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Increased Survival of *Lactococcus lactis* Strains Subjected to Freeze-Drying after Cultivation in an Acid Medium: Involvement of Membrane Fluidity

Cultivation in Acid Medium to Improve Bacteria Survival of Freeze-Drying

Aurore Bodzen[1,2], Audrey Jossier[1], Sébastien Dupont[1], Pierre-Yves Mousset[2], Laurent Beney[1], Sophie Lafay[2] and Patrick Gervais[1*]

[1]University of Burgundy, AgroSup Dijon, PAM UMR A 02.102, F-21000 Dijon, France
[2]Indigo Therapeutics, 5 rue Salneuve, 75017 Paris, France

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SUMMARY

Research background. Freeze-drying is the most widely used dehydration process in the food industry for the stabilization of bacteria. Studies have shown the effectiveness of an acid pre-stress in increasing the resistance of lactic acid bacteria strains to freeze-drying. Adaptation of bacteria to an acid stress is based on maintaining the properties of the plasma membrane. Indeed, the fatty acid composition of lactic acid bacteria membrane is often changed after an acid pre-stress. However, few studies have measured membrane fluidity after an acid stress realized during lactic acid bacteria strains cultivation.

Experimental approach. In order to use two pH profiles, the strains *Lactococcus lactis* NCDO 712 and NZ9000 were cultivated in two media, without any pH control. The two pH profiles obtained were representative of initial media composition, media buffering properties, and strains metabolism. Absorbance at 600 nm and pH were measured during bacterial cultivation. Then, the two strains were freeze-dried and their survival rates determined. Membrane fluidity was evaluated by fluorescence anisotropy measurements using a spectrofluorometer.

Results and conclusions. Cultivation under a more acidic condition significantly increased both strains survival to freeze-drying ($p<0.05$, ANOVA). Moreover, in both strains of *L. lactis*, a more acidic condition during cultivation significantly increased membrane fluidity ($p<0.05$, ANOVA). Our results

*Corresponding author:
Phone: +33 3 80 77 40 08
E-mail: patrick.gervais@u-bourgogne.fr

revealed that cultivation in such condition, fluidifies the membrane and allows a better survival to freeze-drying for the two strains of *L. lactis*. A more fluid membrane can facilitate membrane deformation and lateral reorganization of membrane components, critical for the maintenance of cellular integrity during dehydration and rehydration.

Novelty and scientific contribution: A better understanding of the involvement of membrane properties, especially of membrane fluidity, in bacterial resistance to dehydration is provided in this study.

Key words: freeze-drying, viability, membrane fluidity, acid pre-stress, *Lactococcus lactis*

INTRODUCTION

Freeze-drying is a dehydration process that stabilizes bacteria in order to preserve their long-term viability until they are used. This dehydration process is frequently employed in the food industry for the production of lactic acid bacteria used as starters or probiotics in dehydrated form. It has been widely demonstrated that the technological stresses induced by freeze-drying can cause cell damage and in some cases, cell death. Cellular dehydration, caused in large part by osmotic stress due to the addition of protective solutes and by the freezing itself, is the source of mechanical and structural stresses on cells and cellular components. During severe dehydration, permeabilization of the plasma membrane can be induced and is often reported to be the cause of cell death (*1*). The other critical step in freeze-drying is the rehydration step which precedes the use of the bacteria and which influences both bacterial survival and functionality *(2)*. The influence of different rehydration parameters on the viability and the functionality of bacteria has been discussed in recent studies, in particular the influence of the kinetics (*3*,*4*) and the influence of the presence of oxygen (*5*).

The strategies used to maximize the survival of bacteria during the freeze-drying process and during rehydration are mainly the addition of lyoprotectants (*2*), the application of pre-stresses which prepare the bacteria and the optimization of conventional operating parameters of the process (time - pressure - temperature ranges). The exposure of bacteria to moderate stresses during or after cultivation (thermal, osmotic, acid, *etc*.) has also been described as an effective strategy to increase bacterial viability to technological stresses. More specifically some studies have shown that an acid pre-stress during cultivation has increased the survival of *lactobacillus* strains to freezing or freeze-drying (*6*-*9*). The adaptation of bacteria to an acid stress is based on cell homeostasis (principally *via* an overproduction of ATP) and maintaining the properties of the plasma membrane. To maintain cell homeostasis during acid stress, the ATP produced during glycolysis can be redirected to increase the activity of the F-ATPase pump (proton expulsion from the intracellular medium) (*10*). The cells can also modify the pyruvate pathways and thus produce less lactate (*11*). Finally, the adaptation of

bacteria to acid stress also involves the modification of the protein pool by the production of chaperone proteins or proteins involved in glycolysis (*12*). To maintain the structural and functional integrity of the plasma membrane, the cell can synthesize *de novo* fatty acids by redirecting the acetyl-CoA produced during glycolysis or modify the fatty acids already present in the membrane. Studies have shown that after an acid stress, the fatty acid composition of lactic acid bacteria membrane is often changed (*6*-*8*, *13*-*15*). An increase of the unsaturated fatty acids-to-saturated fatty acids ratio (UFA/SFA) and of cyclopropane fatty acids (CFAs) content were reported in these studies. Indeed, modulating the amount of unsaturated (UFAs) or saturated (SFAs) fatty acids, the amount of CFAs or even the length of the fatty acid chains can thus influence membrane fluidity and bacterial resistance to mechanical stress caused by freeze-drying (*16*,*17*). However, few studies have measured membrane fluidity after an acid stress realized during lactic acid bacteria strains cultivation. Wang *et al.* (*9*) have found that a lower pH during cultivation of a *Lactobacillus acidophilus* strain reduced the loss of acidification activity after freezing at -20 °C and storage for 3 months at -20 °C. The ratio of UFA/SFA and the amount of CFAs were higher after an acid stress. In addition, higher membrane fluidity after cultivation at acidic pH was measured. Nevertheless, the influence of an acid pre-stress during cultivation on the survival of *Lactococcus* spp. strains to freeze-drying has not been studied yet.

Therefore, the aim of our study was to compare the membrane fluidity and the freeze-drying survival of two strains of *Lactococcus lactis* after cultivation under different acidic conditions. The strain *L. lactis* NCDO 712, starter used in the manufacture of cheeses, was first selected for this study. This strain has 6 plasmids including the plasmid pLP 712 with the genes coding for the transport and the metabolism of lactose (*18*). The *L. lactis* NZ9000 strain, which is a strain derived from the *L. lactis* NCDO 712 strain but devoid of plasmids, was also selected for this study. The insertion of the regulatory genes *nisR* and *nisK* into the *pepN* and *napC* genes for the NICE system (gene expression system regulated by nisin) in the *L. lactis* NZ9000 strain and mutations generated differences compared to the *L. lactis* NCDO 712 strain, in particular with regard to the catabolism of sugars. Due to the predominant role of ATP in the resistance to acid stress by increasing the activity of the F-ATPase pump, a greater sensitivity of the *L. lactis* NZ9000 strain to acid stress was hypothesized.

In order to use two pH profiles, the two strains were cultivated in two media commonly used for lactic acid bacteria biomass production, the MRS medium (*19*), and the M17 medium (*20*), without any pH control. As the others culture parameters were controlled (temperature, mixing, *etc.*), pH profiles evolution were characterized and used as fermentation tracer. Indeed, these pH profiles were representative of initial media composition, buffering properties of the media, and strains metabolism. A more acidic profile was obtained for bacterial cultivation with the MRS medium compared to the

M17 medium. After cultivation in these two media, the two strains of *L. lactis* were then freeze-dried and their survival rates determined. The comparison of the membrane fluidities of the two strains was carried out after culture under the different acid conditions in order to further the understanding of the observed differences in survival rates. This approach allows us to directly test the hypothesis that in case of freeze-drying use, the choice of the fermentation medium (more or less acid) could have an impact on two distinct outputs of the fermentation: biomass production and membrane fluidity.

MATERIALS AND METHODS

Bacterial strains and stock solutions

The *Lactococcus lactis* NCDO 712 and *Lactococcus lactis* NZ9000 strains were cultured in gM17 medium (M17 broth supplemented with 0.5 % glucose) at 30 °C for approximately 16 h. The M17 medium was produced according to the manufacturer's instructions (Sigma-Aldrich, Darmstadt, GERMANY) and the glucose (Sigma-Aldrich) was added after sterilization of the medium at 121 °C for 20 min. The pH of the medium gM17 was adjusted before autoclave to pH=7.1±0.2 at 25 °C with sterile NaOH at 1 M. The cultures were then diluted to 20 % (by volume) with sterile glycerol (Honeywell, New Jersey, USA), then aliquoted in 1 mL cryotubes and stored at -80 °C.

Culture conditions

From the cryotubes, strains were inoculated on Petri dishes containing gM17 agar medium: gM17 supplemented with 15 g/L of agar (VWR, Leuven, BELGIUM) by seeding 100 µL of culture. After 24 h at 30 °C, colonies were inoculated in 10 mL of gM17 medium (pre-cultures). Finally, after 24 h at 30 °C, the gM17 and MRS (*Lactobacillus* Broth acc. to De Man, Rogosa and Sharpe, Sigma-Aldrich) media were inoculated at 1 % (by volume) with the pre-cultures and then incubated at 30 °C and 37 °C respectively. The MRS medium was prepared according to the manufacturer's instructions and sterilized at 121 °C for 20 min after the addition of 0.1 % (by volume) Tween 80 (Sigma-Aldrich). The pH was adjusted before autoclave to pH=6.2±0.2 at 25 °C with sterile NaOH at 1 M.

Monitoring of pH and of absorbance during cultivation

In this study, cultures were carried out without pH regulation and acidic conditions was naturally achieved by lactic acid production. The optimum culture pH for strains of *lactococci* is between pH=6.3 and pH=6.9 *(21)*. The initial pH of the M17 medium was pH=7.1±0.2 and contained a buffer solution (sodium glycerophosphate). The culture of the two strains in the M17 medium being carried out without regulation of the pH, acidification of the culture medium was noted progressively with the production of lactic acid. As the initial pH of the MRS medium was lower (pH=6.2±0.2) and as this

medium did not contain a buffer solution, a more acidic condition was observed during the culture of the two strains in this medium in comparison with the M17 medium.

The pH was measured, for the two strains and the two culture media, every hour by means of a pH meter HI1053B provided with a combined pH probe (Hanna Instruments, Tanneries, FRANCE), previously calibrated using buffer solution calibration (pH=4 and pH=7).

Absorbance was measured at 600 nm, for the two strains and the two culture media, every two hours using a spectrophotometer Genesys 20 (Thermo Fisher Scientific, Massachusetts, USA).

Freeze-drying

The biomass produced was harvested in stationary phase and the number of cells present per milliliter was estimated using the method of Colony Forming Unit (CFU) and was expressed in CFU/mL.

The biomass produced was then centrifuged (4000 g - 10 min, Eppendorf 5810 R, Sigma-Aldrich) and the pellets were suspended in 5 % (*m/V*) sucrose (Sigma-Aldrich) in PBS (Phosphate Buffered Saline, Sigma-Aldrich). The sucrose was added so as to concentrate by 10 the number of cells produced per mL. One milliliter of this mixture was introduced into vials (amber glass vials of 5 mL) then frozen at -80 °C (-2 °C/min) before being freeze-dried for 24 h (FreeZone 18-Liter Console Freeze Dry System with Stoppering Tray Dryer, Purge Valve and PTFE-Coated Collector, Labconco, Kansas City, USA). Sublimation was carried out by maintaining the samples for 2 h at -40 °C (condenser temperature at -55 °C and chamber pressure at 10 Pa), then increasing the chamber temperature to 0 °C at a speed of 0.04 °C/min. After about 17 h, the temperature of the chamber was increased to 25 °C at a heating rate of 0.08 °C/min to carry out the secondary desorption. The samples were then sealed under vacuum before rehydration with 1 mL of gM17 medium at 30 °C in order to enumerate the final biomass. The final biomass after freeze-drying was expressed in CFU/mL. The survival rate for the freeze-drying process (%) was expressed by calculating the ratio between the viable biomass after freeze-drying (CFU/mL after freeze-drying) and the biomass produced (CFU/mL before freeze-drying).

Membrane fluidity

The membrane fluidity of the bacterial cells was evaluated by fluorescence anisotropy using a spectrofluorometer (Fluorolog-3, HORIBA Jobin Yvon, Longjumeau, FRANCE) in T format (*22-24*). As before, the cells were harvested in stationary phase after culture in gM17 or MRS media and then washed twice *(*4000 g, 10 min, 25 °C) in 15 mM TRIS-HCl buffer (Trisma hydrochloride, pH=7, Sigma-Aldrich). The cells were then resuspended in the same buffer to reach an optical density of 0.250 at 600 nm (*i.e.* 2.5×10^8 CFU/mL). The hydrophobic fluorescent probe DPH (1,6-diphenyl-1,3,5-

hexatriene, Invitrogen Molecular Probes, Sigma-Aldrich) was used (*23*) and a 10 mM stock solution in tetrahydrofuran was produced. Three milliliters of samples were placed in a quartz cell and 2 µL of the stock solution of DPH (final concentration = 6.7 µM) were added using a Hamilton glass syringe. After incubating the cells for 5 min at 30 °C, the measurements were carried out every 5 °C in a temperature range of + 30 °C (optimal temperature for the growth of the strains) to + 5 °C (temperature allowing the medium to remain liquid for the measurement by fluorescence anisotropy). Two anisotropy measurements were performed for each condition. The excitation and emission wavelengths were 360 nm±5 nm and 430 nm±5 nm respectively (*25*). The anisotropy (r) values were calculated automatically, according to Lakowicz (*26*).

Statistical analysis

An analysis of variance ($p<0.05$, ANOVA) was carried out to determine whether differences in biomass production, freeze-drying survival and biomass present after freeze-drying were significant between the culture media. The experiments were carried out in biological triplicates (3 independent cultures). The results presented are the means and standard deviations for n = 3. The R Software v.3.3.2 was used to statistically analyze data (*27*).

RESULTS AND DISCUSSION

pH profiles obtained during bacterial cultivation in the two media

pH profiles

In order to define the two pH profiles during the culture of the two strains of *L. lactis* in the gM17 and MRS media, pH and absorbance were measured. Fig. 1 presents the monitoring of the pH during the culture of the *L. lactis* NCDO 712 strain (Fig. 1a) and of the *L. lactis* NZ9000 strain (Fig. 1b) as a function of the culture medium (gM17 or MRS).

Fig. 1

According to Fig. 1, the pH of the gM17 medium after sterilization (and therefore at the start of the culture) is close to that before sterilization (after sterilization pH=6.95±0.017). In the case of the MRS medium, the pH of the medium decreases after sterilization. Before sterilization the pH was pH=6.2 and at the start of culture the pH was pH=5.9 (pH=5.86±0.3).

For the *L. lactis* NCDO 712 strain, pH is stable at pH=6.95 for 4 h in gM17 medium. Then, the pH decreases very quickly until reaching minimum values close to 5 (pH=5.15±0.028) from 9 h of culture and thereafter remains stable until harvest. For the *L. lactis* NZ9000 strain, the pH curve obtained during cultivation in the gM17 medium is close to that of *L. lactis* NCDO 712. Concerning the culture

of the two strains in the MRS medium, the pH curves are different from those obtained with the gM17 medium and different between the two strains. For the *L. lactis* NCDO 712 strain, the pH is stable for 3 h at pH=5.9 then decreases rapidly until reaching values close to 5 (pH=4.66±0.01) from 12 h of culture, thereafter remaining stable until harvest. For the *L. lactis* NZ9000 strain, the pH is stable for 10 h at pH=5.9 and then decreases very slowly, reaching pH=5.69 at 16 h of culture. Unlike the case of the *L. lactis* NCDO 712 strain, the pH of the *L. lactis* NZ9000 strain at harvest in the MRS medium is higher than in the gM17 medium. Table 1 presents the contact time with certain pH ranges (optimal pH, acid pH and very acid pH according to Hutkins *et al.* (*21*)) for each strain and each culture medium.

Table 1

For both strains of *L. lactis* during cultivation in the gM17 medium, the same time is spent at optimal pH (5 h between pH=6.3 and pH=7). During cultivation of the strains in the MRS medium, the initial pH was pH=5.9, and thus the start of the growth of the two strains does not take place in an optimal pH range.

In the case of the *L. lactis* NCDO 712 strain, the pH of the extracellular medium reaches values of less than pH=5.3 for 7 h at the end of culture in both culture media. Unlike the *L. lactis* NCDO 712 strain, the extracellular medium of the *L. lactis* NZ9000 strain during cultivation in the two media never reaches values below pH=5.3.

As expected, two pH profiles were observed after cultivation of *L. lactis* NZ9000 and *L. lactis* NCDO 712 in the two media. Both strains of *L. lactis* are subjected to acidic conditions during their cultures in gM17 and MRS media. However, a more acidic condition was obtained for both strains with the MRS medium than with the gM17 medium.

Impact of pH profiles on bacterial growth

Fig. 2 shows the monitoring of growth by measuring the absorbance at 600 nm for the *L. lactis* NCDO 712 strain (Fig. 2a) and for the *L. lactis* NZ9000 strain (Fig. 2b) according to culture medium (gM17 or MRS).

Fig. 2

The same growth phases are observed in both *L. lactis* NCDO 712 and *L. lactis* NZ9000 during cultivation in the gM17 medium. Regarding the monitoring of growth during the cultivation of *L. lactis* NCDO 712 in MRS medium, a latency phase of 2 h is observed. The exponential phase is therefore observed up to 8 h of culture (maximum specific speed of growth being similar between the two

media). The absorbance reached in the stationary phase is higher if the culture is carried out in gM17 medium, which means that the biomass produced in this medium should be greater than that produced in MRS medium. The presence of a latency phase during cultivation in the MRS medium is probably due to the change of culture medium between the pre-culture and the culture and to the time of bacterial adaptation to this new culture medium.

For the *L. lactis* NZ9000 strain, the growth curve in the MRS medium is different from that obtained with gM17 and from that obtained for *L. lactis* NCDO 712. A latency phase of 2 h is observed, followed by a first phase of growth acceleration (up to 10 h of culture) then a second, until the stationary phase is reached after 20 h of culture (results not shown). Harvesting of the bacterial biomass (after 16 h of culture) is therefore carried out when the bacterial cells are in the growth phase. The biomass present during the harvest in the MRS medium should therefore be much lower than that obtained with the gM17 medium. The initial non-optimal pH of the gM17 medium caused a strong acid stress for this strain, which resulted in weak growth and a longer adaptation to this culture medium than that observed in the *L. lactis* NCDO 712 strain.

The growth curves obtained in this study as well as the pH variations during cultivation were very consistent with previous study from the literature. Lui *et al*. (2005), cultivated a strain of *Lactococcus lactis* in a MRS and a M17 medium without pH regulation. After a latency phase of 3 h, the stationary growth phase was reached after 10 h in the M17 medium (an exponential phase of 7 h). Likewise, the pH varied from pH=6.8 to 5 between the start and the end of growth. Concerning the culture in the MRS medium, a lower absorbance at 600 nm (difference of 0.3) as well as a lower pH (pH=4.7) were obtained at the end of the culture in comparison with the M17 medium (*28*). In another study, a strain of *Lactococcus lactis* was grown in a modified MRS medium without pH control. As observed in our study, the stationary phase was reached after 8 h and the pH has varied during growth from pH=6.5 to 4.4 (*29*).

A comparison of Figs. 1 and 2 shows that the pH of the extracellular medium varies as a function of the growth of the strains. Indeed, the acidification of the culture medium is correlated with the onset of bacterial growth and increases in proportion to this growth. The slowing down and then the stop of growth also seem to correlate with the stabilization of the pH at the minimum observed values. As widely documented, during lactic acid bacteria biomass production, acidic pH is the main growth inhibiting factor (*21*). In lactic acid bacteria such as lactococci, the production of ATP for cell multiplication is accompanied by the production of lactic acid. The externalization of lactic acid acidifies the external environment and is responsible for the acidification of the culture media observed. From a certain external pH, the bacterial cells are no longer able to maintain an internal pH at a value allowing for growth (*21*). The internal acidification produced can reduce the activity of

certain enzymes and cause damage to proteins and to DNA. This acid stress can therefore result in slower growth and cell death. This close relationship between pH and growth exists whatever the medium considered. The pH of a culture medium is therefore a predictive variable of the biomass and takes into account the composition of the culture medium.

Moreover, as the growth of *L. lactis* NZ9000 was much more affected than the growth of *L. lactis* NCDO 712 in MRS medium, it would seem that this strain is more sensitive to acidic conditions during cultivation. Our hypothesis concerning a difference in sensitivity to acid stress between the two strains is thus confirmed; the NZ9000 strain is more sensitive to acidic conditions during cultivation than the NCDO 712 strain. This difference can be explained by comparing the genomes of *L. lactis* NCDO 712 and *L. lactis* NZ9000 (*18*,*30*). Thus, mutations observed in the *L. lactis* NZ9000 strain, affecting the function of proteins, code in particular for: glyceraldehyde-3-phosphate dehydrogenase (*gapB*), proteins involved in the transport and metabolism of amino acids (ex: *aroD, gltX*), and the chaperone protein GroEL (*groEL*).

The involvement of proteins for the metabolism of amino acids encoded by the *gltX* and *aroD* genes in the resistance and adaptation to acid stress, *via* their contribution to the synthesis of ATP, can therefore be presumed. Among the genes involved in the acid stress response of *L. lactis*, *groEL* has also been identified (*31*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been recognized as playing an important role in bacterial resistance to acid stress, providing the ATP necessary to maintain homeostasis (*32*). Consequently, mutations affecting the *gltX, aroD, groEL* and *gapB* genes in the *L. lactis* NZ9000 strain may explain its increased sensitivity to acidic conditions during cultivation compared to the *L. lactis* NCDO712 strain.

Influence of pH profiles on the survival of strains to the freeze-drying process

Fig. 3 shows the cultivable produced biomass (CFU/mL) and the final cultivable biomass after freeze-drying (CFU/mL) for the *L. lactis* NCDO 712 strain (Fig. 3a) and the *L. lactis* NZ9000 strain (Fig. 3b) according to culture medium (gM17 and MRS).

Fig. 3

The biomass produced in the gM17 medium is significantly higher ($p<0.05$, ANOVA) in the *L. lactis* NCDO 712 strain (Fig. 3a) than in the *L. lactis* NZ9000 strain (Fig. 3b). In both strains, the biomass produced (CFU/mL) after 16 h of culture in the MRS medium is significantly lower ($p<0.05$, ANOVA) than that present after 16 h of culture in the gM17 medium (Fig. 3), a finding which is in agreement with the results obtained via absorbance (Fig. 2). For the *L. lactis* NCDO 712 strain, the final biomass after freeze-drying (CFU/mL) is not significantly different ($p > 0.05$, ANOVA) between the two culture media (Fig. 3a). For the *L. lactis* NZ9000 strain (Fig. 3b), the final biomass after freeze-drying

(CFU/mL) is significantly lower whenthe culture was carried out in MRS medium (MRS: 5.1 × 10^7 CFU/mL *vs*. gM17: 1.1 × 10^9 CFU/mL).

Fig. 4 shows the freeze-drying survival rate (%) for *L. lactis* NCDO 712 (Fig. 4a) and *L. lactis* NZ9000 (Fig. 4b) according to culture medium (gM17 or MRS).

Fig. 4

Regarding the survival rates in the freeze-drying process (Fig. 4), they are significantly higher ($p<0.05$, ANOVA) with the MRS culture medium (*L. lactis* NCDO 712: 108 %±4 %; *L lactis* NZ9000: 94 %±13 %) than with culture in the gM17 medium (*L. lactis* NCDO 712: 55 %±10 %; *L. lactis* NZ9000: 69 %±12 %). In both strains of *L. lactis*, survival to the freeze-drying process is significantly higher ($p<0.05$, ANOVA) after culture in the MRS medium. Our hypothesis was therefore that culture in the MRS medium, *i.e*. under a more acidic conditions than in the gM17 medium, was responsible for increasing the survival of these *L. lactis* strains to freeze-drying. The components of the MRS and gM17 media may have played a role in the freeze-drying survival of the strains. However, some studies on lactobacilli have shown that an acid stress during cultivation increased their survival to freezing or freeze-drying (*6-9*). Nevertheless, the influence of media components on the survival of these two strains to freeze-drying could be investigated in future experiments.

Although the production of biomass for the *L. lactis* NCDO 712 strain is significantly lower in the MRS medium (MRS: 1.2 × 10^9 CFU/mL vs. gM17: 2.5 × 10^9 CFU/mL), the survival rate to freeze-drying is significantly higher (MRS: 108 % *vs*. gM17: 55 %), and the cultivable biomass after freeze-drying therefore remains high (MRS: 1.2 × 10^9 CFU/mL *vs*. gM17: 1.4 × 10^9 CFU/mL). In this strain, the importance of culture conditions, not in maximizing the production of biomass but in maximizing survival in the freeze-drying process, is therefore verified. The same observation was made in Shao *et al*. (*7*) study where, even if at the end of fermentation, the biomass of the *Lactobacillus delbrueckii* strain was lower for pH=5.1 than for pH=5.7, freeze-drying survival was higher, which resulted in a higher viable biomass after freeze-drying. In the case of *L. lactis* NZ9000, even if culture conditions made it possible to obtain a high survival to freeze-drying (MRS: 94 % *vs.* gM17: 69 %), the biomass produced in the MRS medium is nonetheless very low at the end of culture (MRS: 5.4 × 10^7 CFU/mL *vs.* gM17: 1.6 × 10^9 CFU/mL). It thus seems that for this strain, culture conditions were too stressful (initial pH of the MRS medium<initial pH of the medium gM17) for the production of biomass. Optimization of culture conditions encouraging good production of biomass and survival to the freeze-drying process would therefore be an essential step in any industrial use of this strain. Several strategies may be developed: culture in a medium with a non-optimal but less acidic pH (between 5.9<pH<6.3 with or without pH regulation), an acid shock at the end of the culture, or a culture until

the end of the exponential phase conducted under optimal pH conditions, followed by acid stress for a few hours.

In order to understand the origin of the improved survival of the strains in the freeze-drying process following acidic conditions during cultivation, the structural and functional properties of the bacterial plasma membrane were examined. Although acid stress can also have consequences on cellular homeostasis and in particular on the increased production of ATP, the implication of the plasma membrane in the resistance to the process of freeze-drying seems a stronger hypothesis. Indeed, the plasma membrane is the main target of the cellular damage that occurs during a dehydration/rehydration cycle. The organization of membrane phospholipids in the liquid-crystalline phase or in the gel phase varies depending on temperature and degree of hydration. This is why, during dehydration, the withdrawal of water leads to the destabilization of the membrane structure and a transition from the liquid-crystalline phase to a gel phase, resulting in membrane rigidification. The coexistence of the different lipid phases has been identified as responsible for a reduction in the resistance of the membrane to shear forces and therefore for the possibility of cell permeabilization during dehydration (*16*,*23*). In addition, the cell shrinkage caused by dehydration leads to an increase in the surface area/volume ratio of the cells. Due to the low lateral compressibility of the plasma membrane, the membrane deforms and invagination are formed, leading to the formation of intracellular vesicles responsible for a loss of membrane surface (*33*). This loss of surface is at the origin of a permeabilization of the membrane during the rehydration phase (*34*,*35*). The permeabilization of the membrane during rehydration explains cell death. The deformation of the membrane as well as its mechanical resistance therefore depend on its properties and in particular on its fluidity (*16, 36-38*). A study of membrane fluidity was therefore undertaken.

Modulation of membrane fluidity after cultivation with the two pH profiles and impact on survival to freeze-drying

Estimation of the membrane fluidity of the two strains

Fig. 5 shows the fluorescence anisotropy values according to temperature and culture medium for the strain *L. lactis* NCDO 712 (Fig. 5a) and the strain *L. lactis* NZ9000 (Fig. 5b).

Fig. 5

The anisotropy values vary with temperature in both strains, whatever the culture medium. The lower the temperature, the higher the anisotropy values. The anisotropy values being inversely related to membrane fluidity, the plasma membrane becomes more rigid with the lowering of the temperature.

Influence of pH profiles during cultivation on membrane fluidity

The culture medium has a significant influence ($p<0.05$, ANOVA) on the anisotropy values in both strains, and these values are lower for bacterial strains cultivated in MRS medium. Hence, membrane fluidity of the two *L. lactis* strains was higher after cultivation in the MRS medium than after cultivation in the gM17 medium. Therefore, a more acidic condition during two *L. lactis* strains cultivation increased bacterial membrane fluidity.

Moreover, for *L. lactis* NZ9000, the pH in the MRS medium at harvest time was higher than the pH in the gM17 medium at the harvest time. However, *L. lactis* NZ9000 membrane fluidity was higher after cultivation in the MRS medium than in the gM17 medium. These results reveal that the membrane fluidity of *L. lactis* NZ9000 do not depend solely on the final pH but rather result from the acidic conditionsduring the whole culture.

Influence of membrane fluidity in freeze-drying survival and role of pH profiles

Fig. 6 presents the anisotropy value obtained for each condition as a function of bacterial survival rate to freeze-drying for the two strains.

Fig. 6

A link between the anisotropy values and the survival to freeze-drying is observed: the lower the anisotropy value (*i.e.*, the more fluid the membrane), the higher the freeze-drying survival rate. There even seems to be an anisotropy value threshold (≈ 0.28) below which freeze-drying survival is greater than 80 %. Meneghel *et al*. (*39*,*40*) have also demonstrated that a higher membrane fluidity allowed better survival of *Lactobacillus delbrueckii* strains to osmotic and cold stress, thus a better cryotolerance. In our study, modification of membrane fluidity was induced by means of an acid pre-stress. Culture of the *L. lactis* strains in the MRS medium, *i.e*. under a more acidic condition than in the gM17 medium, resulted in higher bacteria membrane fluidity. These results were in agreement with those of Wang *et al*. (*7*) with a *Lactobacillus acidophilus* strain. An acid stress during cultivation has increased membrane fluidity, which has resulted in an improved cryotolerance of the strain. Others studies have also shown that modulation of fatty acid composition after an acid pre-stress during cultivation have increased *Oenococcus oeni* strains (*14*,*15*) and *Lactobacillus* spp. strains (*6-8*) survival to freezing or freeze-drying.

However, on the contrary, some studies have shown a membrane rigidification and a decrease in the UFA/SFA ratio after an acid stress *(41,42)*. In these studies, acid shocks and strong acid stresses were performed. The decreased of membrane fluidity therefore allowed bacteria to counter the influx of protons and to better resist to acid stress (*41*). Thus, if an acid pre-stress is realized in order to increase the survival of bacterial strains to dehydration, the acid stress should not be too strong or too long to avoid corresponding membrane rigidification. Indeed, a strong membrane rigidification can increase membrane rupture during the mechanical stress caused by dehydration (*16*,*43*,*44*). Therefore, the exposure time/pH scale must be carefully chosen for achieving an acid pre-stress during cultivation. To *et al*. (2011), have shown a decrease of UFA/SFA ratio and an increase of CFAs after a culture at pH=5 of a *Lactococcus lactis* strain, but no significant difference in membrane fluidity compared to a culture at pH=7. Nevertheless, the authors have suggested that *L. lactis* adaptation to stresses depends on membrane fluidity modulation by a balance between the amounts of palmitoleic, cis-vaccenic and lactobacillic acids (*45*). Therefore, in further experiments, it would be interesting to determine the composition of membrane fatty acids, and particularly those three previously mentioned, after culture with our two pH profiles. Some others studies have observed that membrane rigidification allows better bacterial survival to freezing or long term storage (*46*,*47*). However, results from Velly *et al*. (*46*) and Louesdon *et al.* (*47*) are consistent with our study. In these studies, fluorescence anisotropy measurements were performed on cells harvested at different growth phases (from the middle exponential phase to the late stationary phase) and bacterial membrane fluidity was found lower in bacteria harvested in stationary phase than in bacteria harvested in exponential phase. For the authors this membrane rigidification was correlated to a higher bacterial resistance to freeze-drying and storage. Nevertheless, several others morphological and physiological changes in cells occur between the exponential phase and the stationary phase and could be at the origin of such an increased resistance. Better bacteria resistance of cells harvested in the stationary phase to numerous stresses is generally explained by several factors: the activation of the sigma factor, the synthesis of proteins, the modification of the cell wall and cell cytoplasmic membrane, the modification of the protein/lipid ratio in the membrane, the accumulation of compatible solutes, *etc*. (*48*). In our study, cells were always harvested at the same time in stationary phase which prevents from any bias due to the culture growth phase.

The comparison of our results with the literature confirms the main role of acidic conditions during cultivation on the survival of bacterial strains to a freeze-drying process by membrane fluidity modulation. Higher fluidity allows the maintenance of the structural and dynamic properties of the membrane as well as the functionality of membrane proteins during dehydration and rehydration.

CONCLUSIONS

Using an acid medium during cultivation of two strains of *Lactococcus lactis* is an effective strategy to significantly increase their resistance to a freeze-drying process. Our results show that the freeze-drying resistance of the *L. lactis* strains depends on the maintenance of the structural and mechanical properties of the plasma membrane and in particular on the membrane fluidity. The rigidification of the membrane, a consequence of the withdrawal of water during dehydration, reduces the mechanical resistance of the membrane to shear forces and to variations in cell volumes which take place during a freeze-drying/rehydration cycle. Moreover, our results revealed that using an acid medium during the culture of the two strains of *L. lactis* fluidifies the membrane and allows a better survival of both strains to freeze-drying. Optimization of the culture conditions must be made carefully for each bacterium and each medium to maximize the production of biomass while increasing the bacterial resistance to the freeze-drying process. For instance, moderate acidic conditions may be considered in the case of strains which cultivation is sensitive to acid stress such as *L. lactis* NZ9000. Taken together, our results suggest that, for lactic acid bacteria used as starters or probiotics, adapted pH profiles during biomass production can greatly increase their survival to the stabilization process.

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CONFLICT OF INTEREST

None

AUTHORS' CONTRIBUTION

A. Bodzen, A. Jossier, S. Dupont, L. Beney and P. Gervais designed the research. A. Bodzen and A. Jossier performed the experiments. A. Bodzen, S. Dupont and P. Gervais analyzed the data. S. Dupont, L. Beney and P. Gervais helped in writing the manuscript and revised the manuscript. A. Bodzen wrote the manuscript. PY. Mousset, L. Beney, S. Lafay and P. Gervais supervised the work. P. Gervais edited the manuscript.

ORCID ID

A. Bodzen https://orcid.org/0000-0002-2462-6871

A. Josier https://orcid.org/0000-0002-7419-8414

S. Dupont https://orcid.org/0000-0002-3737-3500

L. Beney https://orcid.org/0000-0002-2757-2506

P. Gervais https://orcid.org/0000-0002-4863-7671

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Figures

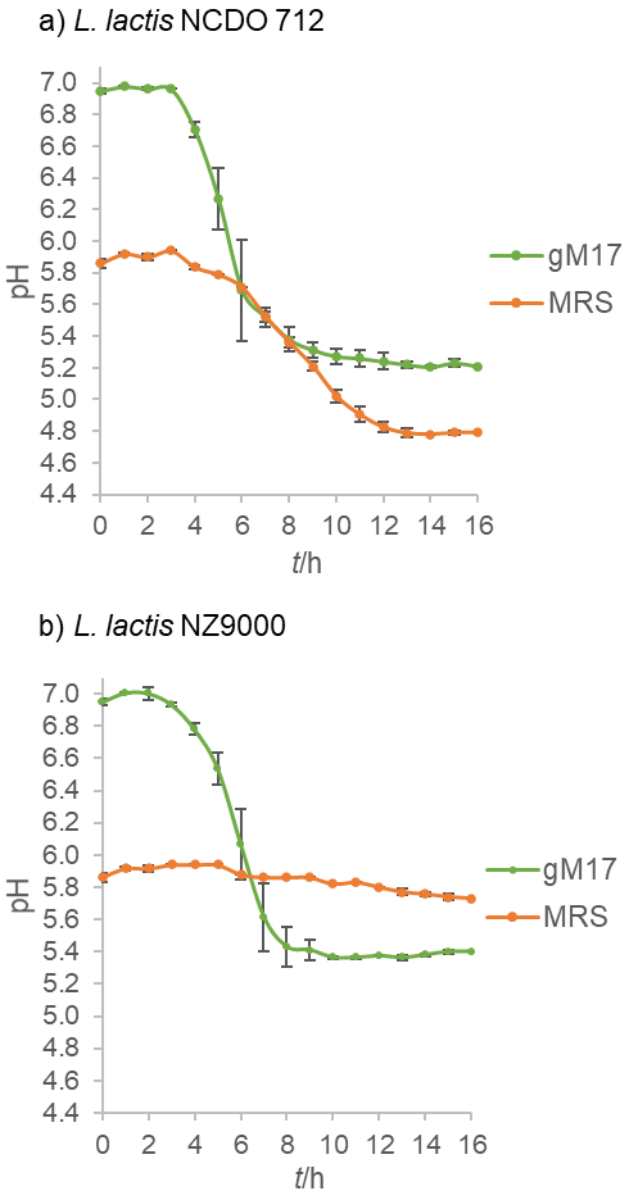

Fig. 1. pH of *Lactococcus lactis* NCDO 712 (a) and of *Lactococcus lactis* NZ9000 (b) over 16h of culture in media gM17 (green) and MRS (orange). Values represent mean±standard deviation obtained from independent triplicates

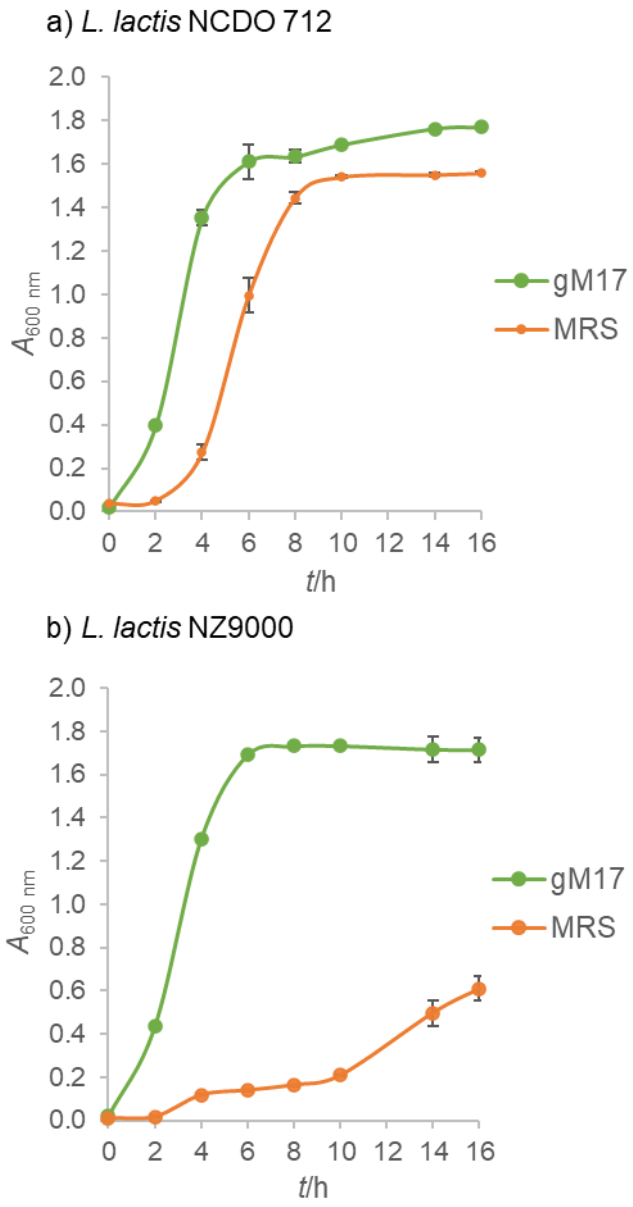

Fig. 2. Absorbance at 600 nm of Lactococcus lactis NCDO 712 (a) and Lactococcus lactis NZ9000 (b) over 16h of culture in media gM17 (green) and MRS (orange). Values represent mean±standard deviation obtained from independent triplicates

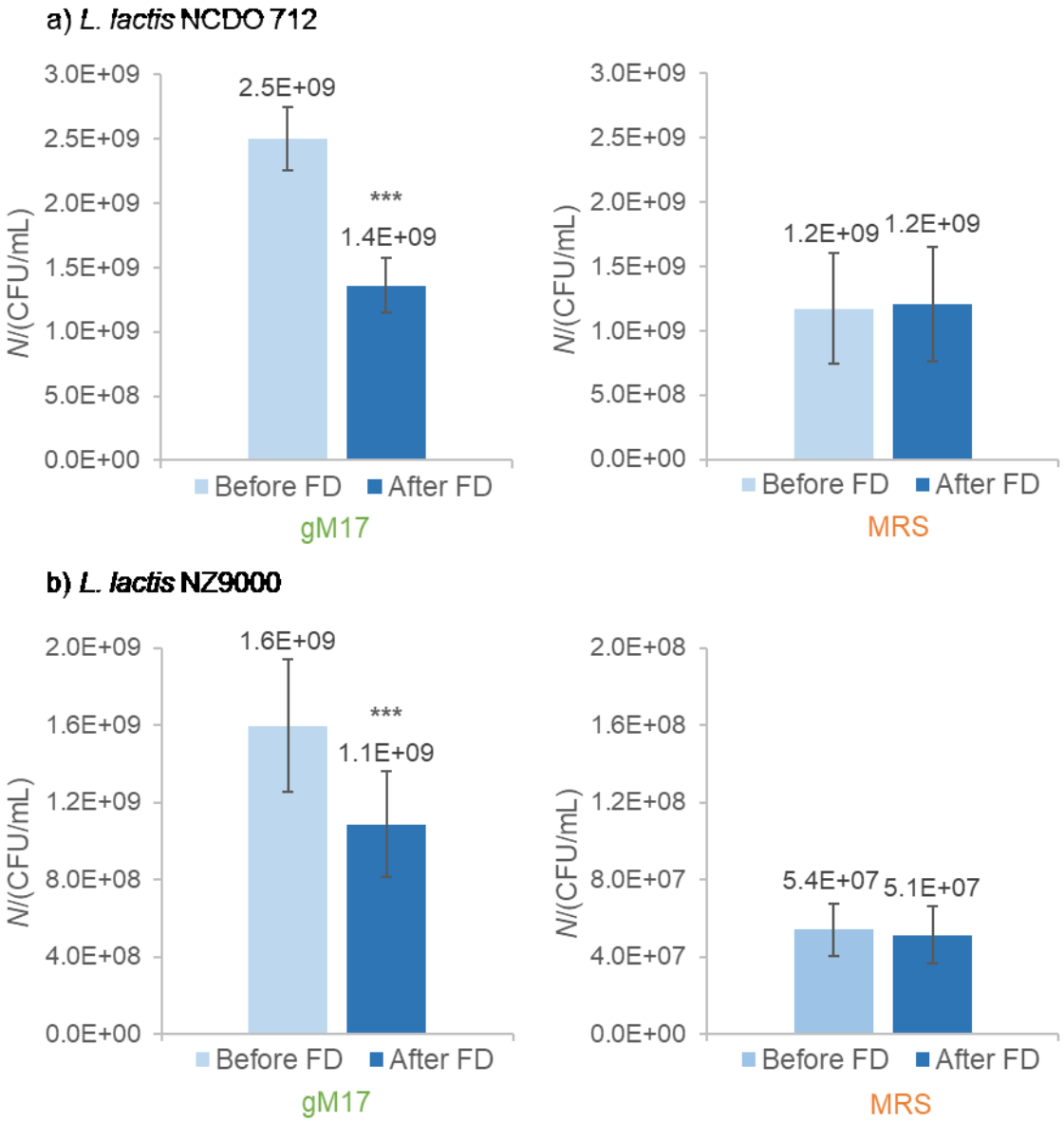

Fig. 3. Cultivable produced biomass in CFU/mL (light blue) and final cultivable biomass after freeze-drying in CFU/mL (dark blue) for *Lactococcus lactis* NCDO 712 (a) and *Lactococcus lactis* NZ9000 (b) as a function of the culture medium (gM17 and MRS). FD: Freeze-Drying. The asterisks indicate a significant difference between produced biomass and final biomass after freeze-drying (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, ANOVA). Values represent mean±standard deviation obtained from independent triplicates

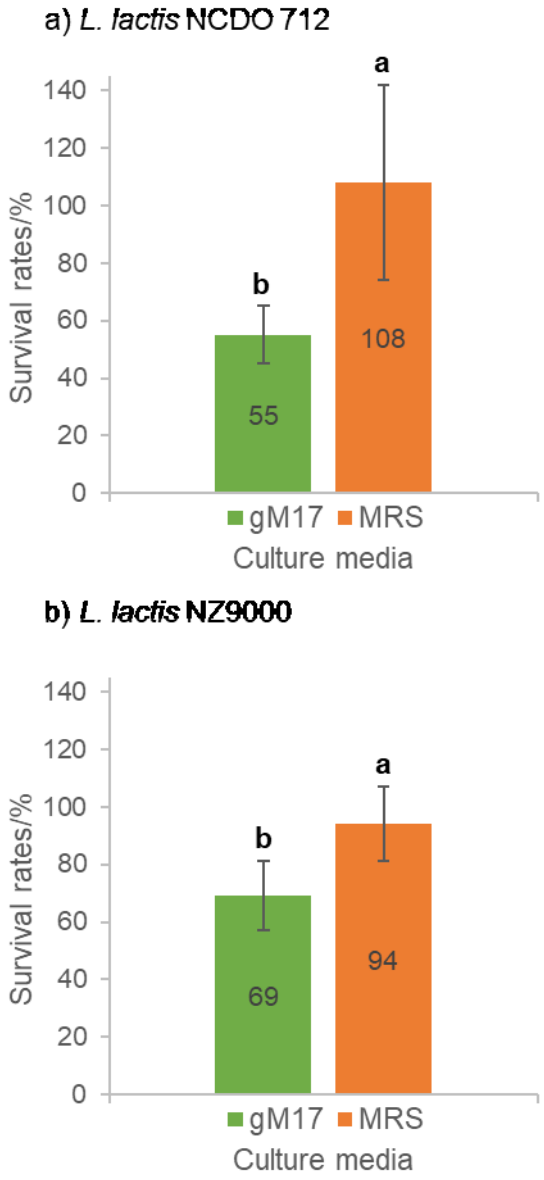

Fig. 4. Freeze-drying survival rate (%) of *Lactococcus lactis* NCDO 712 (a) and *Lactococcus lactis* NZ9000 (b) as function of the culture medium (gM17 in green and MRS in orange). The asterisks indicate a significant difference between the two conditions (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, ANOVA). Values represent mean±standard deviation obtained from independent triplicates

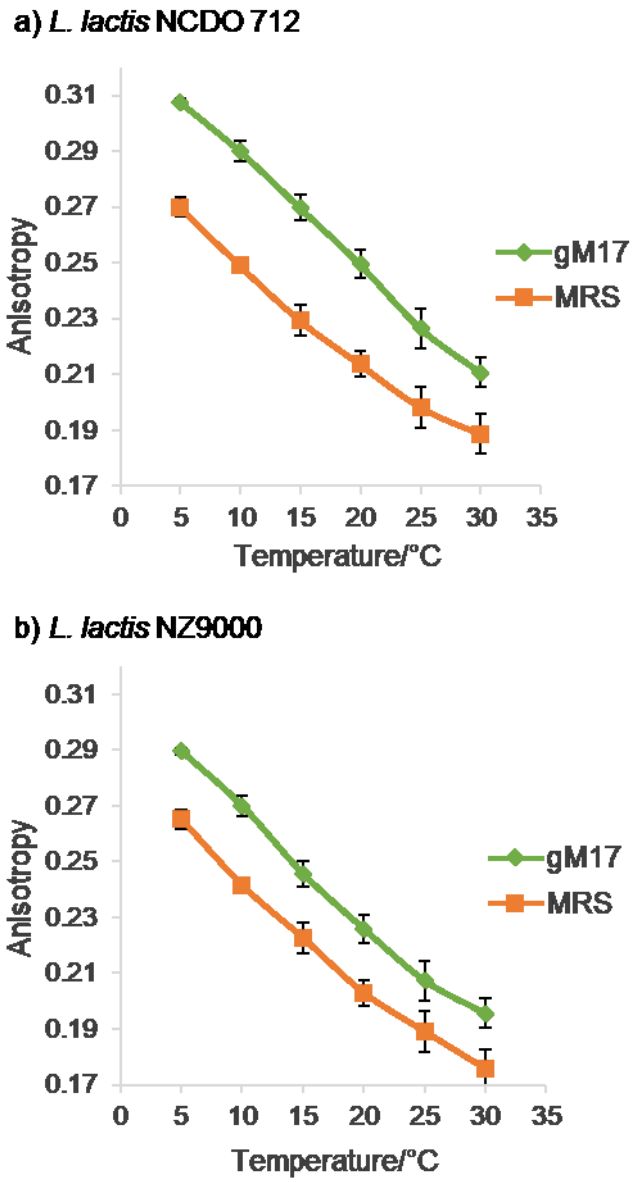

Fig. 5. Anisotropy of *Lactococcus lactis* NCDO 712 (a) and *Lactococcus lactis* NZ9000 (b) as function of the culture medium (gM17 in green and MRS in orange) and temperature (from 5 to 30 °C). Values represent mean±standard deviation obtained from two independent experiments

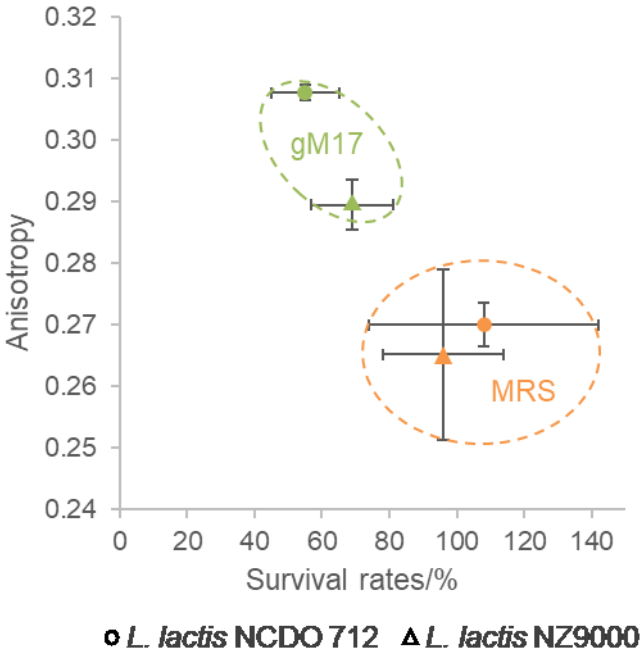

Fig. 6. Fluorescence anisotropy at 5 °C of *Lactococcus lactis* NCDO 712 (circles) and of *Lactococcus lactis* NZ9000 (triangles) according to their survival rate to freeze-drying (%) and to the culture medium (gM17 in green and MRS in orange)

Table 1. Duration of the culture for each medium during which the *Lactococcus lactis* NCDO 712 and *Lactococcus lactis* NZ9000 strains were at optimal pH (6.3<pH<7), at acidic pH but allowing growth (5.3<pH<6.3) and pH below which growth is limited (pH<5.3)

	gM17		MRS	
	NCDO 712	NZ9000	NCDO 712	NZ9000
Time spent at pH 6.3<pH<7 (h)	5	5	0	0
Time spent at pH 5.3<pH<6.3 (h)	4	11	8	16
Time spent at pH<5.3 (h)	7	0	7	0
Total time of culture (h)	16	16	16	16