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The effects of thermal, osmotic and acid stress on *Lactobacillus plantarum* and *Lactobacillus brevis*

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Abstract

Adaptation to environmental stress is an essential process for bacterial survival and growth. The aim of this work is to study the behavior of two *Lactobacillus* strains in osmotic, thermal or acid constraints with a view to a possible technological application. The degree of tolerance conferred on *Lactobacillus plantarum* and *Lactobacillus brevis*, by an osmotic, thermal and acid shock was evaluated, survivors cells were estimated at time 0 and after different times of incubation of stress challenge; the absorbance (OD_{600nm}) values were determined at the same intervals. The lethal treatment was calculated (CFU at T₀ – CFU at T) /CFU at T₀. During hyper osmotic stress, growth capacities are reduced, the acid and thermal stress also decreases the survival cells; at pH=1.5 and 2.5, the survival is strongly affected (absence of viable and cultivable cells from 72 h of incubation). After adaptation of the bacteria to the different stresses, growth and survival are significantly improved, at 2.5% NaCl, 18 ° C and 37 ° C, pH 4.5 and 5.5, the values obtained are similar to those obtained under the favorable conditions of growth. The study of the mortality rate indicates that cells are resistant or not to varying stress degrees from strain and from one stress intensity to another more important. The adaptation of the cells has a positive effect but only at reduced incubation times (optimal tolerance factors). This knowledge is essential in order to promote the proliferation and / or survival of these bacteria used as leaven in hostile environment.

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Introduction

In their natural habitats, microorganisms are frequently exposed to variations in the physicochemical properties of the environment: temperature, osmolarity or acidity of this medium. Dairy industry practices have led to variations in these parameters, and the bacteria used, which are constantly confronted with them, have developed responses adapted to these variations (Özer *et al.*, 2009).

These responses have been the subject of academic studies for more than twenty years to improve industrial manufacturing. In addition to its fundamental interest, the study of the mechanisms of protection against stress in bacteria is also necessary for optimal use of these in industrial processes (Essaid *et al.*, 2009).

Lactic acid bacteria are also found in other areas of application. Thus some members of this family (the Lactobacilli in particular) are used as probiotics, that is to say as a live microbial preparation having a beneficial effect on the host after ingestion (increase in the weight of livestock, reductions in infections) (Bourlioux, 2007).

Some *Lactobacillus* strains are used in food fermentation, and typical examples are found in the dairy industry for the production of cheese, yoghurt and other fermented milk products. The starter strains should resist to adverse conditions encountered in industrial processes (i.e. low and high temperature, low pH, osmotic stress, etc.). Additionally, the formulation and preservation of starter cultures may impose environmental stresses, such as low pH, drying, freezing, thawing, which significantly affect the survival and growth, fermentative capabilities and viability of the cells, decreasing their performance (Zotta *et al.*, 2008).

The growth and survival in these environmental niches depend on the ability of the organism sense and respond to varying conditions such as temperature, pH, nutrient availability and cell population density (Buck *et al.*, 2009).

Understanding the stress regulatory gives information to control these responses in order to achieve desirable robustness of bacteria in relation to various industrial processes. The knowledge of the mechanisms involved in stress adaptation is essential for selecting the most efficient strain for a particular product.

This work consists to characterize the response to various stress in lactobacilli including osmotic, acid and thermal stress. Our work aims to study the behavior of strains of lactobacilli (*Lactobacillus plantarum* and *Lactobacillus brevis*) isolated from camel milk against osmotic, acid and thermal stresses for use in basic research as well as for possible technological applications.

Material and methods

Strains and culture condition

Strains used in this work *Lactobacillus plantarum* and *Lactobacillus brevis* were isolated from camels milk of Illizi and Tindouf and identified by Belkheir *et al.* (2016). The strains were stored frozen at -20°C in MRS broth added of 20% of glycerol as cryoprotective agent. They were routinely reactivated in MRS broth 24h at 30°C .

Stress conditions

Two experimental approaches were followed in this document. The first approach consists to study the ability to resist at a lethal shock and the second approach when the strains were exposed to a low level of stress and develop adaptation strategies to resist a subsequent exposure to a higher level of the same stress and also to a number of different stresses.

Survival during lethal stress

Strains cultures in exponential phase ($\text{DO}_{600\text{nm}} = 0,6$) were centrifuged (7000 g/10min) washed with physiological water and suspended in the same solution. The cells are subjected to three different constraints according to application times and variable intensity.

Thermal stress

Strains (1ml) were suspended in 9ml of MRS broth and incubated at -20°C, 4°C, 18°C, 37°C, 45°C and 60°C; cultures suspensions incubated at 30°C were used as controls.

Osmotic stress

Strains (1ml) were suspended in 9ml of MRS broth added of diverse concentrations of NaCl (2.5%, 4.5% and 6.5% (p/v)), MRS without salt was used as bacterial growth control.

Acid stress

The cells were exposed by acid stress with MRS to pH 1.5, 2.5, 3.5, 4.5 and 5.5 acidified with HCl. Cultures suspensions maintained for pH 6.2 at 30°C were used as controls.

All assays for stress were incubated at 24h, 48h, 72h, 96h, 120h and 144h; survivors cells were estimated plating on MRS at time 0 and after a different times of incubation of stress challenge ;the absorbance (OD_{600nm}) values were determined at the same intervals. The lethal treatment was calculated (CFU at T₀ – CFU at T) /CFU at T₀. Assays were performed by triplicate in independent trials.

Pre-treatment (adaptation) and stress

The pre-treatment was performed in moderate stress to induce an adaptation during t₁; it is then placed under intense stress conditions for a time t₂. The second sample was placed under intense stress conditions for a time t₂. The third sample (control) was a growth control under non-stressful conditions.

The percentages of cells surviving under challenge stress are calculated: N_f final cell count (t₂) / N₀ initial cell count x 100. The tolerance factor was defined as % adapted cells / % nonadapted cells.

Results and discussion

The paragraphe is omitted

Survival during lethal stress

The results obtained are shown in Fig. 1 and 2. In the normal conditions, we note a progressive increase of the DO_{600nm} during the exponential phase, then a stabilization of this OD_{600nm} what implies that cells are in stationary phase and it is from the 2nd day of incubation. Moreover the survivals cells increases 10⁸ to 10⁹ CFU; however after 48 h the survival of cell decreases, six days for exposure to stress the viability is zero.

Table 1. Tolerance Factors of *Lactobacillus brevis*.

Stress	Parameters	24h	48h	72h	96h
Osmotic stress	NaCl				
	2.5%	4.04	17.24	1.25	5.37
	4.5%	2.22	42.4	1440	ND
	6.5%	3.5	473.21	ND	ND
Thermal stress	Temperature				
	-20°C	9.69	2.64	2.3	47.16
	4°C	1.31	0.62	92.3	ND
	18°C	1.14	1.03	16.76	2.28
	37°C	1.15	169.44	58.81	42.2
	45°C	10	52.80	ND	ND
	60°C	1.07	131.7	ND	ND
Acid stress	pH				
	1.5	31.7	10.06	ND	ND
	2.5	30.05	5.72	ND	ND
	3.5	1.1	1.17	68.13	ND
	4.5	2.78	8.69	6.91	11.95
	5.5	0.14	1.79	15.43	173.68

ND: not determined.

During hyper osmotic stress, growth capacities are reduced. Survival also decreased: at 2.5% NaCl, the decrease in survival was less important and was

cancelled after 6 days. However, beyond 2.5% of NaCl, the survival is strongly affected by salt (Fig. 1A, 2A).

Table 2. Tolerance factors of *Lactobacillus plantarum*.

Stress	Parameters	24h	48h	72h	96h
Osmotic stress	NaCl				
	2.5%	33.02	7.45	1	1.88
	4.5%	5.87	21.02	442.3	ND
Thermal stress	Temperature				
	-20°C	9.15	2.68	2.26	1.56
	4°C	2.73	3.89	2.54	ND
	18°C	0.92	1.45	1.76	1.67
	37°C	15.26	193.78	154.67	31.31
	45°C	0.58	5.72	19.54	ND
	60°C	2.39	1.57	ND	ND
Acid stress	pH				
	1.5	155.33	51.64	ND	ND
	2.5	25.67	265.7	ND	ND
	3.5	4.78	4.67	6.16	ND
	4.5	62.5	10.53	0.73	1.07
	5.5	16.06	56.81	11.36	14.76

ND : not determined.

Other authors have found that sub-optimal hyper-osmotic stress of 1 M NaCl improves the survival of *P. pentosaceus* in the stationary phase and prolongs its lifetime (Baliarda *et al.*, 2003). Similar results were obtained in *Lb. sakei* 23K. The authors of this works suppose that the strategies of stress response developed by *Lb. sakei* aim to improve survival rather than growth (Marceau *et al.*, 2003).

Thermal stress affects the survival of cells, the further away from the optimal growth temperature (30°C), the survival is lower until it is nullified (Fig. 1B, 2B).

Studies on *Lactobacillus rhamnosus* showed that the exhibition of cells in a temperature of 50°C improves the survival cell during the stationary phase for a few days (Prasad *et al.*, 2003).

Gwenola *et al.* (2001) studied the influence of the thermal stress on the viability of two strains of

Lactobacillus bulgaricus and found that the viability of cells was affected only beyond 55°C. Similar results were observed with *Lactobacillus helveticus* (Di Cagno *et al.*, 2006), *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, *Oenococcus oeni* (Jobin *et al.*, 1998) and *Lactobacillus delbrueckii ssp. bulgaricus* (Silva *et al.*, 2005).

The acid stress also decreases the survival cells (Fig. 1C and 2C), at pH=1.5 and 2.5, the survival is strongly affected where there is a rapid death of cells (absence of viable and cultivable cells from 72 h of incubation). At pH=3.5, the lifetime of the cells increases (4 days), until it reaches 6 days at 4.5 and 5.5, which is equal to 6.5 (optimum pH).

Guillouard *et al.*, (2004) showed that the resistance of five strains of *Lactobacillus bulgaricus* was variable according to the strains and also different from one strain to another.

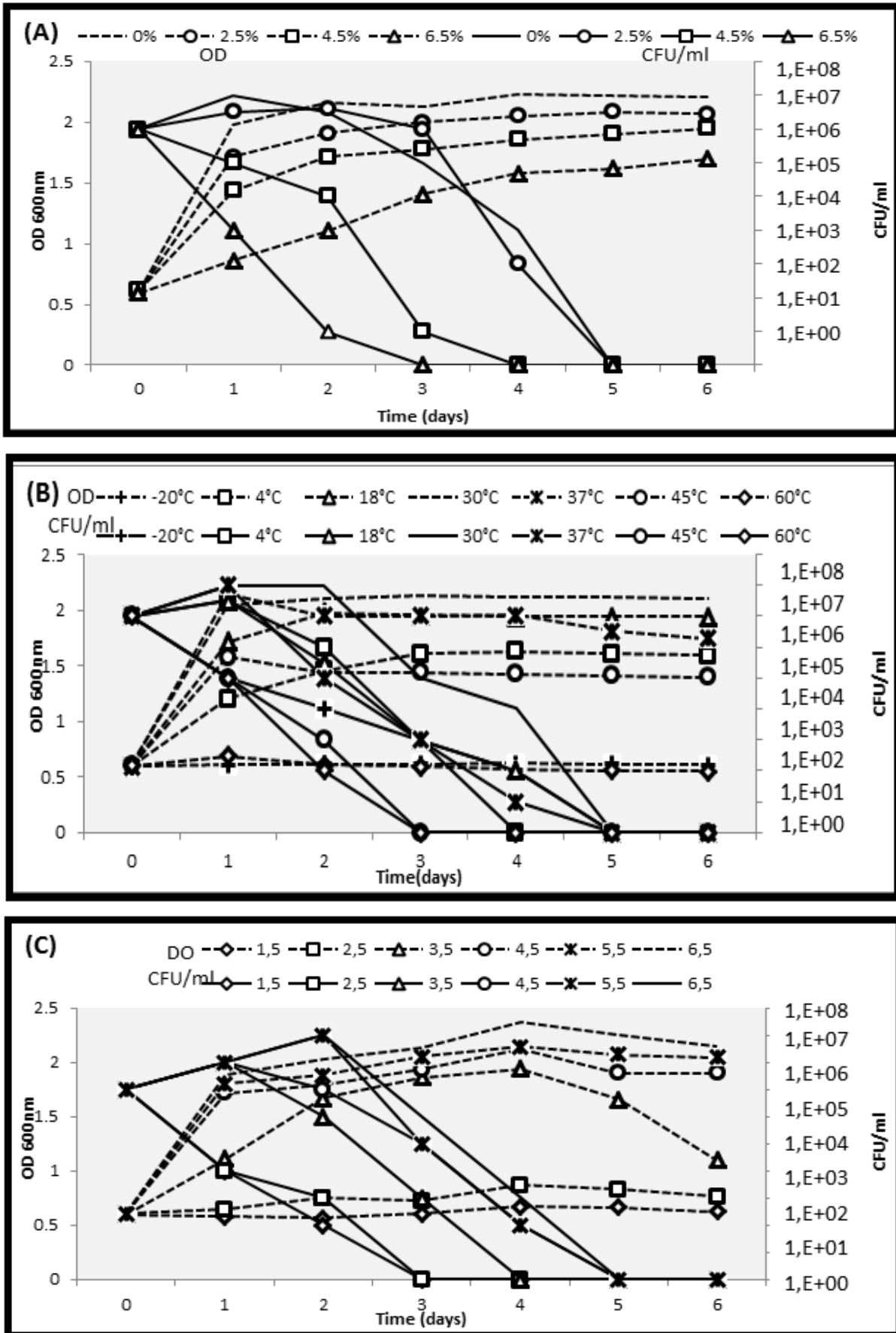


Fig. 1. Evolution of growth and survivors cells of *Lactobacillus brevis* under stress (A): Osmotic stress NaCl (B): Thermal stress °C (C): Acid stress Ph.

Survival after adaptation of cells

The representation of the DO_{600nm} and the number of viable and cultivable cells shows that the adaptation of the cells affects positively the DO_{600nm} (growth) and the number of CFU/ml, it is noted that the values are

directly increased until it is almost equal to the controls values: 2.5% NaCl, 18°C, 37°C, pH4.5 and 5.5. However, in extreme values (-20°C, 60°C, pH1.5 and 2.5), adaptation did not greatly alter DO_{600nm} and the survivals of cells (Fig. 3 and 4).

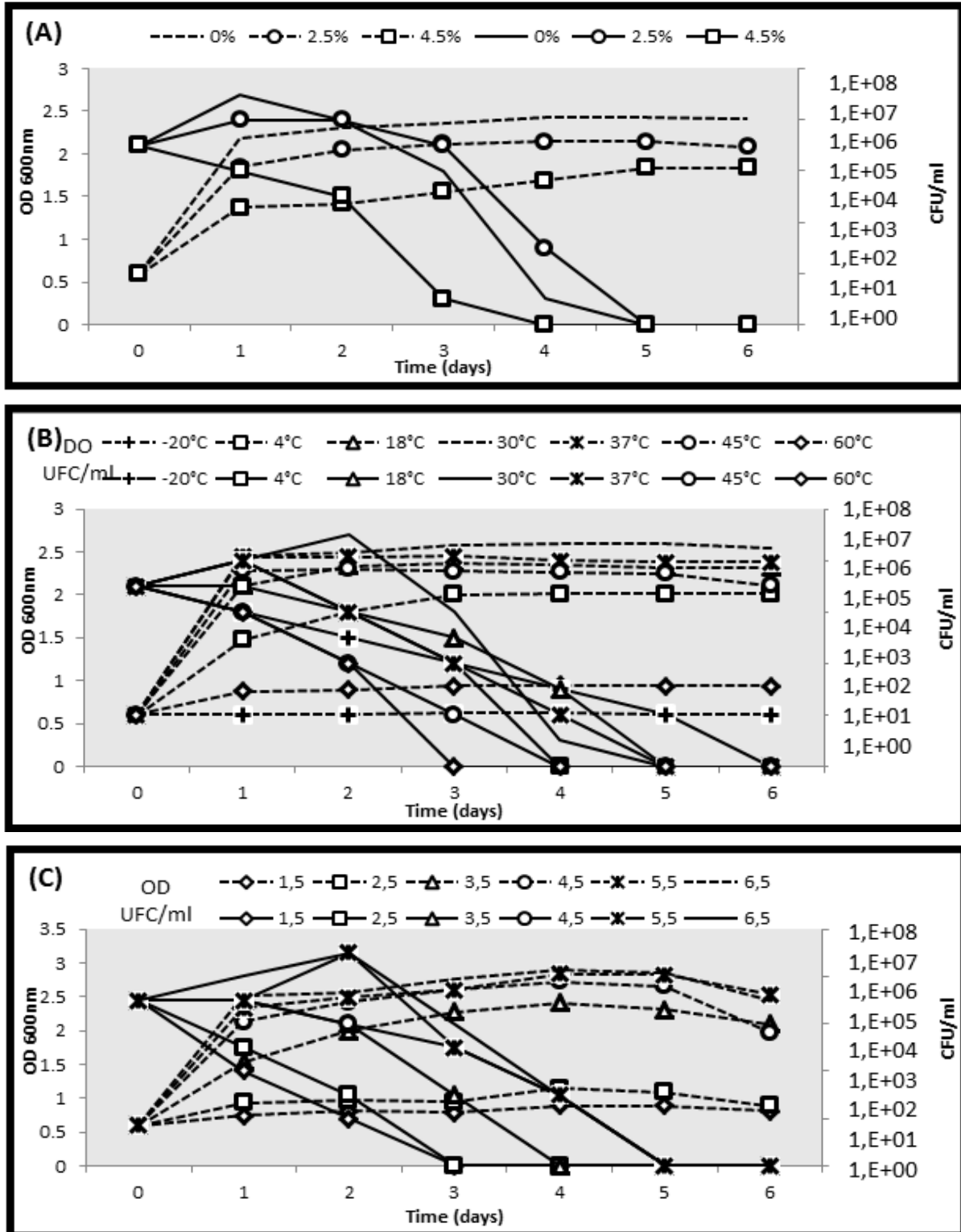


Fig. 2. Evolution of growth and survivors cells of *Lactobacillus plantarum* under stress (A): Osmotic stress NaCl (B): Thermal stress °C (C) :Acid stress pH.

In *Lactobacillus bulgaricus*, cells acquire a thermotolerance against heat shock at 65°C. during 10 min, that is a greater viability, after exhibition in a thermal pretreatment moderated in 50°C (Gwenola *et al.*, 2001). Other strains of *Lactobacillus bulgaricus* became about 250 times more tolerant to acid shock when they undergo a preliminary adaptation to a pH of 4.75. Strains of *Lactobacillus bulgaricus* underwent

adaptation to the acidity gave higher survival rate than those observed with strains that had not undergone any adaptation (Guillouard *et al.*, 2004). The temperature and concentration of NaCl are the most important factors inducing the loss of capacity to form colonies (Besnard *et al.*, 2000). Omitted another paragraphe.

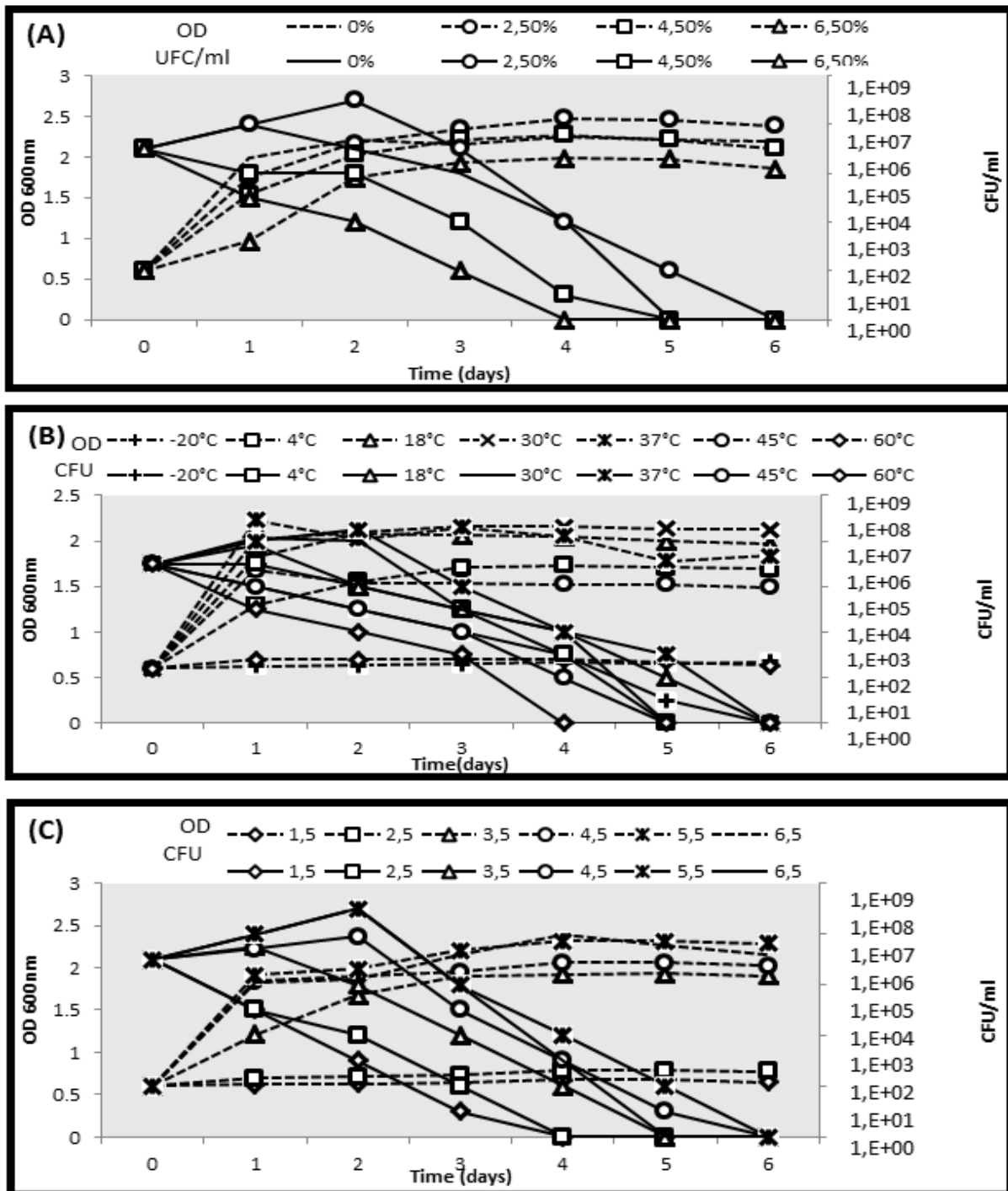


Fig. 3. Evolution of growth and survivors cells of *Lactobacillus brevis* after adaptation condition under stress (A): Osmotic stress NaCl (B): Thermal stress °C (C): Acid stress pH.

Effect of different stress on the mortality of cells

The percentages of mortality associated to every treatment processing are represented in the following Fig. 5 and 6.

Hyperosmotic shock: at the beginning of the experiment (24-48h), the concentration of 2.5% NaCl had little effect on the viability of the stressed cells

(between 0% and 2.5% mortality for *Lactobacillus brevis* and *Lactobacillus plantarum*). A concentration of 4.5% of NaCl causes more mortality (*Lactobacillus plantarum* is rather resistant); values are quite high (between 92.26% and 99.99% of mortality). For *Lactobacillus brevis* at 6.5%, we note that the mortality rate is too high (99.70% after 24h of incubation) omitted the sentence.

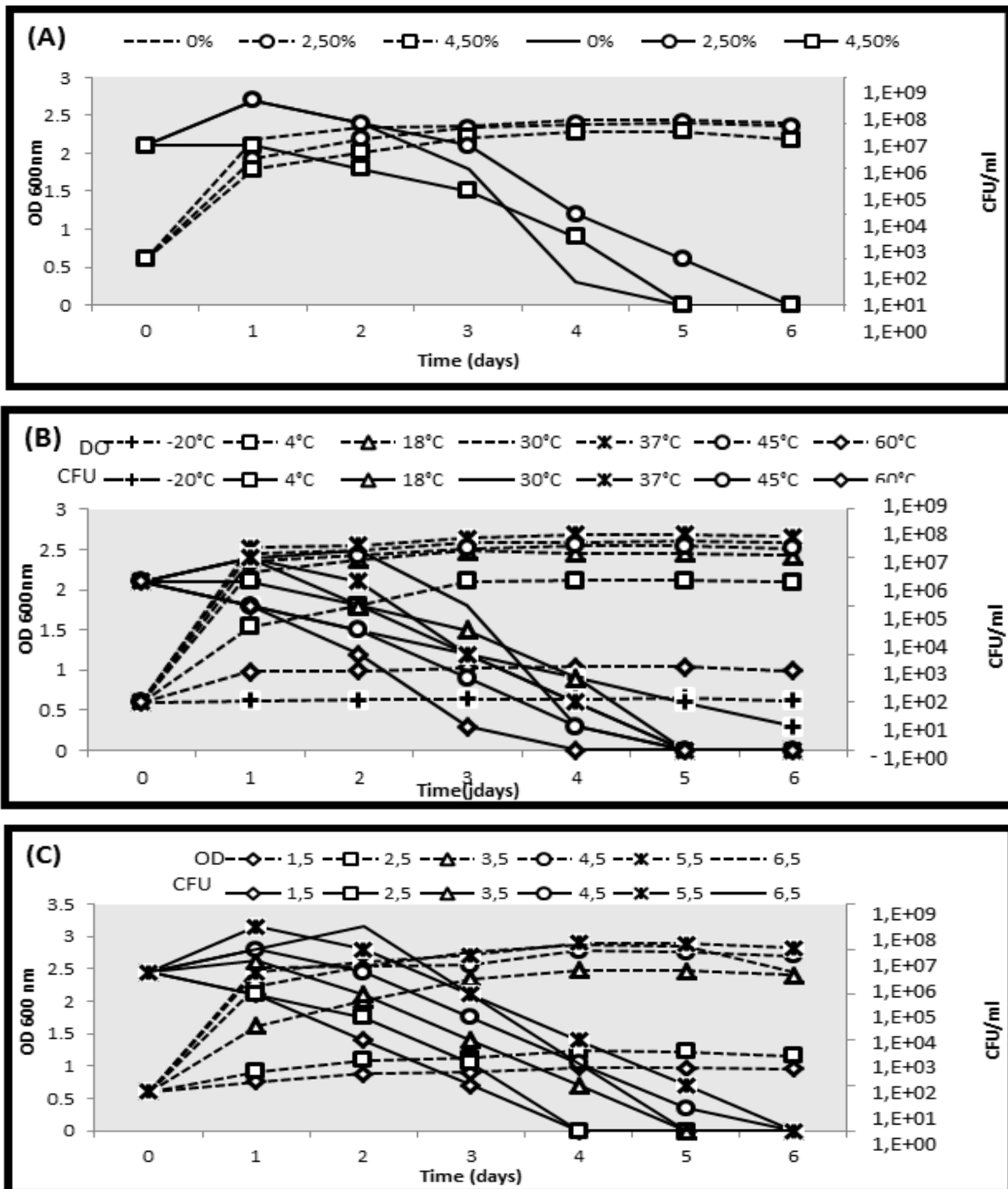


Fig. 4. Evolution of growth and survivors cells of *Lactobacillus plantarum* after adaptation under stress (A): Osmotic stress NaCl (B): Thermal stress °C (C): Acid stress pH.

Studies indicate that hyper-osmotic shock (2 M NaCl and 4 M NaCl) has not much effect on the viability of the put under stress cells. No mortality was observed after 6h, 20h or 40h of exposure to 2 M NaCl. A 45% of mortality is obtained after 20 h of exposure to 4 M NaCl. These data suggest that *P. pentosaceus* cells are particularly resistant to hyperosmotic shock compared to *Enterococcus faecalis* (45% mortality after 2 h exposure to 1.1 M in BHI medium (Flahaut *et*

al., 1996), *Lb. acidophilus* CRL639 (99.6% mortality after 24h exposure to 3 M NaCl (Lorca and Font de Valdez, 2001), *Lb. acidophilus* LA1-1 (54% of mortality after 2 h exposure to 3 M NaCl in MRS medium (Kim *et al.*, 2001) and *Lc. lactis ssp cremoris* NCDO712 (99.7% of mortality after 2 h exposure to 3.5 M NaCl (O'Sullivan and Condon, 1997).

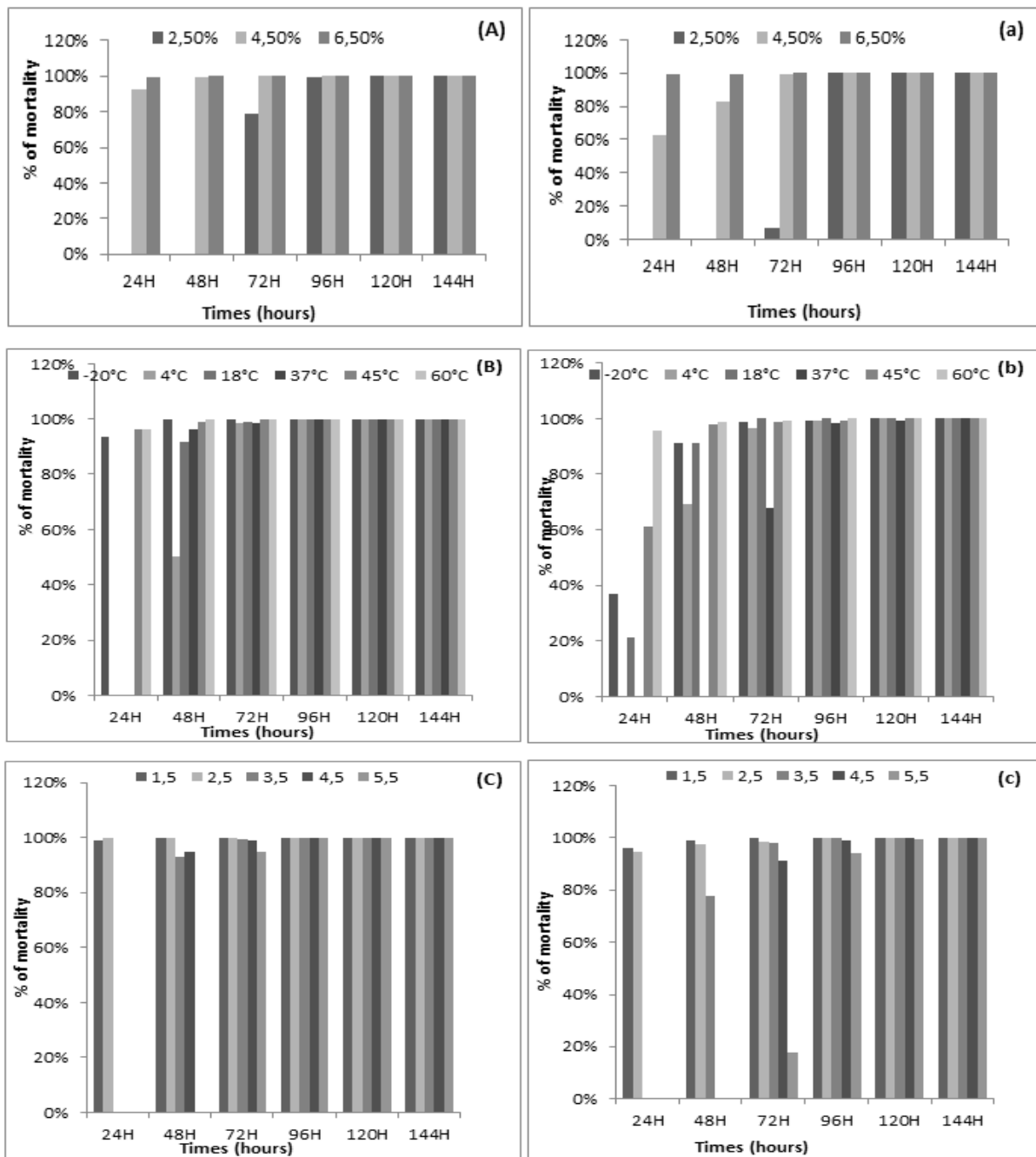


Fig. 5. Effect of stress on the mortality rate of *Lactobacillus brevis* strain (A): hyperosmotic stress (a) hyperosmotic stress (adapted assay) (B): thermal stress (b) thermal stress (adapted assay) (C): acid stress (c) acid stress (adapted assay).

Thermal shock: the rough increase of temperature at 60°C results in high cell mortality and from 24 hours of incubation (99.60% for *Lactobacillus brevis* and 74.90% for *Lactobacillus plantarum*).

The effect of the incubation at 45°C begins to appear better at 48h (at 24h, the mortality rates are less drastic than at 60°C).

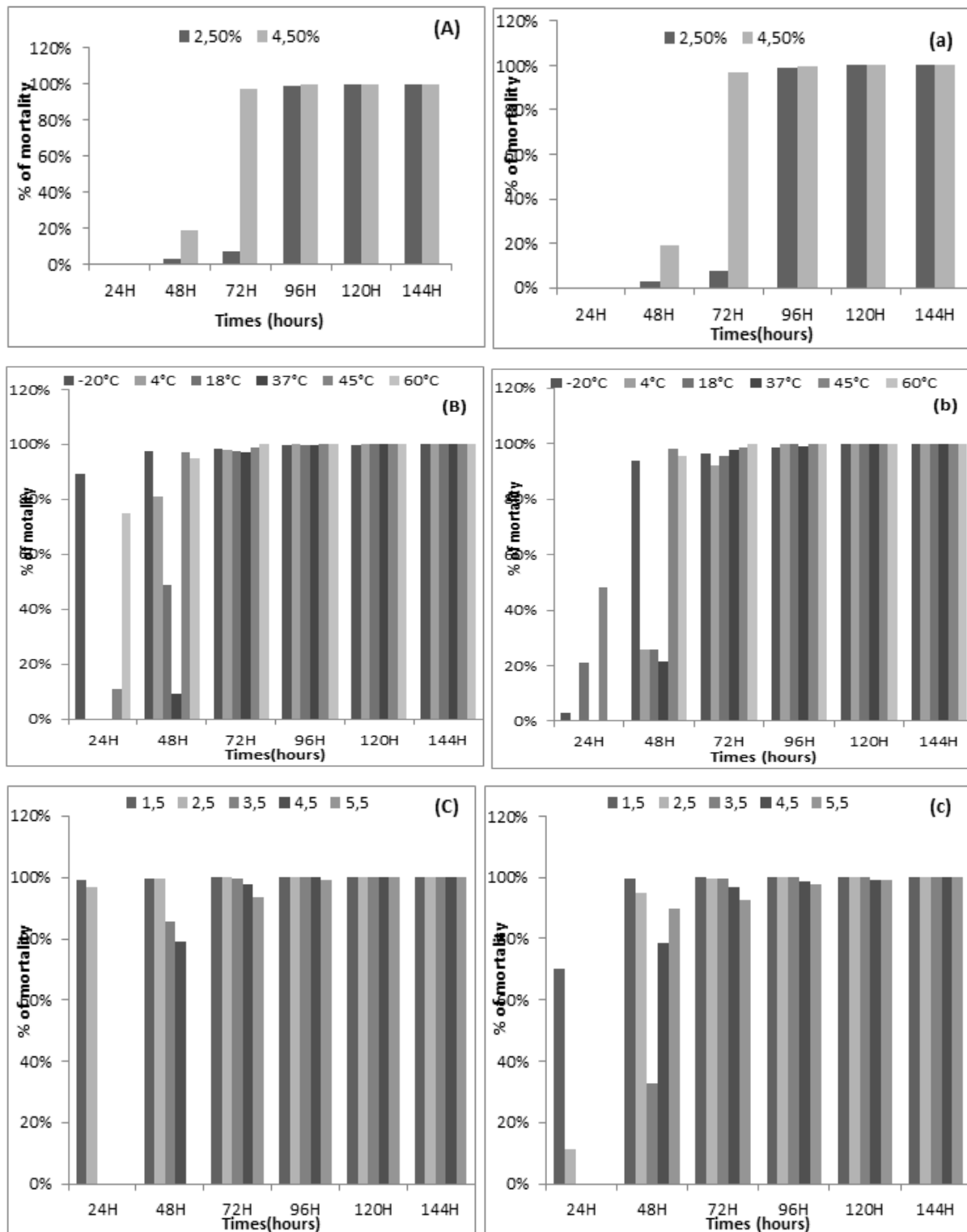


Fig. 6. Effect of stress on the mortality rate of *Lactobacillus plantarum* strain (A) : hyperosmotic stress (a) hyperosmotic stress (adapted essay) (B) :thermal stress (b) thermal stress (adapted essay)(C) : acid stress (c) acid stress (adapted essay).

The cells appear to be less sensitive to temperatures of 37°C, 18°C and 4°C. However, this resistance observed at 24 and 48h of incubation, is not valid any more from 72h.

At -20°C, there was a rather important mortality, which is unexpected, as the bacterial cells are usually conserved at -20°C, this can be explained by the fact that the MRS medium does not contain cryoprotectants, substances usually used to protect the bacterial cells from freezing.

These results are similar to those found with *P. pentosaceus*. A thermal shock consisting in brutally increasing the temperature of incubation from 37°C to 42°C or 50°C led to a low mortality while the increase of the temperature to 53°C produced high mortality. The tolerance of *P. pentosaceus* ATCC33316 to heat (98.2% of mortality after treatment at 53°C) is close to that observed in *O. oeni* LOD004 (99.6% of mortality after treatment at 53°C (Garbay and Lonvaud-Funel, 1996). While *Lb. acidophilus* LA1-1 is more resistant (no mortality after a treatment of 30 min at 53°C) (Kim *et al.*, 2001).

An acid shock consisting in reducing the pH of the culture medium from pH=6.5 to 5.5 was without effect on the cells of the two strains after 24 and 48h. Its effect begins to appear only from 72h where the mortality rate increases until reaching 99.99% at the end of the experiment. pH of 4.5 and 3.5 also appear to have no effect at 24h, a low effect at 48h and a lethal concentration from 72h. On the other hand, the pH values of 1.5 and 2.5 give the most important mortality rates and from 24 hours only (mortality ranging from 96.70% for *Lactobacillus plantarum* to 99.81% for *Lactobacillus brevis*). This indicates that the strains can resist to acid stress but not to very acid medium.

Works realized on *P. pentosaceus* showed that reducing the pH of the culture medium pH 6.5 to pH 4.0, was very weakly lethal for *P. pentosaceus* (5% mortality), but when the pH of the culture medium was lowered to pH 3.0, mortality increased with incubation time (Baliarda *et al.*, 2003).

P. pentosaceus presents a good tolerance to acid stress compared to *Lc. lactis spp lactis* LL41-1, *Lb. acidophilus* CRL639 (Lorca and Font de Valdez, 2001) and *Enterococcus faecalis* ssp *faecalis* ATCC19433 (Flahaut *et al.*, 1996).

Adapted assay

The purpose of this part is to define if pre-exposure of cells to moderate stress can improve the response of cells to subsequent intense stress.

The efficiency of adaptation is variable from one strain to another and from one stress to another. However, this adaptation does not give good results except at incubation times of 24, 48 and sometimes 72 h, but from 96 h, the mortality rate of the cells is always high (Fig. 5 and 6). To better explore of the results, we must refer to the factors of tolerance.

A reduction or increase in the temperature of the culture relative to the optimum temperature of bacteria, especially for thermophilic bacteria, affects their stability during freezing, freeze-drying and storage. This effect was demonstrated by Wang *et al.* (2005) for the *Lb acidophilus* RD758 strain. With fermentations conducted at three different temperatures (30°C, 37°C and 42°C), the authors observed that the cells cultured at 30°C (temperature lower than their optimal temperature of 37°C) were more resistant to freezing and maintained high acidifying activity after 365 days of storage at -20°C.

Murga *et al.* (2000) also showed that *Lb acidophilus* CRL640, grown at 25°C are more resistant to freezing than cells grown at 37°C. Their survival rate is 67% instead of 16%.

Some authors observe that the resistance of cells to preservation stages depends on the pH at which the culture is carried out. The cells of *Lb. reuteri* ATCC 55730 cultivated at pH 5 are more resistant to freeze-drying than cells cultured at their optimum pH (pH 6) (Palmfeldt and Hahn-Hägerdal, 2000). A similar result was obtained for this strain.

Wang *et al.* (2005) showed that the cells of *Lb. acidophilus* RD758 grown at pH 5 are more resistant to freezing and storage in frozen form than cells cultured at pH 6 (pH optimum) or pH 4.5. These results indicate that fermentation carried out at an acidic pH, far from the optimal pH of the cells, has a negative effect on their resistance. This is because a decrease in extracellular pH prevents cells from maintaining their intracellular pH at an appropriate level for metabolic reactions (Hutkins and Nannen, 1993).

The study of the mortality rate indicates that cells are resistant or not to varying stress degrees from strain and from one stress intensity to another more important. The adaptation of cells have an effect but only at reduced incubation times.

Tolerance Factors

From the results obtained, the tolerance factors for each strain are calculated and at each stress, the tolerance factor is defined as the percentage of survival of cells adapted after a stress / percentage of survival after stress to the non-adapted cells. The results obtained are shown in Tables 1 and 2.

It can be said that the efficiency of adaptation varies according to the nature of the stress: optimal tolerance factors are about 473, 169 and 173 for hyperosmotic, acid and thermal stresses respectively for *Lactobacillus brevis*; and 442, 193 and 265 for *Lactobacillus plantarum*.

From these results, it was concluded that the adaptation performed on *Lactobacillus plantarum* gave the best results.

It is also noted that adaptation is a transient phenomenon: the optimal factors fall if the incubation of the cells is prolonged. On the other hand, the intensity of the stress is also an important parameter; one notes that for each intensity, it is necessary to precise time for the adaptation to be highlighted. The adaptation of the cells gives variable results from constraint to another, it is also transient. The sentence is omitted.

Conclusion

Lactobacillus plantarum is very tolerant to osmotic, acid and thermal stress and this resistance is improved by adaptation to hostile conditions. This strain could be selected as a potential candidate for a technological and probiotic use. It will be interesting to highlight the mechanisms of fight against these stresses developed by this bacterial genus.

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