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ORIGINAL ARTICLE

Mg^{2+} improves the thermotolerance of probiotic *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8

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Significance and Impact of the Study: In order to improve the productivity and stability of live probiotics, extensive investigations have been carried out to improve thermotolerance of probiotics. However, most of these studies focused on the effects of carbohydrates, proteins or amino acids. The roles of inorganic salts in various food materials, which have rarely been reported, should be considered when incorporating probiotics into these foods. In this study, Mg^{2+} was found to play a significant role in the thermotolerance of probiotic lactobacilli. A novel strategy may be available in the near future by employing magnesium salts as protective agents of probiotics during manufacturing process.

Keywords
inorganic salts, magnesium, probiotics, recovery, thermotolerance.

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Abstract

Food-related carbohydrates and proteins are often used as thermoprotectants for probiotic lactobacilli during industrial production and processing. However, the effect of inorganic salts is rarely reported. Magnesium is the second-most abundant cation in bacteria, and commonly found in various foods. Mg^{2+} homeostasis is important in *Salmonella* and has been reported to play a critical role in their thermotolerance. However, the role of Mg^{2+} in thermotolerance of other bacteria, in particular probiotic bacteria, still remains a hypothesis. In this study, the effect of Mg^{2+} on thermotolerance of probiotic lactobacilli was investigated in three well-documented probiotic strains, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8, in comparison with Zn^{2+} and Na^{+}. Concentrations of Mg^{2+} between 10 and 50 mmol l^{-1} were found to increase the bacterial survival upon heat challenge. Remarkably, Mg^{2+} addition at 20 mmol l^{-1} led to a 100-fold higher survival of *L. rhamnosus* GG upon heat challenge. This preliminary study also showed that Mg^{2+} shortened the heat-induced extended lag time of bacteria, which indicated the improvement in bacterial recovery from thermal injury.

Introduction

Lactobacillus is an important food-related bacterial genus comprising various species used as starter cultures for human applications with a long history of safe use. In recent decades, a large number of lactobacilli strains have been reported to confer beneficial effects on the host when ingested in an adequate dose (Hill *et al.* 2014). These lactobacilli strains are known as 'probiotics', which are of great interest nowadays in pharmaceutical and functional food industries. Apart from the flavour-forming properties and health effects, stress tolerance is also an essential characteristic when selecting lactobacilli for food and pharmaceutical applications. Indeed, stress tolerance determines the ability of bacteria to adapt to different ecosystems and thereby maintain a high level of viability during processing, storage and digestion (Hussain *et al.* 2013).

There is a growing interest in inducing a thermotolerant phenotype in lactobacilli for industrial applications. The reason mainly relates to the feasibility of producing them with satisfying viability via spray drying (Fu and

Chen 2011). Freeze drying is currently the most widespread industrial drying method to produce starter or probiotic cultures. However, spray drying represents a more cost-effective, energy-efficient and productive drying alternative compared to freeze drying (Fu and Chen 2011)). Nevertheless, the high temperatures during spray drying typically lead to lower viability of bacteria than freeze drying. Hence, by improving the thermotolerance of bacteria, spray drying could be a suitable process for sustainable production of starter and probiotic cultures.

Food-grade carbohydrates and proteins are extensively employed as protective agents to improve bacterial viability upon heat treatment and spray drying (Fu and Chen 2011). However, the effect of inorganic salts on thermotolerance of *Lactobacillus* has been rarely reported. Previously, Ca^{2+} was found to influence the thermotolerance of lactic acid bacteria (Huang and Chen 2013). Apart from Ca^{2+}, Mg^{2+} is the second-most abundant cation in bacteria (Romani and Scarpa 2000). The roles of bacterial Mg^{2+} in homeostasis, sensing and transport were extensively investigated (mostly in Gram-negative bacteria *Salmonella enterica* serovar Typhimurium and *Escherichia coli*), including: acting as a cofactor in ATP-dependent phosphorylation and a variety of other enzymatic reactions, stabilizing ribosome and membranes, influencing RNA folding, the nucleic acid–protein interactions and bacterial virulence (Groisman *et al.* 2013). O'Connor *et al.* (2009) first reported the function of Mg^{2+} in the regulation of bacterial thermotolerance. Increased expression of Mg^{2+} transport proteins was found to enhance survival of *S. enterica* upon heat treatment. However, the role of Mg^{2+} in the regulation of thermotolerance in other organisms still remains unclear.

In this study, we investigated the influence of Mg^{2+} concentration on thermotolerance of three well-documented probiotic lactobacilli strains, *Lactobacillus rhamnosus* GG (LGG in brief), *Lactobacillus casei* Zhang (LCZ in brief) and *Lactobacillus plantarum* P-8 (LP in brief).

Results and discussion

Bacterial survival after heat challenge

Bacterial survival rate is expressed as log N/N_0, where N is the bacterial population following heat challenge and N_0 is the population before heat challenge. The survival of three probiotic strains after heat challenge is shown in Fig. 1. Treatment of bacteria with a low concentration of $MgCl_2$ (5–100 mmol l^{-1}) in a 10% lactose solution was found to improve bacterial tolerance upon heat treatment. More specifically, 20 mmol l^{-1} $MgCl_2$ was the optimal concentration to enhance thermotolerance of LGG and LP (Fig. 1a,c), whereas it was 10 mmol l^{-1} for LCZ (Fig. 1b).

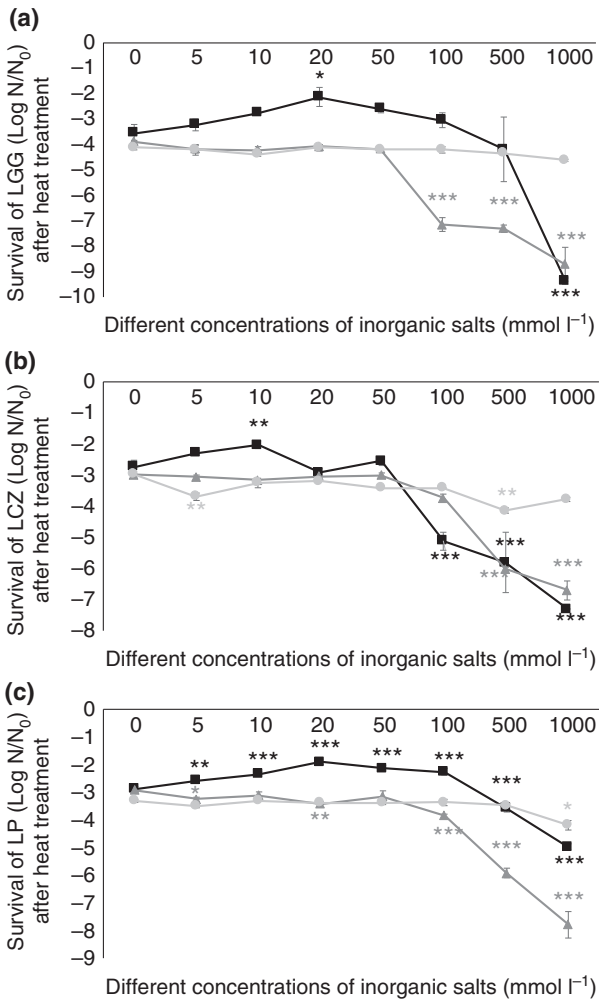

Figure 1 The survival (log N/N_0) of (a) LGG, (b) LCZ and (c) LP in lactose solutions supplemented with different concentration of $MgCl_2$ (—■—), $ZnCl_2$ (—▲—) and NaCl (—●—) after heat treatment. Significant difference was compared between the salt-treated samples and the samples without adding salt, i.e. 0 mmol l^{-1} added (*: $0{\cdot}01 < P < 0{\cdot}05$, **: $0{\cdot}001 < P < 0{\cdot}01$, ***: $P < 0{\cdot}001$).

In LGG, 20 mmol l^{-1} $MgCl_2$ addition led to a 100-fold higher survival rate upon heat challenge. However, a negative effect on thermotolerance of all three probiotic strains was observed when the concentration of $MgCl_2$ was higher than 500 mmol l^{-1}. In contrast, treatment of bacteria with $ZnCl_2$ and NaCl did not enhance bacterial thermotolerance despite the use of high or low concentrations. Moreover, bacterial viability dropped sharply when being treated with a high concentration of $ZnCl_2$ (>100 mmol l^{-1}).

The results in Fig. 1 display the different effects of $MgCl_2$, $ZnCl_2$ and NaCl on the survival rate of three bacterial strains after heat challenge. The comparison suggests that the effects of $MgCl_2$ on bacterial thermotolerance should be attributed to the cation Mg^{2+}, instead of the anion chloride. The osmolality caused by salt

supplementation (mostly by NaCl) may trigger thermotolerance in bacteria in lactose solution (De Angelis and Gobbetti 2004). However, this cross protection phenomenon was not induced by a high concentration of salts in this study. It may be explained by the insufficient duration of the 30-min salt treatment to induce the cellular stress response (Huang *et al.* 2016). Moreover, a high concentration of salts (500 mmol l^{-1}) displayed negative effects on the survival of bacteria upon heat treatment. This may be caused by the extra osmotic stress apart from heat stress. Since there was only lactose in the suspension, the bacteria were not able to uptake the compatible solutes from the environment to reach cellular homeostasis.

The enhanced thermotolerance of *Lactobacillus* strains in this study is in agreement with the speculation proposed by O'Connor *et al.* (2009), who found that overproduction of the *Bacillus subtilis* MgtE protein led to enhanced thermotolerance of *Salmonella*. The genes encoding similar magnesium-transport proteins can be found in most of the lactobacilli species and strains, *mgtA*, *mgtC* and *corA* in LGG for instance (Morita *et al.* 2009). It indicates that the effect of Mg^{2+} on bacterial thermotolerance may be of interest for other lactobacilli species and strains used as starter and adjunct cultures for many food fermentations. However, the possible interactions between magnesium transport, homeostasis and heat shock response in *Lactobacillus* require further transcriptomics and proteomics investigation.

Besides, the effect of Mg^{2+} on improving bacterial thermotolerance observed in this study was similar to the Ca^{2+} effect reported previously regarding the close range of effective concentration (Huang and Chen 2013). Indeed, Mg^{2+} is similar to Ca^{2+} regarding their roles in lactic acid bacteria, especially for their interactions with enzyme activity, cell division, surface proteins and nucleic acids (Boyaval 1989). This indicates that the distinct physicochemical properties of Mg^{2+} and Ca^{2+} may play significant roles in thermotolerance of *Lactobacillus*, particularly their abilities in binding and stabilizing biomolecules such as nucleic acids, proteins and phospholipid bilayers, and their influence on cell adhesion on environmental matrices or cell mutual adhesion (Burgain *et al.* 2014; Gao and Yang 2016). These physicochemical properties may allow the effect of Mg^{2+} to be applicable to other bacterial species.

Recovery of heat-injured probiotics

The re-growth activity of bacteria is characterized by lag phase (λ), and asymptotic value (A) from the re-growth curves (Table 1). The definition of re-growth corresponds to the ability of *Lactobacillus* to grow after being exposed to a heat treatment for 1 min at 75°C. The methods of obtaining λ and A are explained in the Materials and Methods section. Heat treatment caused extended λ and decreased A in the absence of salt addition in de Man, Rogosa and Sharpe (MRS) broth. Interestingly, as a reference, MRS supplementation with $MgCl_2$ or NaCl can significantly shorten the heat-induced extension of λ (Table 1 and Fig. S1). The optimal concentrations differed depending on the strain. For $ZnCl_2$ supplementation, only a low concentration (5 mmol l^{-1}) showed a positive effect on the λ of LP strain, but not for LGG and LCZ strains. Regarding the asymptotic value (A) in the re-growth curves, the effects of inorganic salts differed largely depending on different strains. Specifically, MRS supplementation with 5–50 mmol l^{-1} $MgCl_2$ and 10–20 mmol l^{-1} $MgCl_2$ showed significantly enhanced A values for LCZ and for LP, respectively, whereas no effect of $MgCl_2$ was shown on LGG. In contrast, a low concentration of $ZnCl_2$ increased the A values of LGG and LP, whereas NaCl supplementation increased the A values of LGG (5 mmol l^{-1}) and LCZ (5, 10 and 100 mmol l^{-1}).

Recovery of bacteria from sub-lethal injury requires *de novo* synthesis ribosomes and membrane (Wu 2008). Mg^{2+} can influence the nitrogen metabolism and enzyme activity of bacteria, which may involve in protein synthesis during the repair process from thermal injury. after thermal injury (Boyaval 1989; O'Connor *et al.* 2009). Besides, Mg^{2+} can stabilize bacterial cell morphology and membrane integrity, which was found to relate to the heat shock response in *L. plantarum* WCFS1 (Capozzi *et al.* 2011). For instance, Rayman and MacLeod (1975) found that Mg^{2+} strengthens the bacterial cell wall by influencing the structure of peptidoglycan.

Mg^{2+} was also reported to promote the division of Gram-positive bacteria at certain concentrations, which may explain the better re-growth after being treated with Mg^{2+} (Hayek *et al.* 2013). However, the effect of Mg^{2+} on bacterial re-growth was strain-dependent (Table 1). Moreover, certain concentrations of Na^{+} and low concentration of Zn^{2+} also displayed a strain-dependent effect on promoting the re-growth of heat-injured bacteria. We speculated that the reason may be the interactions between Na^{+} and Zn^{2+}, and bacterial enzyme activity and membrane integrity, which possibly affect the metabolism and transmembrane transport of nutrients in bacteria as suggested by Korkeala *et al.* (1992) and Omburo *et al.* (1992).

Materials and methods

Bacterial strains and culture conditions

Lactobacillus rhamnosus GG (LGG) was obtained from a commercial product (Culturelle, CVS Pharmacy).

Table 1 The heat-induced changes in lag time $\lambda - \lambda_0$ and asymptotic value $A - A_0$ between the re-growth curves of *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus casei* Zhang (LCZ) and *Lactobacillus plantarum* P-8 (LP) in MRS with and without inorganic salt supplementation

	Concentrations of mineral ions (mmol l^{-1})						
	0	5	10	20	50	100	500
LGG							
$\lambda-\lambda_0$ (h)							
$MgCl_2$	0	0·1 ± 0·1^{c}	0·2 ± 0·0^{cd}	**−0·2 ± 0·1^{d}**	**−0·5 ± 0·1^{b}**	0·3 ± 0·1^{a}	ND
$ZnCl_2$		0·6 ± 0·1^{a}	5·0 ± 0·1^{b}	ND	ND	ND	ND
NaCl		**−0·5 ± 0·1^{a}**	0·5 ± 0·0^{d}	0·2 ± 0·1^{c}	**−0·2 ± 0·1^{b}**	**−0·3 ± 0·1^{b}**	1·6 ± 0·1^{e}
$A-A_0$							
$MgCl_2$	0	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	−0·2 ± 0·0^{a}	ND
$ZnCl_2$		**0·1 ± 0·0^{b}**	0·0 ± 0·0^{a}	ND	ND	ND	ND
NaCl		**0·1 ± 0·0^{c}**	−0·1 ± 0·0^{a}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	−0·1 ± 0·0^{a}
LCZ							
$\lambda-\lambda_0$ (h)							
$MgCl_2$	0	**−0·7 ± 0·1^{b}**	**−0·6 ± 0·1^{b}**	**−2·5 ± 0·2^{a}**	**−0·8 ± 0·1^{b}**	1·7 ± 0·1^{c}	ND
$ZnCl_2$		0·9 ± 0·1^{a}	2·5 ± 0·1^{b}	ND	ND	ND	ND
NaCl		**−1·2 ± 0·2^{c}**	**−0·6 ± 0·2^{d}**	**−2·4 ± 0·3^{a}**	**−1·7 ± 0·2^{b}**	**−1·4 ± 0·2^{bc}**	**−0·6 ± 0·2^{d}**
$A-A_0$							
$MgCl_2$	0	**0·2 ± 0·0^{c}**	**0·3 ± 0·0^{d}**	**0·1 ± 0·0^{b}**	**0·1 ± 0·0^{b}**	−0·1 ± 0·0^{a}	ND
$ZnCl_2$		0·0 ± 0·0^{b}	−0·1 ± 0·0^{a}	ND	ND	ND	ND
NaCl		**0·2 ± 0·0^{e}**	**0·2 ± 0·0^{e}**	−0·1 ± 0·0^{a}	0·0 ± 0·0^{c}	**0·2 ± 0·0^{d}**	−0·1 ± 0·0^{b}
LP							
$\lambda-\lambda_0$ (h)							
$MgCl_2$	0	0·0 ± 0·1^{d}	**−0·8 ± 0·1^{a}**	**−0·5 ± 0·1^{b}**	**−0·3 ± 0·1^{c}**	**−0·1 ± 0·1^{d}**	ND
$ZnCl_2$		**−0·6 ± 0·1^{a}**	5·2 ± 0·1^{b}				
NaCl		**−0·5 ± 0·1^{a}**	**−0·3 ± 0·1^{bc}**	**−0·4 ± 0·1^{ab}**	**−0·2 ± 0·1^{c}**	**−0·5 ± 0·1^{a}**	0·5 ± 0·1^{d}
$A-A_0$							
$MgCl_2$	0	−0·1 ± 0·0^{a}	**0·1 ± 0·0^{c}**	**0·2 ± 0·0^{d}**	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	ND
$ZnCl_2$		**0·1 ± 0·0^{b}**	0·0 ± 0·0^{a}	ND	ND	ND	ND
NaCl		0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	0·0 ± 0·0^{b}	−0·1 ± 0·0^{a}

Different superscript letters represent significant difference from the values in the same lines, i.e. the samples treated with the same salts ($P < 0·05$).
'ND' represents undeterminable due to non-growth of bacteria after heat treatment.
Data in bold mean positive effect in re-growth (i.e. less extended lag time and less decreased biomass yield).

Lactobacillus casei Zhang (LCZ) and *L. plantarum* P-8 (LP) were provided by the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, China. These three *Lactobacillus* strains were routinely activated and cultivated statically at 37°C for 24 h in an MRS broth.

Heat challenge

Preparation of bacterial suspension and heat challenge experiments were performed as described previously (Huang and Chen 2013). Lactose solution (10% w/v) was used as the medium due to its excellent drying behaviour and potential protection on bacteria during spray drying. Briefly, 500 μl of bacterial culture after 24 h incubation was washed with peptone water and then re-suspended in 500 μl 10% w/v lactose solution with 0, 5, 10, 20, 50, 100 and 500 mmol l^{-1} of $MgCl_2$ respectively. Besides, the same concentrations of NaCl and $ZnCl_2$ were also used as a control to compare the effects of Mg^{2+}. The suspensions were moderately shaken at 25°C for 30 min prior to heat treatment at 70°C for 2 min. The viable and cultivable population of *Lactobacillus* was determined by CFU counting, on plates of MRS medium, solidified by 10 g l^{-1} agar, for both heated and unheated cultures. The survival was thus expressed as log N/N_0, where N is the population following the heat treatment and N_0 is the population before the heat challenge.

Recovery from heat injury

As in the heat challenge experiment, 1 ml of bacterial suspension was prepared by re-suspending bacterial pellets in 1 ml 10% w/v lactose solution. Heat treatment was performed at 75°C for 1 min. After the heat treatment, the heated suspension was inoculated (1% inoculum) into the

MRS broth supplemented with different concentrations of inorganic salts in 48-well plates. The bacterial re-growth was monitored by measurement of the change in optical density (OD_{600}) at 37°C for 30 h (curves are shown in the supporting information, Fig. S1) using a microplate reader (SpectraMax M5; Molecular Devices, CA, USA). The modified Gompertz model was used to fit the OD_{600} curves (Zwietering *et al.* 1990). The growth lag phase (λ) and asymptotic value (A, indicates the final biomass yield) were calculated to characterize the re-growth activity of bacteria (an example of fitting is shown in Fig. 2 and Table 2). The R-square values between all experimental curves and fitting equations were calculated by Origin Pro 8 SR02 (OriginLab, MA, USA). The goodness of fit of all curves was found to be higher than 0·995. To compare the effects of salts on the re-growth activity of bacteria, the difference between heat-induced changes in lag phase and asymptotic value were compared by $\lambda - \lambda_0$ and $A - A_0$. λ_0 and A_0 refer to the lag phase and asymptotic value of bacteria re-growth in MRS without inorganic salt supplementation after heat treatment, while λ and A refer to that of in MRS with inorganic salt supplementation after heat treatment respectively.

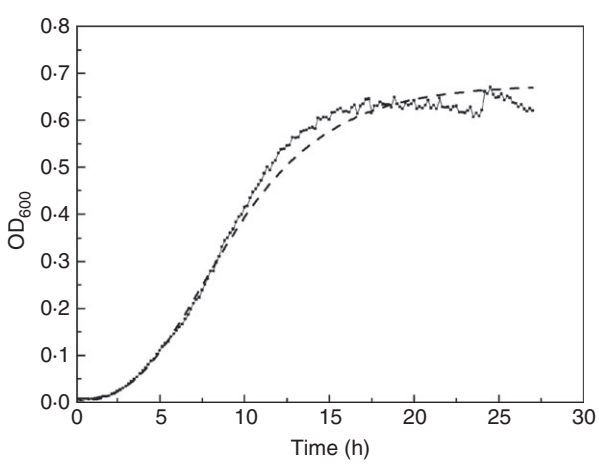

Figure 2 An example to describe the growth curve of *Lactobacillus rhamnosus* GG at 37°C fitted with the Gompertz (Gom) model. The solid curve is the experimental curve, while the dotted one represents the fitted curve.

Table 2 The values of μ_m, λ and A from the curve fitted by the Gompertz model in Fig. 2

Lactobacillus rhamnosus GG	Equation	$Y = a*\exp(-\exp(-k*(x - x*c)))$
Fitted value	a	0·67503
	$x*c$	7·48637
	k	0·24523
Formula	$\lambda = (k*x*c - 1)/k$	λ = 3·963933
	$A = a$	A = 0·64727

Statistical analysis

The heat challenge experiments were repeated three times. Bacterial re-growth experiments were performed with duplicate samples. The data were presented as mean values ± standard deviation. The difference between the mean values was compared by the Tukey test using R software in couple with R commander (Rcmdr package; R Development Core Team, Vienna, Austria).

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Conflict of Interest

All authors report no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Growth curves of LGG, LCZ and LP in MRS supplemented with different concentrations of $MgCl_2$, $ZnCl_2$ and NaCl.