Short communication: Salt tolerance of *Lactococcus lactis* R-604 as influenced by mild stresses from ethanol, heat, hydrogen peroxide, and UV light

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ABSTRACT

Lactococcus lactis is a culture widely used in salt-containing dairy products. Salt hinders bacterial growth, but exposure to environmental stress may protect cells against subsequent stress, including salt. The objective of this study was to evaluate the salt tolerance of *L. lactis* R-604 after exposure to various stresses. The culture was subjected to 10% (vol/vol) ethanol for 30 min, mild heat at 52°C for 30 min, 15 mM hydrogen peroxide for 30 min, or UV light (254 nm) for 5 min and compared with a control. Starting with 5 log cfu/mL for all treatments, growth was determined in M17 broth with 5 NaCl concentrations (0, 1, 3, 5, and 7% wt/vol). Plating was conducted daily for 5 d. Salt tolerance was enhanced with mild heat exposure before growth in M17 broth with 5% (wt/vol) NaCl on d 3, 4, and 5, and with exposure to hydrogen peroxide and ethanol stresses before growth in M17 broth with 5% (wt/vol) NaCl on d 4 and 5. Exposure of this culture to mild heat, hydrogen peroxide, or ethanol before growth in M17 broth containing 5% (wt/vol) salt can enhance its survival, which could be beneficial when using it in salt-containing dairy products.

Key words: salt tolerance, *Lactococcus lactis*, cheese culture, cross-protection, bacterial growth

Short Communication

Previous exposure of *Lactococcus lactis* to low-intensity stress encourages resistance to the same stressors at lethal levels, because this first exposure initiates cell defense mechanisms (Hartke et al., 1994; Sanders et al., 1999). Exposure to an environmental stress can encourage resistance to different types of stressors because the many stress-induced proteins and low-molecular-weight compounds produced under more than one stress con-

dition create an effect of cross-protection (Pichereau et al., 2000).

Ethanol is known to cause changes in the composition and function of bacterial cells, inhibit cell division, decrease viability and sugar transport systems, and affect the function of mitochondria and vacuoles. Exposure to 4% (vol/vol) ethanol for 30 min confers heat resistance (50°C for 25 min) to gram-positive bacteria such as *Bacillus cereus* by stimulating the production of heat-stress proteins such as GroEL, DnaK, and FtsH (Periago et al., 2002).

Heat is one of the most damaging stressors to bacteria. When most bacterial cells are under heat stress, they encode several proteins that act as chaperones to repair or destroy damaged structures (Yousef and Courtney, 2003). Some examples of heat-induced proteins are RecA, GroEL, grpE, DnaK, and hflB, which are involved in DNA repair and other protein synthesis (van de Guchte et al., 2002). These proteins are also associated with increasing resistance to oxidative stress, acidity, and cold temperature shock (Smith et al., 2010). Exposure to heat above the optimal growth temperature of microorganisms can activate resistance mechanisms that may increase their tolerance to other lethal stressors (Boutibonnes et al., 1991). Under osmotic stress with 2.5% (wt/vol) NaCl, *L. lactis* produced heat-stress proteins such as DnaK, GroEL, and GroES. This overlap in protein production enhanced resistance to heat after a short osmotic shock (Kilstrup et al., 1997).

Oxidative stress can be defined as cells' response to an excessive accumulation of reactive oxygen compounds in the environment (Rochat et al., 2005); *L. lactis* and other lactic acid bacteria are very vulnerable to oxidative stress, because they lack the catalase activity to reduce hydrogen peroxide into water and oxygen (Rochat et al., 2005).

The viability of *L. lactis* can be reduced by almost 90% after 30 min exposure to 100 kJ/m² UV light at 254 nm, and stress-induced proteins such as GroEL and GroES are produced. Exposure of *L. lactis* to UV light

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increases its resistance to lethal challenges of other stresses such as acid, ethanol, heat, and hydrogen peroxide (Hartke et al., 1995). Pre-exposure to UV light encourages production of U1 protein, which protects the cell DNA from other stresses (Hartke et al., 1995). Similar to oxidative stress, UV light attacks the cell's DNA and is used as a mutagenic tool in several microorganisms (Miller, 1985). For gram-negative bacteria, 4 protection mechanisms have been proposed: RecA heat-induced protein synthesis to protect DNA; chemical modification of the cell membrane; permeability changes in the cell membrane; and reduction in cell division, which saves DNA for other uses. Some of these mechanisms may be used by *L. lactis* when it is exposed to UV light (Duwat et al., 1997).

One may question whether cultures widely used in salt-containing dairy products can become more resistant to one type of stress after previous application of another. For example, it is not known if the salt tolerance of *L. lactis* R-604 can be enhanced by previously subjecting it to other mild stress conditions. Our objective was to determine the effect of exposing *L. lactis* R-604 in M17 broth to different stressors (ethanol, mild heat, oxidative stress, and UV light) on its subsequent growth in M17 broth containing 0, 1, 3, 5, or 7% (wt/vol) NaCl.

The conditions consisted of ethanol stress (30-min incubation in M17 broth at 30°C with 10% vol/vol ethanol), mild heat stress (52°C for 30 min), oxidative stress (30-min incubation in M17 broth at 30°C with 15 mM H₂O₂), UV light exposure (254 nm for 5 min), and control (no stress). After each treatment, the culture was inoculated and incubated in M17 broth with 1 of 5 NaCl concentrations (0, 1, 3, 5, and 7% wt/vol) without stirring for 5 d. Bacterial counts were determined every 24 h for 5 consecutive days. Three replications of the experiments were conducted, and plate counts were performed in duplicate.

We used M17 broth for all samples and prepared it according to manufacturer specifications by diluting 37.25 g of M17 broth powder (Fisher Scientific, Fair Lawn, NJ) in 1 L of distilled water. This suspension (94 mL) was poured into dilution bottles and sterilized at 121°C for 15 min. A 10% (wt/vol) lactose solution (Fisher Scientific) was prepared separately and sterilized under the same conditions. After autoclaving, 5 mL of the lactose solution was aseptically added to the sterile M17 bottles. The bottles were then tempered at 30°C in an aerobic incubator.

We used 1 g of pure *L. lactis* DVS culture R-604 (Chr. Hansen, Milwaukee, WI) for the control and the ethanol, mild heat, and oxidative stress treatments. The R-604 culture contained defined mesophilic strains of *Lactococcus lactis* without biovar *diacetylactis* (Chr.

Hansen, 2016). For the control, the culture was thawed and inoculated into 99 mL M17 broth supplemented with 0.5% (wt/vol) lactose without subjecting the culture to stress. The ethanol treatment consisted of thawing the culture and inoculating it into 99 mL M17 broth supplemented with 0.5% (wt/vol) lactose and 10% (vol/vol) ethanol before incubation at 30°C for 30 min. The mild heat stress treatment consisted of thawing the culture and aseptically transferring it into 99 mL M17 broth supplemented with 0.5% (wt/vol) lactose before heating at 52°C for 30 min in a water bath and rapidly chilling in ice water. The oxidative stress treatment consisted of thawing the culture and inoculating it into 99 mL of M17 broth supplemented with 0.5% (wt/vol) lactose and 15 mM hydrogen peroxide before incubation at 30°C for 30 min.

The UV light stress experiment was conducted according to Hartke et al. (1995) and Duwat et al. (1997) with some modifications. A 10-mL sample of culture was aseptically poured into a sterile Petri dish and left uncovered 65 cm from the UV source (Germicidal 30W fluorescent tube; Sylvania, Wilmington, MA) at 254 nm for 5 min in laminar flow cabinet (Purifier class II; Labconco Corp., Kansas City, MO). Then, 1 g of this culture was inoculated into 99 mL of M17 broth supplemented with 0.5% (wt/vol) lactose.

After subjecting the cultures to each stress condition (ethanol, mild heat, oxidative stress, and UV light) or no stress, 1 mL of each sample was transferred to 5 different bottles containing sterile M17 broth with 0, 1, 3, 5, and 7% (wt/vol) NaCl (Fisher Scientific). The broths containing the cultures were immediately plated in M17 agar supplemented with 0.5% (wt/vol) lactose and incubated aerobically at 30°C (Kim et al., 2002) for 3 d before enumeration. The cultures in the M17 broths were incubated without stirring and drawn daily for 5 d for plating. All fluid samples and subsequent dilutions in dilution bottles were thoroughly shaken (American Public Health Association, 1992) before plating.

Data were analyzed as a completely randomized split-plot design with repeated measures over time using Proc Glimmix of SAS (version 9.3; SAS Institute Inc., Cary, NC). Significant differences were tested with least squares means at $P < 0.05$ for main effects (stress conditions, time, and salt concentration), the interaction effect (stress conditions $\times$ salt concentrations $\times$ time of exposure), and paired comparisons.

Counts of *L. lactis* R-604 in M17 broth containing various salt concentrations over 5 d of salt exposure are presented in Table 1. However, these counts may have included potential lactococci chains in which each chain was counted as 1 colony. The 3-way interaction between stress conditions $\times$ salt concentrations $\times$ days was significant ($P < 0.05$), meaning that *L. lactis* cell

Table 1. Least squares means of cell counts (log cfu/mL) of *Lactococcus lactis* R-604 in M17 broth containing various salt concentrations to show its salt tolerance as influenced by exposure to stressors

Exposure	% Salt (wt/vol)	Days					
		0	1	2	3	4	5
Control	0	5.17 ^{D,a}	9.46 ^{A,a}	9.06 ^{A,a}	8.07 ^{B,cde}	6.90 ^{C,bcdef}	6.26 ^{C,bcdef}
	1	5.13 ^{D,a}	9.48 ^{A,a}	9.01 ^{A,a}	7.90 ^{B,de}	7.09 ^{BC,bcde}	6.45 ^{C,bcdef}
	3	5.14 ^{D,a}	9.40 ^{A,a}	9.06 ^{A,a}	7.76 ^{B,de}	6.24 ^{C,defg}	6.04 ^{CD,defg}
	5	5.10 ^{D,a}	8.48 ^{AB,ab}	9.16 ^{A,a}	8.10 ^{B,bcde}	6.75 ^{C,defg}	5.02 ^{D,g}
	7	5.11 ^{B,a}	4.83 ^{B,d}	5.15 ^{B,c}	6.38 ^{A,fg}	7.27 ^{A,bcd}	6.93 ^{A,abcde}
Ethanol	0	5.19 ^{C,a}	9.31 ^{A,a}	9.23 ^{A,a}	8.51 ^{A,abcd}	7.32 ^{B,bcd}	7.32 ^{B,ab}
	1	5.19 ^{D,a}	9.37 ^{A,a}	9.09 ^{A,a}	8.03 ^{B,de}	6.87 ^{C,bcdefg}	6.86 ^{C,abcde}
	3	5.15 ^{D,a}	9.36 ^{A,a}	9.34 ^{A,a}	7.81 ^{B,de}	6.12 ^{C,efg}	6.10 ^{C,bcdef}
	5	5.15 ^{C,a}	6.72 ^{B,c}	9.14 ^{A,a}	9.14 ^{A,abc}	8.45 ^{A,a}	7.06 ^{B,abcd}
	7	5.17 ^{A,a}	4.65 ^{A,de}	4.31 ^{AB,c}	3.56 ^{BC,i}	3.18 ^{C,h}	2.73 ^{C,h}
Mild heat	0	4.82 ^{D,a}	9.49 ^{A,a}	9.13 ^{A,a}	8.19 ^{B,bcde}	7.04 ^{C,bcde}	6.74 ^{C,abcde}
	1	4.82 ^{D,a}	9.54 ^{A,a}	9.05 ^{A,a}	7.55 ^{B,de}	6.71 ^{BC,defg}	6.62 ^{C,bcde}
	3	4.83 ^{D,a}	9.39 ^{A,a}	9.26 ^{A,a}	7.53 ^{B,de}	5.96 ^{C,fg}	6.04 ^{C,defg}
	5	4.81 ^{D,a}	6.41 ^{C,c}	9.11 ^{AB,a}	9.47 ^{A,a}	8.38 ^{B,a}	6.10 ^{C,bcdef}
	7	4.82 ^{C,a}	3.60 ^{D,e}	4.49 ^{CD,c}	5.09 ^{BC,h}	5.83 ^{B,g}	6.60 ^{A,bcde}
Hydrogen peroxide	0	5.25 ^{D,a}	9.39 ^{A,a}	9.31 ^{AB,a}	8.43 ^{B,abcd}	7.31 ^{C,bcd}	7.18 ^{C,abc}
	1	5.33 ^{D,a}	9.42 ^{A,a}	9.18 ^{A,a}	8.23 ^{B,bcde}	6.95 ^{C,bcdef}	6.74 ^{C,abcde}
	3	5.33 ^{D,a}	8.53 ^{A,ab}	9.43 ^{A,a}	8.46 ^{B,abcd}	6.45 ^{C,defg}	5.88 ^{CD,defg}
	5	5.30 ^{C,a}	6.17 ^{BC,c}	6.61 ^{B,b}	7.70 ^{A,de}	7.93 ^{A,abc}	7.77 ^{A,a}
	7	5.33 ^{A,a}	5.08 ^{AB,d}	4.39 ^{B,c}	3.20 ^{C,i}	2.09 ^{D,i}	1.14 ^{E,i}
UV light	0	5.19 ^{C,a}	9.41 ^{A,a}	9.09 ^{A,a}	7.53 ^{B,de}	6.99 ^{B,cdef}	6.79 ^{B,abcde}
	1	5.24 ^{C,a}	9.48 ^{A,a}	8.99 ^{A,a}	7.35 ^{B,ef}	6.80 ^{B,defg}	6.63 ^{D,bcde}
	3	5.20 ^{D,a}	9.42 ^{A,a}	9.16 ^{A,a}	7.56 ^{B,de}	6.32 ^{C,defg}	5.77 ^{CD,efg}
	5	5.19 ^{C,a}	8.20 ^{B,b}	9.21 ^{A,a}	9.17 ^{A,ab}	7.83 ^{B,abc}	5.42 ^{C,fg}
	7	5.15 ^{B,a}	5.14 ^{B,d}	5.38 ^{B,c}	6.05 ^{AB,gh}	6.62 ^{A,defg}	6.86 ^{A,abcd}

^{A-E}LSM without a common uppercase letter within a row were significantly different ($P < 0.05$).
^{a-i}LSM without a common lowercase letter within a column were significantly different ($P < 0.05$).

counts' dependence on the stress condition applied at a specific salt concentration over the days of exposure was not predictable.

Log counts for *L. lactis* were approximately 5 log cfu/mL immediately after inoculation into each broth for each salt concentration and were not significantly different from each other. For 0, 1, and 3% salt concentrations, the *L. lactis* counts usually increased by 4 to 5 log units from d 0 to 1, and these log counts showed a tendency to decrease progressively from d 1 to 5. The log counts for the 5% salt concentration reached a peak between d 2 and 4 before decreasing to d 5. Variable trends for the effect of various stresses on *L. lactis* counts during storage occurred for the 7% salt concentration. The control did not show a consistent pattern during storage. Previous exposure to ethanol and hydrogen peroxide resulted in decreasing counts during storage. However, previous exposure to UV light and mild heat led to increasing counts after 1 d of storage. Although the 1 and 3% salt concentrations did not adversely affect *L. lactis* growth during storage compared with the control, the 5% salt concentration, and especially the 7% salt concentration, adversely affected its growth after 1 d of storage.

The effect of NaCl on *L. lactis* growth has been reported in other studies. Uguen et al. (1999) reported

significant reduction (70%) in the growth of *L. lactis* ADRIA 85L030 in 0.4 M NaCl and inhibition of growth at 0.5 M NaCl [equivalent to 3% (wt/vol) NaCl] and higher. Obis et al. (2001) reported that several strains of *L. lactis* ssp. *lactis* and *cremoris* could grow at 6.5% (wt/vol) NaCl, but for some other strains, growth was negatively affected at 2% (wt/vol) NaCl and above. The difference in these results compared with our results could have been caused by the different strains used in each study.

The effects of ethanol, heat, oxidative stress, and UV light on growth and resulting changes have also been reported in other studies. According to van Bokhorst-van de Veen et al. (2011), ethanol pre-exposure can only confer cross-protection against heat stress because the damage that ethanol causes to the cell membrane mainly affects its permeability and fluidity, and it destabilizes enzymes on the membrane but increases the transcription of heat-stress-associated proteins. Exposure to heat (39°C) can increase resistance to NaCl, because heat-stress-induced proteins such as htrA are also used under osmotic stress as a defense mechanism (Foucaud-Scheunemann and Poquet, 2003). Kilstrup et al. (1997) showed that after 4 h of salt exposure [2.5% (wt/vol) NaCl concentrations and higher], *L. lactis* growth was negatively affected. This study showed

that several heat-stress genes, such as GroES, GroEL, and DnaK, can also be produced under osmotic stress. Desmond et al. (2002) showed that heat resistance can be achieved by previous sub-lethal exposure to salt [approximately 2% (wt/vol) NaCl], because some genes produced under both stresses are the same. The lack of catalase activity in *Lactococcus* made it vulnerable to oxidative stress, which often leads to DNA and cell membrane damage that may weaken the bacterial cells and prevent them from growing in 7% (wt/vol) NaCl (Rochat et al., 2005). Hartke et al. (1995) showed that *L. lactis* exposed to 100 J/cm² UV light can develop resistance to ethanol (20% vol/vol) and acid (pH 4.0) stress due to an overlap in stress-protein expression. In another study, Hartke et al. (1997) reported that 30 min of exposure to UV light at 254 nm was necessary to start the production of heat-stress proteins such as GroEL and GroES, which can also lead to a cross-protection effect.

Unlike the log counts of *L. lactis* R-604 grown in 1, 3, and 7% salt concentrations, some of the log counts of cells grown in 5% salt concentration were significantly higher than log counts for the corresponding control. Specifically, log counts of *L. lactis* R-604 grown in 5% salt and previously subjected to mild heat on d 3, d 4, and d 5 or to ethanol or hydrogen peroxide on d 4 and d 5 were significantly higher than the corresponding log counts of control *L. lactis* R-604 in 5% salt. We can conclude that previous exposure to the stresses of ethanol, hydrogen peroxide, or mild heat before inoculation into M17 broth containing 5% (wt/vol) salt provided resistance to *L. lactis* for enhancing subsequent growth after 3 or 4 d of storage.

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