Broek, M.F.L., Allonsius, C.N., Martens, K., Wuyts, S., Wittouck, S., Cauwenberghs, E., Jokicevic, K., Vandenheuvel, D., Spacova, I., Lemarcq, M., De Rudder, C., Thys, S., Timmermans, J.-P., Vroegop, A. V., Verplaetse, A., Van de Wiele, T., Kiekens, F., Hellings, P., Vanderveken, O.M., Lebeer, S., 2019. Lactobacilli Have a Niche in the Human Nose. SSRN Electron. J. https://doi.org/10.2139/ssrn.3422481

Doron, S., Snydman, D.R., Gorbach, S.L., 2005. Lactobacillus GG: Bacteriology and Clinical Applications. Gastroenterol. Clin. North Am. 34, 483–498. https://doi.org/10.1016/j.gtc.2005.05.011

FAO/WHO, 2002. Guidelines for the Evaluation of Probiotics in Food. Rome and Geneva.

Ilić, I., Govedarica, B., Šibanc, R., Dreu, R., Srčič, S., 2013. Deformation properties of pharmaceutical excipients determined using an in-die and out-die method. Int. J. Pharm. 446, 6–15. https://doi.org/10.1016/j.ijpharm.2013.02.001

Jokicevic, K., Broeckx, G., Vandenheuvel, D., De Boeck, I., Allonsius, C., Lebeer, S., Kiekens, F., 2020. Processing of potential upper respiratory tract probiotics by spray drying. Dry. Technol.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P.A., Lebeer, S., De Keersmaecker, S.C.J., Vanderleyden, J., Hamalainen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjarvi, T., Auvinen, P., de Vos, W.M., 2009. Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein. Proc. Natl. Acad. Sci. 106, 17193–17198. https://doi.org/10.1073/pnas.0908876106

Kiekens, S., Vandenheuvel, D., Broeckx, G., Claes, I., Allonsius, C., De Boeck, I., Thys, S., Timmermans, J., Kiekens, F., Lebeer, S., 2019. Impact of spray-drying on the pili of Lactobacillus rhamnosus GG. Microb. Biotechnol. 12, 849–855. https://doi.org/10.1111/1751-7915.13426

Kosin, B., Rakshit, S.K., 2006. Microbial and processing criteria for production of probiotics: A review. Food Technol. Biotechnol. 44, 371–379.

Lebeer, S., Claes, I., Tytgat, H.L.P., Verhoeven, T.L.A., Marien, E., von Ossowski, I., Reunanen, J., Palva, A., de Vos, W.M., De Keersmaecker, S.C.J., Vanderleyden, J., 2012. Functional Analysis of Lactobacillus rhamnosus GG Pili in Relation to Adhesion and Immunomodulatory Interactions with Intestinal Epithelial Cells. Appl. Environ. Microbiol. 78, 185–193. https://doi.org/10.1128/AEM.06192-11

Lebeer, S., Claes, I.J.J., Verhoeven, T.L.A., Vanderleyden, J., De Keersmaecker, S.C.J., 2011. Exopolysaccharides of Lactobacillus rhamnosus GG form a protective shield against innate immune factors in the intestine. Microb. Biotechnol. 4, 368–374. https://doi.org/10.1111/j.1751-7915.2010.00199.x

Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C.J., 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat. Rev. Microbiol. 8, 171–184. https://doi.org/10.1038/nrmicro2297

Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C.J., 2008. Genes and Molecules of Lactobacilli Supporting Probiotic Action. Microbiol. Mol. Biol. Rev. 72, 728–764. https://doi.org/10.1128/MMBR.00017-08

Lebeer, S., Verhoeven, T.L.A., Francius, G., Schoofs, G., Lambrichts, I., Dufrene, Y., Vanderleyden, J., De Keersmaecker, S.C.J., 2009. Identification of a Gene Cluster for the Biosynthesis of a Long, Galactose-Rich Exopolysaccharide in Lactobacillus rhamnosus GG and Functional Analysis of the Priming Glycosyltransferase. Appl. Environ. Microbiol. 75, 3554–3563. https://doi.org/10.1128/AEM.02919-08

Levenberg, K., Arsenal, F., 1943. A Method for the Solution of Certain Non-Linear Problems in Least Squares. Q. Appl. Math. 1, 536–538. https://doi.org/10.1090/qam/10666

Man, W.H., De Steenhuijsen Piters, W.A.A., Bogaert, D., 2017. The microbiota of the respiratory tract: Gatekeeper to respiratory health. Nat. Rev. Microbiol. 15, 259–

270. https://doi.org/10.1038/nrmicro.2017.14

Marquardt, D.W., 1963. An Algorithm for Least-Squares Estimation of Nonlinear Parameters Author (s): Source : Journal of the Society for Industrial and Applied Mathematics , Vol . 11 , No . 2 Published by : Society fDonald W . Marquardtor Industrial and Applied Mathematics S. J. Soc. Ind. Appl. Math. 11, 431–441.

More, J.J., 1978. The Levenberg-Marquard Algorithm; Implementation and Theory. Numer. Anal. 105–116.

Muller, C., Mazel, V., Dausset, C., Busignies, V., Bornes, S., Nivoliez, A., Tchoreloff, P., 2014. Study of the Lactobacillus rhamnosus Lcr35® properties after compression and proposition of a model to predict tablet stability. Eur. J. Pharm. Biopharm. 88, 787–794. https://doi.org/10.1016/j.ejpb.2014.07.014

Roopwani, R., Buckner, I.S., 2011. Understanding deformation mechanisms during powder compaction using principal component analysis of compression data. Int. J. Pharm. 418, 227–234. https://doi.org/10.1016/j.ijpharm.2011.05.040

Velez, M.P., Verhoeven, T.L.A., Draing, C., Von Aulock, S., Pfitzenmaier, M., Geyer, A., Lambrichts, I., Grangette, C., Pot, B., Vanderleyden, J., De Keersmaecker, S.C.J., 2007. Functional Analysis of D-Alanylation of Lipoteichoic Acid in the Probiotic Strain Lactobacillus rhamnosus GG. Appl. Environ. Microbiol. 73, 3595–3604. https://doi.org/10.1128/AEM.02083-06

Wuyts, S., Wittouck, S., De Boeck, I., Allonsius, C.N., Pasolli, E., Segata, N., Lebeer, S., 2017. Large-Scale Phylogenomics of the Lactobacillus casei Group Highlights Taxonomic Inconsistencies and Reveals Novel Clade-Associated Features. mSystems 2. https://doi.org/10.1128/mSystems.00061-17

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. Int. J. Syst. Evol. Microbiol. 70, 2782–2858. https://doi.org/10.1099/ijsem.0.004107

Tables

Formulation	Spray drying medium	Filler-binder
1	Lactose	Tablettose® 100
2	Lactose + HPMC	Tablettose® 100
3	Trehalose	Tablettose® 100
4	Trehalose + HPMC	Tablettose® 100
5	Lactose	Xylisorb® XTAB 240
6	Lactose + HPMC	Xylisorb® XTAB 240
7	Trehalose	Xylisorb® XTAB 240
8	Trehalose + HPMC	Xylisorb® XTAB 240

Table 1. The studied probiotic formulations

	Tablettose® 100			**Xylisorb® 240**		
	40 MPa	80 MPa	120 MPa	40 MPa	80 MPa	120 MPa
Lactose	54,63 ± 7,89	20,59 ± 2,55	10,63 ± 3,19	17,34 ± 0,79	17,65 ± 3,23	12,28 ± 0,14
Lactose + HPMC	5,75 ± 0,61	2,69 ± 1,15	2,01 ± 0,41	83,82 ± 6,25	41,16 ± 3,59	32,08 ± 1,48
Trehalose	82,26 ± 8,83	70,92 ± 9,37	46,24 ± 7,27	76,85 ± 0,74	42,78 ± 6,04	26,14 ± 2,71
Trehalose + HPMC	27,28 ± 4,16	17,09 ± 1,64	6,88 ± 0,54	85,42 ± 6,07	52,72 ± 4,39	26,23 ± 4,89

Table 2. The survival rate (%) of *L. rhamnosus* GG in function of the applied pressure (MPa)

	Tablettose® 100			**Xylisorb® 240**		
	40 MPa	80 MPa	120 MPa	40 MPa	80 MPa	120 MPa
Lactose	42,94 ± 6,99	36,14 ± 5,33	13,50 ± 2,34	13,38 ± 0,88	8,95 ± 1,96	8,02 ± 4,26
Lactose + HPMC	7,31 ± 1,14	3,75 ± 1,48	2,02 ± 0,17	25,15 ± 9,02	9,03 ± 1,03	8,23 ± 1,60
Trehalose	64,43 ± 11,28	51,43 ± 6,82	32,86 ± 9,77	43,20 ± 15,78	34,62 ± 8,86	18,00 ± 2,93
Trehalose + HPMC	34,02 ± 2,70	22,29 ± 4,88	9,81 ± 1,41	66,01 ± 14,20	51,53 ± 3,61	19,92 ± 9,29

Table 3. The survival rate (%) of the *welE* mutant in function of the applied pressure (MPa)

	Tablettose® 100			**Xylisorb® 240**		
	40 MPa	80 MPa	120 MPa	40 MPa	80 MPa	120 MPa
Lactose	73,14 ± 4,86	35,00 ± 2,86	23,62 ± 2,09	67,42 ± 4,32	65,29 ± 7,58	24,52 ± 1,19
Lactose + HPMC	56,17 ± 17,42	23,93 ± 6,79	12,27 ± 1,31	36,78 ± 10,67	16,45 ± 2,91	10,92 ± 0,89
Trehalose	72,93 ± 4,83	38,75 ± 2,56	31,35 ± 3,86	61,86 ± 5,34	41,43 ± 4,37	24,88 ± 3,18
Trehalose + HPMC	70,91 ± 7,87	49,37 ± 0,96	29,41 ± 2,95	63,35 ± 3,31	38,24 ± 3,25	13,86 ± 1,03

Table 4. The survival rate (%) of the *dltD* mutant in function of the applied pressure (MPa)

	Tablettose® 100			**Xylisorb® 240**		
	40 MPa	80 MPa	120 MPa	40 MPa	80 MPa	120 MPa
Lactose	21,71 ± 3,11	13,36 ± 2,02	6,89 ± 1,72	94,90 ± 3,92	82,84 ± 5,52	64,02 ± 1,87
Lactose + HPMC	13,63 ± 0,33	5,52 ± 7,29	2,80 ± 6,59	9,60 ± 1,66	6,08 ± 0,73	2,63 ± 0,37
Trehalose	57,19 ± 2,00	30,64 ± 2,90	27,28 ± 2,03	55,13 ± 3,25	50,24 ± 4,99	43,52 ± 2,57
Trehalose + HPMC	91,60 ± 4,06	51,61 ± 5,67	39,16 ± 5,26	60,21 ± 2,62	24,27 ± 0,80	13,55 ± 2,05

Table 5. The survival rate (%) of *L. casei* AMBR2 in function of the applied pressure (MPa)

	Tablettose® 100			Xylisorb® 240		
	d	*e* (MPa^{-1})	ω	*d*	*e* (MPa^{-1})	ω
Lactose	0,2784 ± 0,0293	0,0010 ± 0,0003	0,0058 ± 0,0004	0,4823 ± 0,0282	0,0013 ± 0,0006	0,0080 ± 0,0007
Lactose + HPMC	0,2762 ± 0,0153	0,0011 ± 0,0002	0,0066 ± 0,0005	0,3931 ± 0,0084	0,0008 ± 0,0002	0,0076 ± 0,0004
Trehalose	0,1920 ± 0,0503	0,0004 ± 0,0002	0,0067 ± 0,0013	0,3344 ± 0,0529	0,0000 ± 0,0003	0,0088 ± 0,0006
Trehalose + HPMC	0,2558 ± 0,0206	0,0006 ± 0,0003	0,0069 ± 0,0007	0,4143 ± 0,0721	0,0011 ± 0,0004	0,0077 ± 0,0007

Table 6. Calculated means and standard deviations for the parameters of the 3-D modelling in function of the spray drying media

Figures

Figure 1. The survival rate (%) of *L. rhamnosus* GG in function of applied pressure

Figure 2. The survival rate (%) of the *welE* mutant in function of applied pressure

Figure 3. The survival rate (%) of the *dltD* mutant in function of applied pressure

Figure 4. The survival rate (%) of the *L. casei* AMBR2 mutant in function of applied pressure

Figure 5. The survival rate (%) of the studied probiotic strains at 80 MPa. The formulations with a significant higher or lower survival rate (%) are indicated with a star (*) (two-way ANOVA, Turkey's and Sidak's multiple comparisons test).