Annals of Microbiology, **57 (4)** 537-544 (2007)

Preservation by freezing of potentially probiotic strains of *Lactobacillus rhamnosus*

Mariantonietta SUCCI*, Patrizio TREMONTE, Anna REALE, Elena SORRENTINO, Raffaele COPPOLA

Dipartimento di Scienze e Tecnologie Agro-alimentari, Ambientali e Microbiologiche, Università degli Studi del Molise, Via De Sanctis, 86100 Campobasso, Italy

Received 30 May 2007 / Accepted 22 October 2007

Abstract - The aim of this work was to detect the best conditions to preserve by freezing potentially probiotic strains of *Lactobacillus rhamnosus* isolated from food. Four strains isolated from Parmigiano Reggiano cheese, the commercial strain *Lactobacillus* GG and the type strain ATCC 7469T were used in the present study. Two different pre-incubation times (5 and 24 h), three protective media (Skim milk, Skim milk plus glucose and MRS plus glycerol) and two storage temperatures (-20 and -80 °C) were used for a preservation period of 90 days. A sensible loss of survival of the strains was detected and the acidifying activity decreased depending on the different factors analysed. Moreover, plate counts performed in MRS plus bile salts evidenced that a considerable percentage of cells suffers damages deriving from cold. This study showed that the growth phase of the cells plays an important role for the resistance to the storage by freezing. Finally, Skim milk had the best protective action, showing the highest activity at –80 °C.

Key word: cryotolerance, freezing, protective media, probiotic, lactobacilli.

INTRODUCTION

Lactic acid bacteria are involved in many technological processes regarding fermented foods. The low viability of micro-organisms ascertained in many commercial preparations have led researchers to study new methods for their preservation, so that the highest number of strains results alive at the moment of their use as starter cultures.
Actually, freezing represents an easy and not very costly tool for the starter preservation (Coppola *et al.*, 1996) and especially the dairy industry frequently makes use of frozen starters because of the possibility of their direct use in fermenter or in vat, without risks of contamination (Gilliland and Speck, 1974; Monnet *et al*., 2003).

Nowadays, selection criteria of strains for their use as starters in the production of fermented foods also include the ability to survive stress factors, such as low storage temperatures, preserving their biochemical and technological characteristics (Van De Guchte *et al.*, 2002).

Over the past years, particular attention was paid to bacterial cell damages deriving from the freezing-thawing, including a lethal effect, an inhibition of the growth and a reduction or loss of a metabolic activity (sublethal effect) (Fernández Murga *et al.*, 2000). As a result, a frozen starter could consist of viable cells (active after freezing-thawing process), damaged cells (alive but characterised by a reduction or a loss of a particular activity) and dead cells (Smittle *et al.*, 1972; Gilliland and Lara, 1988).

* Corresponding author. Phone: +390874404871;
Fax +390874404652; E-mail: succi@unimol.it

Gram-positive bacteria react to the freezing in different manners but differences can also be appreciated among strains belonging to the same species (Gibson *et al.*, 1965; Smittle *et al.*, 1972; Leach and Sandine, 1976; Thunnell *et al.*, 1984). Moreover, the same strain could display a different cold-shock response depending on its physiological state or on the composition of the preservation medium (Smittle *et al.*, 1972; Smittle *et al.*, 1974; Golberg and Eschar, 1977; Klaenhammer and Kleeman, 1981; Wright and Klaenhammeret, 1983; Johnson *et al.*, 1984).

Many studies focusing on the ascertainment of effects deriving from preservation temperatures established the lowest storage temperature, the highest cell survival. Moreover, the mortality of the cells increases with the cryo-preservation time (Gibson *et al.*, 1965; Smittle *et al.*, 1972) while the cooling rate plays a fundamental role in the definition of possible damages deriving from cold (Mazur, 1977; Tomás *et al.*, 2004).

However the bacterial resistance to freezing temperatures is strictly correlated to the medium composition used for the cryo-preservation: different substances, such as Skim milk (de Antoni *et al.*, 1989; Foschino *et al.*, 1996; Kim and Dunn., 1997), glycerol (Accolas and Auclair, 1967; Smittle *et al.*, 1972; Font de Valdéz *et al.*, 1983; Fonseca *et al.*, 2000) and sugars (Stadhouders *et al.*, 1971; Chavarri *et al.*, 1988) play protective roles; on the other hand, low pH values and high saline concentrations exert negative effects on the cells (Van De Guchte *et al.*, 2002).

Nowadays these themes are more and more important because of the utilisation in the food industry of starters containing probiotic micro-organisms preserved by freeze-

drying or by freezing. These starter cultures have to be strictly investigated for possible changes in physiological and biochemical features due to the freezing and the frozen storage.

The present study aimed to ascertain the freezing effect on *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese at the end of the ripening and selected on the basis of probiotic features, such as the antibiotic susceptibility, the ability to produce antimicrobial substances, and the capability to survive at low pH and in presence of bile salts (Coppola *et al.*, 1996, 2005; Succi *et al.*, 2005). The reason for focusing on *Lactobacillus rhamnosus* species is tied to many studies, in which their occurrence during cheese ripening (Zago *et al.*, 2007) or their probiotic characteristics are reported (Goldin *et al.*, 1992; Saxelin *et al.*, 1993; Millar *et al.*, 1993; Salminen *et al.*, 1996; Korpela *et al.*, 1997; Majamaa and Isolauri, 1997, Saxelin, 1997).

MATERIALS AND METHODS

Bacterial strains. Four *Lactobacillus rhamnosus* strains (CT1, GT1/1, OT1/3 and VT1/1) from the DISTAAM collection (University of Molise), isolated from Parmigiano Reggiano cheese and selected on the basis of their potentially probiotic features (Coppola *et al.*, 1997, 2000, 2005; Succi *et al.*, 2005), *L. rhamnosus* GG, isolated from a pharmaceutical preparation (Valio LTD, Helsinki, Finland), and *L. rhamnosus* type strain ATCC 7469^T were used in the present study. Strains were maintained in tubes of MRS agar (5 g/L, Oxoid, Milan, Italy) at 4 °C and propagated twice in MRS broth (Oxoid, Milan, Italy) for 16 h at 37 °C before the use.

Preservation conditions. Each strain from overnight cultures was 1% inoculated into 3 tubes containing 20 mL of MRS broth. After incubation for 5 h at 37 °C (IT5), cells were harvested by centrifugation at 9000 rpm for 10 min and washed twice in Ringer's solution. Pellet from the 3 tubes was suspended into 3 tubes containing respectively 20 mL of Skim milk (Oxoid) or MRS broth plus 20% glycerol (v/v) (Sigma-Aldrich, Milan, Italy) or Skim milk plus 0.5% glucose (v/v) (Sigma-Aldrich). Before the use, glycerol was sterilised by autoclaving at 121 °C for 15 min while glucose was sterilised by filtration using 0.20 mm filters (Schleicher and Shuell, Germany).

After a drastic agitation on vortex aliquots of 2 mL from the homogenised suspensions for each medium were transferred in cryogenic vials. Therefore, for each strain at IT5, were obtained ten vials for each preservation medium: five were stored at −20 °C and other five at −80 °C.

An identical procedure was adopted on the same strains incubated for 24 h at 37 °C (IT24) instead of IT5.

Experiments were replicated three times for each strain.

Survival during freezing and frozen storage. The survival of the assayed strains was determined in MRS agar (15 g/L) before storage and 1, 30, 60 and 90 d after storage at -20 and -80 °C in the substrates described above. For this purpose, cryogenic vials were thawed at room temperature and the subsequent microbial counts were performed in MRS agar plates incubated at 37 °C for 48-72 h. Each experiment was replicated three times for each strain and results were expressed (as the mean of measurements) in terms of mortality.

The occurrence of damages to the cells during the storage was evaluated by plate counts in MRS agar supplemented with 1% bile salts (Oxoid) as described above. Plates were incubated at 37 °C for 7 d. Microbial counts were reported as ratio between values registered at each observation time and values detected before freezing in MRS agar added of 1% bile salts. Values were reported as percentages of cells unable to grow in the presence of bile salts at time zero. Each experiment was replicated three times for each strain and results were expressed (as the mean of measurements) in terms of mortality.

Evaluation of the acidifying ability. *Lactobacillus rhamnosus* strains were evaluated for their acidifying activity before storage and after 1, 30, 60 and 90 days of storage at −20 or −80 °C in the different media. Acidfying ability was evaluated after incubation at 37 °C for 24 h in MRS broth (pH 6.2) and in MRS broth plus 1% bile salts (1% inoculum) using a pH-meter (Crison). The results were expressed in terms of ΔpH (difference between the initial value of non-inoculated medium and final values after acidification). Each experiment was replicated three times for each strain and results were expressed as the mean of measurements and standard error.

RESULTS AND DISCUSSION

Survival freezing and frozen storage

Results illustrated in the Fig. 1 highlighted a strain-specific behaviour. Generally, *L. rhamnosus* type strain ATCC 7469^T showed the highest mortality independently by the different conditions of storage, while the lowest one was evidenced by strain GT1/1.

In particular, after one day, the strain ATCC 7469 had the lowest mortality in Skim milk or in Skim milk plus glucose after IT24 and preservation at -80 °C. On the other hand, the storage in MRS plus glycerol at −20 °C, IT24, produced the highest mortality, more than 90% starting from the 30th d until the end of the storage.

The strain GG showed the lowest mortality for IT5 and preservation in Skim milk at -80 °C; the highest rate was observed for IT24 and storage in Skim milk plus glucose at -20 °C.

Until the 60th d, the strain CT1 had the lowest mortality for IT5 and storage in Skim milk at -80 °C, whereas after 60 d the lowest rate was registered for IT24 and storage at -80 °C in Skim milk. During the entire storage, the highest mortality was reached in MRS plus glycerol at -20 °C and IT24 for the entire storage.

The strain GT1/1 had the lowest mortality for IT5 and storage in Skim milk at -80 °C. After 1 d and between 60 and 90 d of storage, the highest rate was evidenced in Skim milk plus glucose, IT5 and storage at -20 °C, whereas during the period 1-30 d, it was observed for IT24 and preservation in Skim milk plus glucose at -80 °C.

Until the 30th d, the strain OT1/3 showed the lowest mortality for IT24 and storage in Skim milk plus glucose at -80 °C; after 60 d, the lowest value was observed in Skim milk at -80 °C for IT24. Finally, at 90 d the lowest rates were observed in Skim milk at -80 °C, for both IT5 and IT24. The highest mortality was always registered for IT5 and storage in MRS plus glycerol at -20 °C.

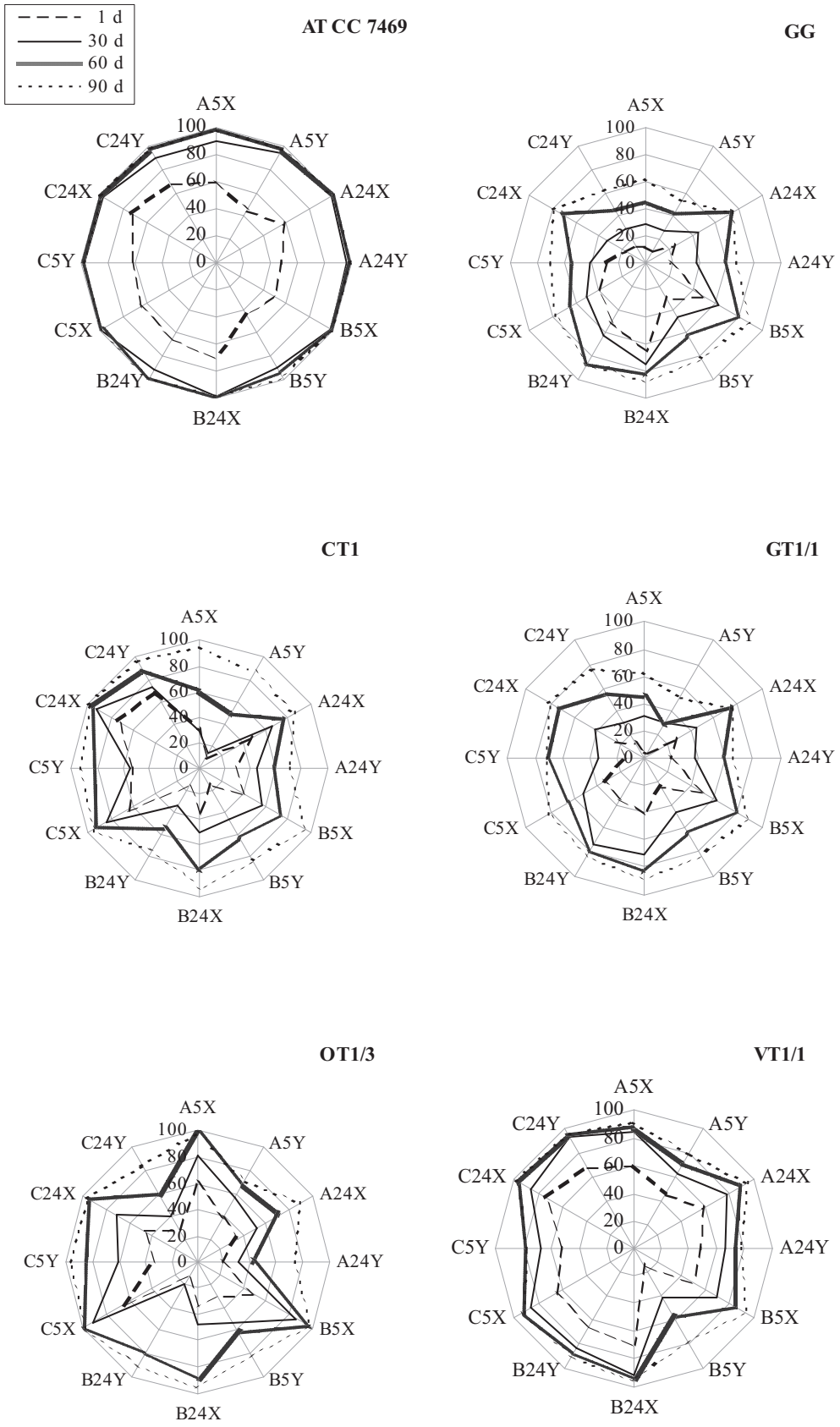

FIG. 1 - Mortality (% of died cells at time t / counts ascertained before freezing) of *Lactobacillus rhamnosus* strains stored in Skim milk (A), Skim milk plus glucose (B) or MRS plus glycerol (C), at -20 °C (X) or -80 °C (Y) after growth for 5 h (5) or 24 h (24) at 37 °C (mean of triplicate results).

During the entire storage, the strain VT1/1 showed the lowest mortality in Skim milk plus glucose stored at -80 °C after IT5. The highest mortalities were instead evidenced in MRS plus glycerol after IT24 and storage at -20 or -80 °C.

Figure 2 illustrates the mortalities during the storage, expressed in terms of mean of values for all the strains, in order to highlight common traits between the assayed strains. Counts were also performed in MRS in the presence of Bile salts to ascertain possible cold induced injuries to the permeability of cell membrane, as previously reported (Succi *et al*., 2005).

After freezing (counts carried out at 1 d of storage) it was possible to ascertain in MRS mortalities between 26 and 56%. In particular, the lowest one was observed after IT5 and subsequent storage at –80 °C in Skim milk or in Skim milk plus glucose (26 and 27% respectively); the highest mortality at this time was found out at –20 °C in MRS plus glycerol after IT5 (56%).

During the subsequent 60 d of storage, a moderate mortality was ascertained, whereas after 90 d the percentage of survivors was very low. Specifically, at 30 d values resulted between 41 and 66%: the lowest rate was obtained in Skim milk stored at -80 °C after IT5; the highest one was detected in MRS plus glycerol stored at -20 °C after IT5. At 60 d of storage, a similar behaviour was noticed: the lowest mortality (45%) was ascertained in Skim milk stored at -80 °C after IT5, whereas the highest one (77%) was found out in MRS plus glycerol stored at -20 °C after IT5. At the end of the storage (90 d), the mortality resulted very high for all the experimental conditions, with values between 84 and 97%. Lowest values were ascertained in Skim milk stored at -80 °C after IT5 (84%) or IT24 (85%).

The results evidenced that storage temperatures, as well as the different media adopted, were able to influence the survival of *L. rhamnosus* strains. In particular, the lowest mortalities were always ascertained for cells stored at -80 °C in Skim milk or in Skim milk plus glucose, after IT5. Highest values were ascertained in MRS plus glycerol stored at -20 °C after IT5.

The finding that the lowest temperature (-80 °C) is able to guarantee the highest survival is in agreement with Tomás *et al*. (2004). Fonseca *et al*. (2001) showed in their work, performed on two lactic acid bacteria utilised in the production of starter culture, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, the higher the freezing rate and the lower the storage temperature, the better the bacterial resistance to freezing and frozen storage. In another work (Fonseca *et al*., 2006), they also demonstrated that a very high cooling rate was able to increase cells viability, but only a low storage temperature (-80 °C) preserved this state. In contrast, low cooling rates would be preferred if a high storage temperature (-20 °C) was employed.

As for the media assayed in our study, Skim milk seemed to guarantee protective features higher than those ensured by Skim milk plus glucose or by MRS plus glycerol. In a study performed on *Lactobacillus delbrueckii* ssp. *bulgaricus*, Panoff *et al*. (2000) demonstrated the possibility to induce phenotypic cryoadaptation by adaptive pretreatment with osmotica known as cryoprotective chemicals, such as lactose, trehalose, and sucrose. They found that cells treated with glycerol became more sensitive to freezing and thawing, whereas the other cryoprotectants tested in their study increased the survival rate. In this experiment, the possible mechanism of cryoresistance, enhanced by pretreatment with sugars, may be a partial dehydration of the cells.

Finally, the lowest mortalities were always ascertained for cells in the exponential growth phase (5 h of incubation before freezing), even if this datum appears in contrast with other reports, showing a higher bacterial resistance when the culture is stopped in the stationary phase (Brashears and Gilliland, 1995; Fonseca *et al*., 2001).

In order to ascertain the effective number of viable and not damaged cells at different days of freezing, microbial counts were also performed in the presence of bile salts (Fig. 2).

Counts performed until the 30th d of storage resulted very similar to those performed without bile salts, generally demonstrating low injuries during this period. However, after 60 d, values found out resulted sensibly higher than those ascertained without bile salts. In particular, the lowest injury was ascertained in Skim milk stored at -20 °C after IT24 (55%) whereas the highest rates were pointed out after IT24 and storage at -80 °C in Skim milk plus glucose (82%) or in MRS plus glycerol (89%). Finally, at 90 d of storage mortalities with or without bile salts were very similar again, with percentages of mortality ranging from 84 to 99%.

Generally, the injury detected in Skim milk was lower than that detected in Skim milk plus glucose and the latest one was lower than in MRS plus glycerol.

Preservation of the acidifying ability

Values reported in Fig. 3 highlighted a moderate decrease of the acidifying ability during the storage period. In particular, the lowest loss of this activity was detected in Skim milk, the highest one in MRS plus glycerol.

Considering the acidifying activity evaluated in the presence of bile salts, it is interesting to note the sensible loss of the activity in each evaluated case, probably due to the presence of cold-stressed cells. Moreover, the addition of bile salts to the standard medium well highlighted the different protective action of media adopted in this study. In fact, after 90 d of storage, the loss of the acidifying ability was lower in Skim milk or in Skim milk plus glucose than that ascertained in MRS plus glycerol.

CONCLUSIONS

Starter cultures to be employed in the food industry are generally available in frozen or freeze-dried form. Normally, are "ready to use" cultures and their survival to the storage, as well as the preservation of their features (e.g. acidifying ability), is of great importance in order to guarantee the success of final products. Moreover, nowadays the market offers a great number of probiotic preparations, in which probiotic micro-organisms are added to the "traditional starter". Considering that the health benefits of these products are due to the presence of "healthy bacteria", it is particularly important to assay the survival of these micro-organisms in order to guarantee a high number of viable and non-stressed cells at the moment of their use.

The present work illustrated some important results regarding the preservation by freezing of potentially probiotic *Lactobacillus rhamnosus* strains. In particular, data

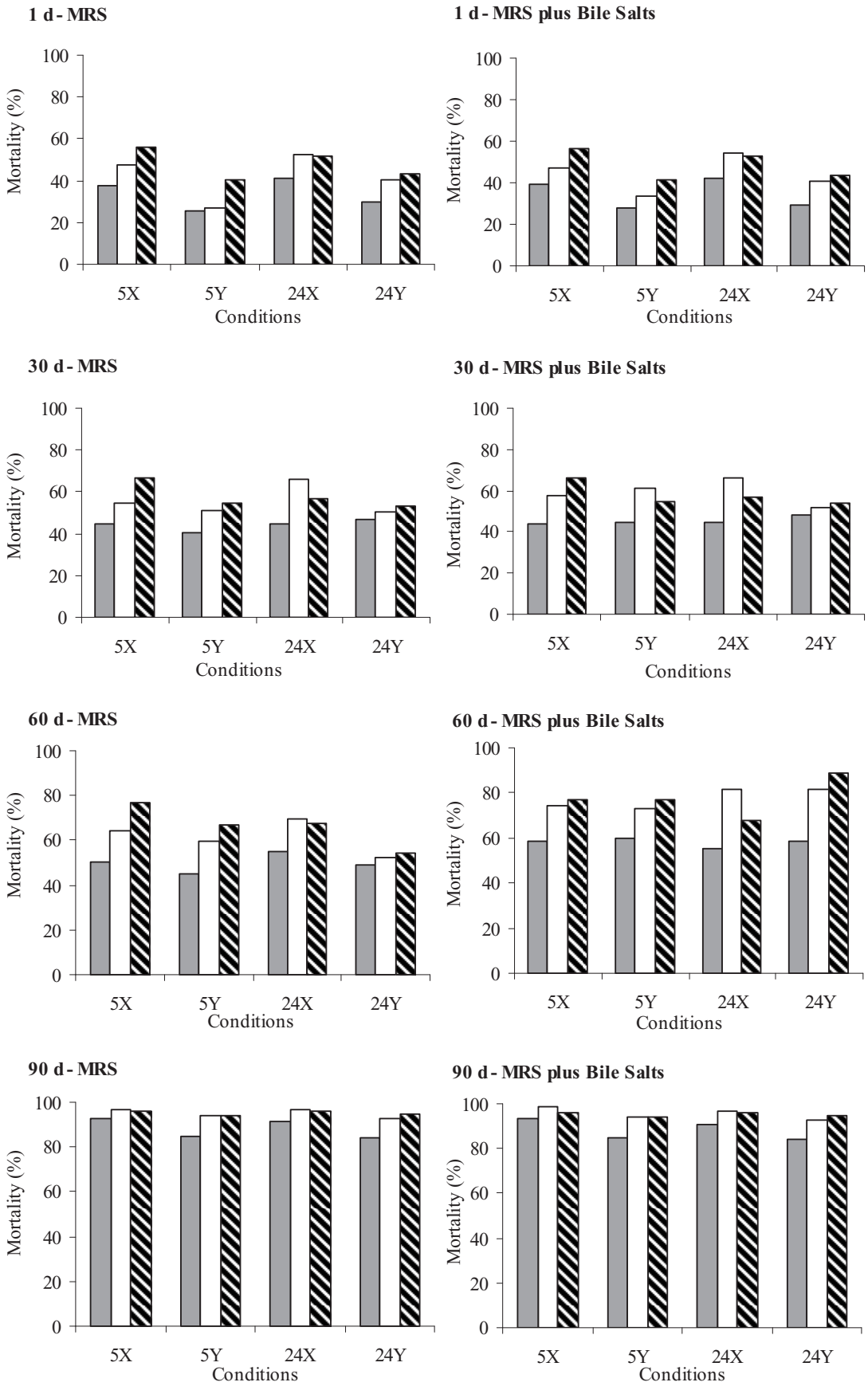

FIG. 2 - Mortality (% of died cells at time t / counts ascertained before freezing) of *Lactobacillus rhamnosus* strains evaluated in MRS or in MRS plus 1% bile salts during the storage in Skim milk (■), Skim milk plus glucose (□) or MRS plus glycerol (◪), at -20 °C (X) or -80 °C (Y) after growth for 5 h (5) or 24 h (24) at 37 °C (mean of triplicate results obtained on 6 different strains).

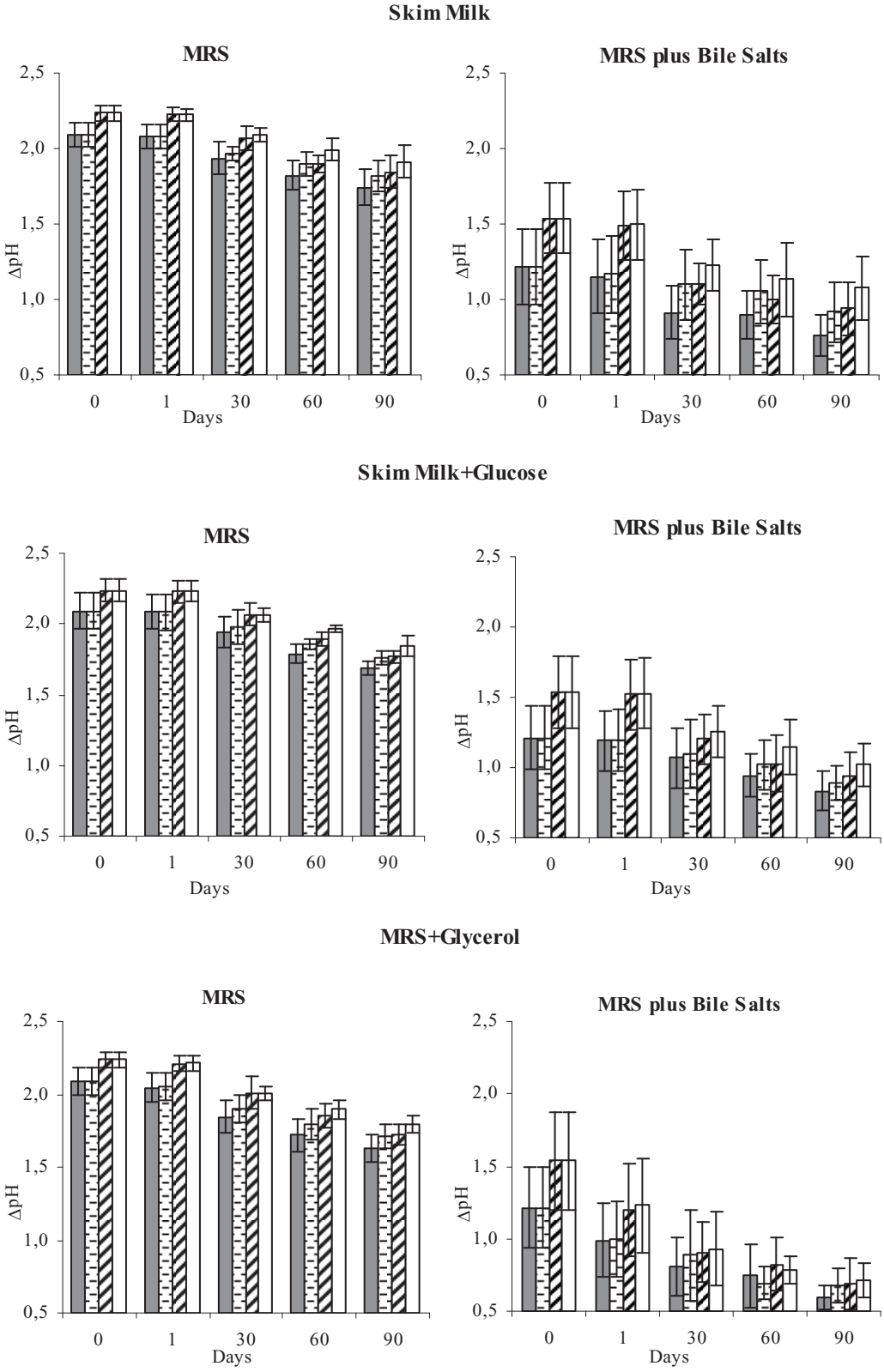

FIG. 3 - Acidifying ability in MRS broth or in MRS broth plus 1% bile salts (measured as pH lowering after 24 h) of 6 *Lactobacillus rhamnosus* strains after the storage in Skim milk, Skim milk plus glucose or MRS plus glycerol at -20 °C (X) or -80 °C (Y) after growth for 5 h (5) or 24 h (24) at 37 °C. ▩ 5X, ▦ 5Y, ▨ 24X, □ 24Y (results expressed as the mean of 3 measurements and standard error).

obtained made possible to detect a strain-specific response to the different conditions adopted in this study that demonstrate the importance to test each strain before the use as starter culture. However it was possible to highlight some common factors to preserve the assayed strains. In fact, between the adopted media, Skim milk showed the highest protective action and, amongst freezing temperatures, the lowest one (-80 °C) seemed to be the best. Moreover, the IT5 instead of 24 generally gave a better survival.

The storage time also demonstrated to be of great importance. In fact, the highest rates of mortality were ascertained after one (probably due to the freezing) and 90 d of storage, whereas similar counts were generally detected at 30 and 60 d. Finally, the highest damages appeared after 60 d of storage and the stress resulted particular evident in MRS plus glycerol.

REFERENCES

Accolas J.P., Auclair J. (1967). Storage of highly concentrated suspensions of lactic acid bacteria in the frozen state. Lait, 47: 253-260.

Brashears M.M., Gilliland S.E. (1995). Survival during frozen and subsequent refrigerated storage of *Lactobacillus acidophilus* cells as influenced by the growth phase. J. Dairy Sci., 78, 2326-2335.

Chavarri F.J., De Paz M., Nuñez M. (1988). Cryoprotective agents for frozen concentrated starters from non-bitter *Streptococcus lactis* strains. Biotechnol. Lett., 10: 11-16.

Coppola R., Iorizzo M., Sorrentino A., Sorrentino E., Grazia L. (1996). Resistenza al congelamento di lattobacilli mesofili isolati da insaccati e paste acide. Industrie Alimentari, 35: 349-356.

Coppola R., Nanni M., Iorizzo M., Sorrentino A., Sorrentino E., Grazia L. (1997). Survey of lactic acid bacteria isolated during the advanced stages of the ripening of Parmigiano Reggiano cheese. J. Dairy Res., 64: 305-310.

Coppola R., Nanni M., Iorizzo M., Sorrentino A., Sorrentino E., Chiavari C., Grazia L. (2000). Microbiological characteristics of Parmigiano Reggiano cheese during the cheesemaking and the first months of the ripening. Lait, 80: 479-480.

Coppola R., Succi M., Tremonte P., Reale A., Salzano G., Sorrentino E. (2005). Antibiotic susceptibility of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. Lait, 85: 1-12.

de Antoni G.L., Pérez P., Abraham A., Anon M.C. (1989). Trehalose, a cryoprotectant for *Lactobacillus bulgaricus*. Cryobiology, 26: 149-153.

Fernández Murga M.L., Cabrera G.M., Font de Valdéz G., Disalvo A., Seldes A.M. (2000). Influence of growth temperature on cryotolerance and lipid composition of *Lactobacillus acidophilus*. J. Appl. Microbiol., 88: 342-348.

Fonseca F., Béal C., Corrieu G. (2000). Method of quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. J. Dairy Res., 67: 83-90.

Fonseca F., Béal C., Corrieu G. (2001). Operating conditions that affect the resistance of lactic acid bacteria to freezing and frozen storage. Cryobiology, 43: 189-198.

Fonseca F., Marin M., Morris G.J. (2006). Stabilization of frozen *Lactobacillus delbrueckii* subsp. *bulgaricus* in glycerol suspensions: freezing kinetics and storage temperature effects. Appl. Environ. Microbiol., 72: 6474-6482.

Font de Valdéz G., de Giori G., de Ruiz Holgado A., Oliver G. (1983). Protective effect of adonitol on lactic acid bacteria subjected to freeze-drying. Appl. Environ. Microbiol., 45: 302-304.

Foschino R., Fiori E., Galli A. (1996). Survival and residual activity of *Lactobacillus acidophilus* frozen cultures under different conditions. J. Dairy Res., 63: 295-303.

Gibson C.A., Landerkin G.B., Morse P.M. (1965). Survival of strains of lactic streptococci during freezing storage. J. Dairy Res., 32: 151-156.

Gilliland S.E., Lara R.C. (1988). Influence of storage at freezing and subsequent refrigeration temperatures on b-galactosidase activity of *Lactobacillus acidophilus*. Appl. Environ. Microbiol., 54: 898-902.

Gilliland S.E., Speck L.M. (1974). Frozen concentrated cultures of lactic acid bacteria - A review. J. Milk Food Technol., 37: 107-111.

Golberg I., Eschar, L. (1977). Stability of lactic acid bacteria to freezing as related to their fatty acid composition. Appl. Environ. Microbiol., 33: 489-496.

Goldin B.R., Gorbach S.L., Saxelin M., Barakat S., Gualtieri L., Salminen S. (1992). Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. Dig. Dis. Sci., 37: 121-128.

Johnson M., Ray B., Speck M.L. (1984). Freeze injury in cell wall and its repair in *Lactobacillus acidophilus*. Cryoletters, 5: 171-176.

Kim W.S., Dunn N.W. (1997). Identification of a cold shock gene in lactic acid bacteria and the effect of cold shock on cryotolerance. Curr. Microbiol., 35: 59-63.

Klaenhammer T.R., Kleeman E.G. (1981). Growth characteristics, bile sensitivity and freeze damage in colonial variants of *Lactobacillus acidophilus*. Appl. Environ. Microbiol., 41: 1461-1467.

Korpela R., Peuhkuri K., Lahteenmaki T., Sievi E., Saxelin M., Vapaatalo H. (1997). *Lactobacillus rhamnosus* GG shows antioxidative properties in vascular endothelial cell culture. Milchwissenschaft, 52: 503-505.

Leach R.D., Sandine E. (1976). Numerical relationship between strains in frozen concentrates of lactic streptococcal starter cultures. J. Dairy Sci., 59: 1392-1397.

Majamaa H., Isolauri, E. (1997). Probiotics: a novel approach in the management of food allergy. J. Allergy Clin. Immunol., 99: 179-185.

Mazur P. (1977). The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology, 14: 251-272.

Millar M.R., Bacon C., Smith S.L., Walker V., Hall M.A. (1993). Enteral feeding of premature infants with *Lactobacillus* GG. Arch. Dis. Child., 69: 483-487.

Monnet C., Béal C., Corrieu G. (2003). Improvement of the resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* to freezing by natural selection. J. Dairy Sci., 86: 3048-3053.

Panoff J.M., Thammavongs B., Guéguen M. (2000). Cryoprotectants lead to phenotypic adaptation to freeze-thaw stress in *Lactobacillus delbrueckii* ssp. *bulgaricus* CIP 10102T. Cryobiology, 40: 264-269.

Salminen S., Isolauri E., Salminen E. (1996). Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Van Leeuwenhoek, 70: 347-358.

Saxelin M. (1997). *Lactobacillus* GG - a human probiotic strain with thorough clinical documentation. Food Rev. Int., 13: 293-313.

Saxelin M., Ahokas M., Salminen S. (1993). Dose response on the faecal colonisation of *Lactobacillus* strain GG administered in two different formulations. Microbiol. Ecol. Health Dis., 6: 119-122.

Smittle R.B., Gilliland S.E., Speck M. (1972). Death of *Lactobacillus bulgaricus* resulting from liquid nitrogen freezing. Appl. Microbiol., 24: 551-554.

Smittle R.B., Gilliland S.E., Speck M., Walter W.M.J. (1974). Relationship of cellular fatty acid composition to survival of *Lactobacillus bulgaricus* in liquid nitrogen. Appl. Microbiol., 27: 738-743.

Stadhouders J., Hup G., Jansen L.A. (1971). A study of the optimum conditions of freezing and storing concentrated mesophilic starters. Neth. Milk. Dairy J., 25: 229-239.

Succi M., Tremonte P., Reale A., Sorrentino E., Grazia L., Pacifico S., Coppola R. (2005). Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. FEMS Microbiol. Letters, 244: 129-137.

Thunnell R.K., Sandine W.E., Bodyfelt F.W. (1984). Frozen starters from internal-pH-control-grown cultures. J. Dairy Sci., 67: 24-36.

Tomás M.S.J., Ocaña M.E., Nader-Macías M.E. (2004). Viability of vaginal probiotic lactobacilli during refrigerated and frozen storage. Anaerobe, 10: 1-5.

Van De Guchte M., Serror P., Chervaux C., Smokvina T., Ehrlich S.D., Maguin E. (2002). Stress response in lactic acid bacteria. Antonie van Leeuwenhock, 82: 187-216.

Wright C.T., Klaenhammer T.R. (1983). Survival of *Lactobacillus bulgaricus* during freezing and freeze-drying after growth in the presence of calcium. J. Food Sci., 48: 773-777.

Zago M., Fornasari M.E., Rossetti L., Bonvini B., Scano L., Carminati D., Giraffa G. (2007). Population dynamics of lactobacilli in Grana cheese. Ann. Microbiol., 57 (3): 349-353.