

Full Length Research Paper

## Effect of growth conditions on glutathione accumulation, *gshR* gene expression and resistance to the lyophilization process in *Lactococcus lactis* LVA. 2

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The functionality of glutathione (GSH) and the effect of growth conditions on GSH accumulation, *gshR* gene expression and viability following lyophilization were examined in *Lactococcus lactis* LVA. 2. The accumulation of GSH under anaerobic and continuously stirred aerobic conditions enabled a higher GSH accumulation than a static aerobic culture, which is the growth condition normally used. The *gshR* gene expression under the growth conditions in the anaerobic, static aerobic and continuously stirred aerobic media was similar. Although, GSH failed to provide an apparent protection to *L. lactis* LVA. 2 following the lyophilization process, the fact that this strain accumulates GSH and expresses the *gshR* gene makes it a promising strain to cope with other stress situations.

**Key words:** *Lactococcus lactis*, reduced glutathione, lyophilization, survival.

### INTRODUCTION

Lyophilization is the method most commonly used in the preservation of lactic acid bacteria intended for cheese, yogurt and fermented-milk production (Champagne et al., 2009; Turchi et al., 2013). Although the lyophilization process is used to maintain the viability of bacteria for a long storage period, much of the cell viability is compromised. Lyophilization exposes cells to stress conditions due to freezing and to osmotic stress caused

by water loss (Santivarangkna et al., 2008). Various treatments are applied to the cell suspension to protect it from the damages caused by lyophilization, the most common being the addition of cryoprotectant substances and the application of sublethal stress treatments (Savini et al., 2010).

Glutathione ( $\gamma$ -glutamyl-cysteine-glycine [GSH]) is a tripeptide found in eukaryotes and Gram-negative bacteria

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and to a lesser extent in Gram-positive bacteria (Copley and Dhillon, 2002). GSH is found in the reduced (GSH) and oxidized (GSSG) forms in cells. An increased proportion of GSSG is found when cells are exposed to oxidative stress conditions. One of the main functions of GSH in cells is the maintenance of intracellular homeostasis. In addition to GSH accumulation, the presence of glutathione reductase (GshR), whose role is to convert GSSG into GSH to maintain intracellular homeostasis, is also important (Pophaly et al., 2012).

The ability to transport GSH into the intracellular environment and the presence of glutathione reductase have already been noted in some strains of *Lactococcus lactis* (Li et al., 2003). The functionality of GSH in *L. lactis* was identified as contributing to protection from conditions of oxidative stress (Li et al., 2003), acid stress (Zhang et al., 2007) and osmotic stress (Zhang et al., 2010). However, the functionality of GSH has not been evaluated in *L. lactis* regarding protection from the lyophilization process.

Thus, the purpose of this study was to evaluate whether GSH has a protective effect following the lyophilization process in *L. lactis* LVA. 2. Furthermore, the effect of several growth conditions on GSH accumulation, expression of the *gshR* gene (which encodes the glutathione reductase enzyme), and resistance to lyophilization was evaluated.

## MATERIALS AND METHODS

### Detection of synthesis and/or transport of GSH

*L. lactis* LVA. 2 is a strain isolated from cow's milk, belonging to the collection of cultures from the Laboratory of Industrial Microbiology, Federal University of Viçosa (Universidade Federal de Viçosa - UFV), Brazil. The culture was transferred from the stock at -80°C into M17 broth containing 5 g/L of glucose and incubated at 30°C for 14 h to prepare the preculture. The preculture was inoculated (1%, v/v) in a chemically defined medium (CDM) supplemented and unsupplemented with reduced GSH (Sigma-Aldrich Co., St Louis). The final concentrations of GSH in the CDM were 2, 4 and 6 mM in the supplemented treatments. The inoculated media were statically incubated at 30°C for 7 h. The detailed composition of CDM is described by Li et al. (2003).

### Growing conditions for physiological characterization

M17 supplemented with GSH (final GSH concentration of 4 mM) and without GSH supplementation was incubated statically and in a rotary shaker at 200 rpm at 30°C for 7 h. Anaerobic M17 medium (supplemented and unsupplemented with GSH) obtained by heating and injecting nitrogen gas for 30 min was prepared in addition to those conditions. The inoculated media were incubated at 30°C for 7 h.

### Cell-free extract preparation

The cells derived from the different growth conditions were recovered by centrifugation (8,000  $\times$ g, 10 min, 4°C). The cell pellet was washed twice with 0.1 M potassium phosphate buffer supple-

mented with EDTA (5 mM), pH 7.5 (KPE) and re-suspended in 4 mL of KPE buffer. Glass beads (Sigma-Aldrich Co., St Louis) were added to the cell suspension for cell lysis in Precellys 24 (Bertin Technologies®, Bioamerica Inc.) at 4,000 rpm for 15 s. The cell extracts were pelleted by centrifugation for 10 min (10,000  $\times$ g, 4°C), and the supernatant was used to assess the GSH. The protein concentration in the supernatant was assessed using the Lowry method, and bovine serum albumin was used as standard.

### GSH assessment

Total GSH, which includes both the reduced form (GSH) and the oxidized form (glutathione disulfide - GSSG), and GSSG were quantified using the enzymatic recycling method (Tietze, 1969), and the absorbance at 412 nm was assessed in a 96-well microdilution plate in an enzyme-linked immunosorbent assay (ELISA) reader (Biotek) (Rahman et al., 2006). The amount of GSH was calculated as the difference between total glutathione and GSSG (total glutathione-GSSG = GSH). The glutathione content was expressed as nmol/mg protein in the sample.

### *gshR* gene detection

The primer pair GSHRf/GSHRr, based on the nucleotide sequence of *L. lactis* subsp. *lactis* Il1403 (NC\_002662.2) available in the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) database, was designed to amplify the *gshR* gene encoding the protein glutathione reductase. The Primer-Blast tool, available at NCBI, was used for primer selection, and the primer quality was assessed using the OligoAnalyzer 3.1 software (<http://www.idtdna.com>). The PCR product obtained was sequenced. The primer sequence is shown in Table 1.

### Analysis of *gshR* gene expression

The *L. lactis* LVA. 2 cells derived from growth in M17 medium (static growth, in a rotary shaker, in anaerobic medium) were recovered by centrifugation at 8,000  $\times$ g and 4°C for 5 min. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA purity was assessed by measuring the absorbance values at 260 and 280 [nm] (approximately 1.8 to 2.0) in a Nanodrop spectrophotometer (Thermo Scientific, San Jose, CA, USA), and the RNA integrity was evaluated using electrophoresis. The contaminant genomic DNA present in the total RNA was removed upon treatment with RNase-free DNase (Promega, Madison, WI) at 37°C for 60 min, and the resulting RNA was used to synthesize cDNA using the ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI) according to the manufacturer's instructions.

The partial sequence of the *gshR* gene, previously obtained by sequencing, was used to design the GS912f/GS912r primer pair to evaluate the *gshR* gene expression. The GYRB4f/GYRB4r primer pair was used to evaluate the expression of the *gyrB* gene, which encodes the protein DNA gyrase, used as an internal control. GenScript Real-time PCR tools were used for primer selection, and the primer quality was assessed using the OligoAnalyzer 3.1 software (<http://www.idtdna.com>). The primer sequences are shown in Table 1.

The quantitative analyses were performed on a CFX96™ Real-Time PCR detection system and a C100™ Thermal Cycler (Bio-Rad) thermocycler to evaluate the *gshR* gene expression at a transcriptional level, using Platinum® SYBR® Green qPCR supermix-UDG (Invitrogen) to monitor the synthesis of double-strand DNA. The melting curve was analyzed to verify the absence of non-specific amplification of the PCR product. All the samples

**Table 1.** Sequences of primers used.

Gene	Primer (5'-3')	Amplicon size	Use
<i>gshR</i>	GSHRf - TGATGGCAGTTGTCTGAAGCC	917	PCR
	GSHRr - ATGAATCCCCTCACCTGCCA		
<i>gshR</i>	GS912f - CGCTTGAATTTCCCGCTCA	91	qRT-PCR
	GS912r - TCGCTTCCAACCGCTTTAGCA		
<i>gyrB</i>	GYRB4f - GGGATGATGTCCGCGAAGGA	143	qRT-PCR
	GYRB4r - TGTTGTCAAAGCTTCCGCAAAGA		

were analyzed in triplicate, and the mean values were used in the subsequent calculations. The reaction efficiencies were measured (Bustin et al., 2005; Pfaffl, 2001) and the amplification specificity was assessed through analysis of dissociation curves. The *gshR* gene expression was normalized using the expression of the internal control (*gyrB*) gene. The relative expression was determined using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

### Lyophilization

Aliquots (1 mL) from the *L. lactis* LVA. 2 culture grown in CDM and M17 media, under different growth conditions, were centrifuged at 10,000  $xg$  and 4°C for 5 min. The pellet formed was washed twice with 0.1% peptone water, re-suspended in 1 mL of peptone water and stored at -80°C for 3 h. Subsequently, the samples were dehydrated in a lyophilizer (Enterprise II, Terroni) for 7 h. After the lyophilization process, the dehydrated cells were reconstituted in 1 mL of 0.1% peptone water for 15 min at room temperature. The viability was assessed using the flow cytometry method immediately after reconstitution.

### Analysis of viable cells

The cell integrity following the lyophilization process was analyzed using two dyes, which stain nucleic acids, in combination with the flow cytometry analyses. The fluorescent dyes syto9 and propidium iodide (PI) from the bacterial viability kit LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> (Molecular probes, Invitrogen) were used for that purpose. The syto9 dye stains the DNA of living and damaged cells. The PI dye only stains the DNA of cells with compromised plasma membranes. The cell-staining protocol provided by the manufacturer was modified in a few steps. Aliquots of stock solutions of the Syto (3.34 mM) and PI (20 mM) dyes were diluted in 0.85% saline to prepare a 0.83 and 5 mM concentration of Syto9 and PI, respectively. The dehydrated cells reconstituted in 0.1% peptone water were diluted in 0.85% saline to reach  $10^4$  to  $10^5$  colony-forming units (CFU) mL<sup>-1</sup>. Aliquots (100  $\mu$ L each) of the diluted cell suspension were supplemented with 900  $\mu$ L of 0.85% saline and 1.5  $\mu$ L of each dye. The samples were incubated for 15 min at room temperature and in the dark and were then centrifuged (13,000  $xg$ , 3 min, 4 °C), and the pellet was resuspended in 300  $\mu$ L of 0.85% saline prior to the flow cytometry analysis. The analyses were performed in a Guava EasyCyte Plus<sup>®</sup> flow cytometer. The red fluorescence of the PI-stained cells and the green fluorescence of the syto9-stained cells were detected using 583/26 and 525/30 nm filters, respectively. The gates of the forward scatter/side scatter (FSC/SSC) graphs were used to distinguish the bacteria. The analyses were performed using low flow (0.24  $\mu$ l sec<sup>-1</sup>), and the event data (10,000 events per sample counted in the bacterial gate in the FSCxSSC plots) were collected and analyzed using Cytosoft 5.3 Guava<sup>®</sup>. The results found are derived from three replicates.

### Nucleotide sequence accession number

The nucleotide sequence for the *gshR* gene described in this study was deposited in GenBank under accession number KC821740.

## RESULTS

### Presence of GSH in *L. lactis* LVA. 2

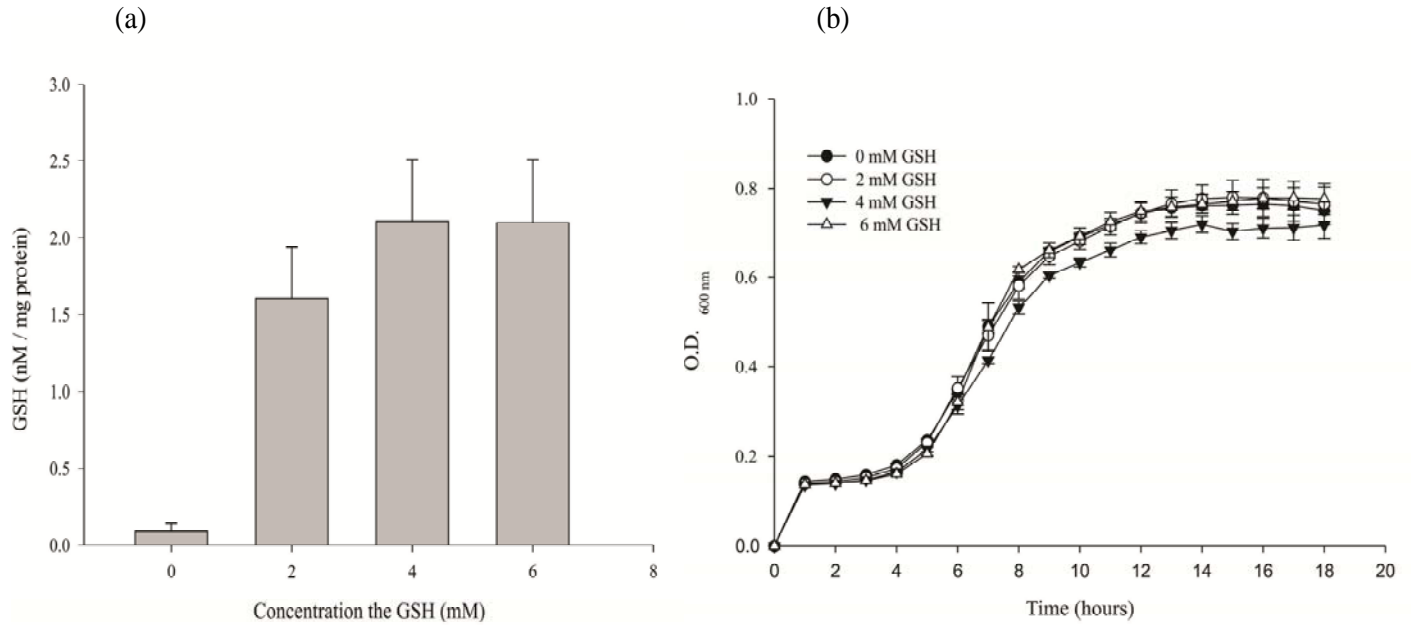
The intracellular GSH content of *L. lactis* LVA. 2 grown in CDM with and without GSH supplementation was evaluated (Figure 1). The absence of intracellular GSH in *L. lactis* LVA. 2 in the CDM without GSH supplementation indicated that this strain is not able to synthesize GSH and is only able to transport it when the CDM is supplemented with GSH (Figure 1a). It was also observed that the cells accumulated almost the same amount of GSH that was found in the CDM at the 4-mM GSH concentration when the final concentration of GSH in the CDM was 6 mM. Furthermore, the tested strain does not use GSH from the culture medium as a source of that nutrient for its growth (Figure 1b).

### Increased concentration of intracellular GSH

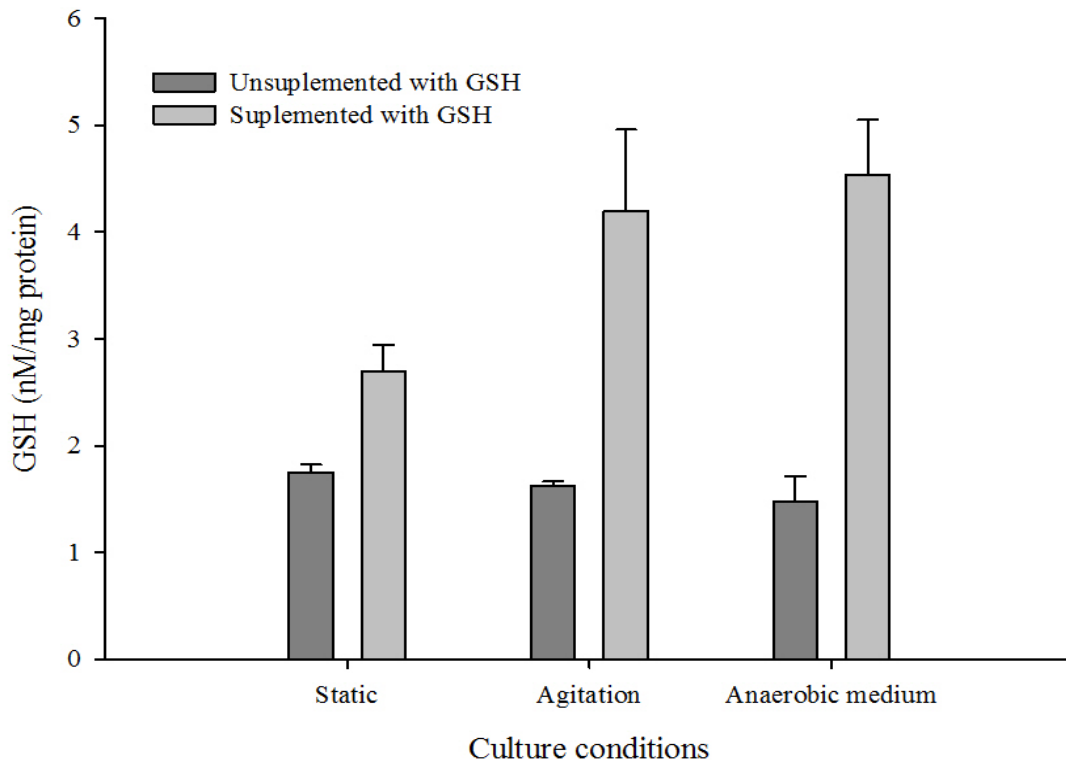
Different growth conditions were evaluated to assess their effect on the intracellular GSH concentration in *L. lactis* LVA. 2 (Figure 2). A low concentration of intracellular GSH was obtained when the cells were grown in M17 medium without GSH supplementation, regardless of the growth condition used, and no noticeable difference was found. The supplementation of the M17 medium with GSH increased the intracellular GSH concentration in the three growth conditions, although such increase was more marked in the anaerobic medium and in the aerobic growth in a rotary shaker.

### *gshR* gene expression in different growth conditions

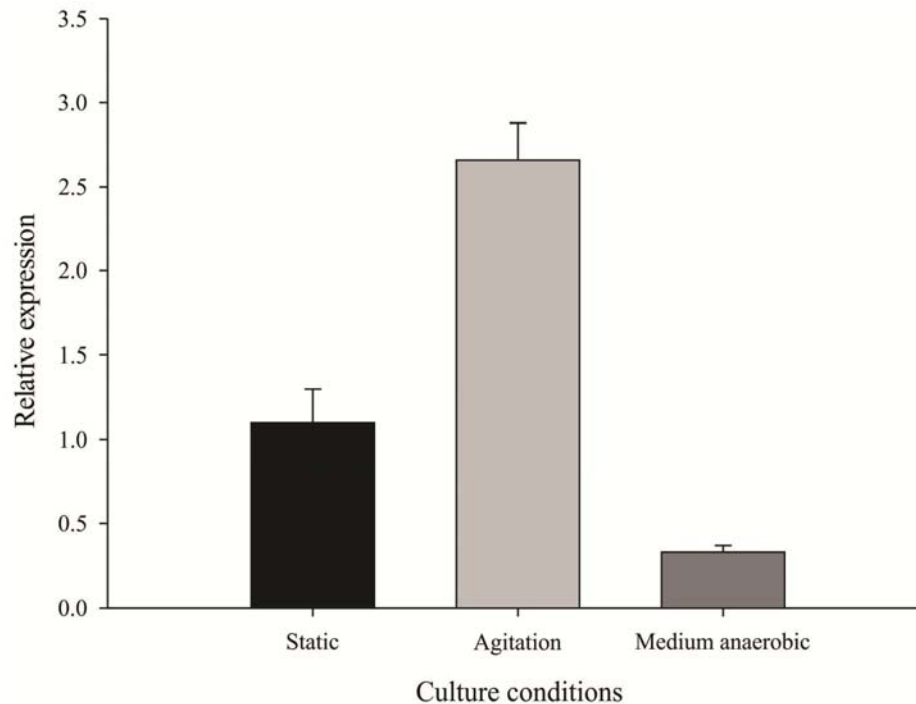
The relative expression analysis using real-time quantitative polymerase chain reaction (RT-qPCR) of the *gshR* gene was evaluated to assess whether the gene's expression is altered in the different growth conditions in



**Figure 1.** Effect of adding different GSH concentrations to CDM on the intracellular GSH concentration (a) and growth of *L. lactis* LVA. 2 (b) cultured at 30°C for 7 h. The intracellular GSH concentration and cellular growth were assessed using a spectrophotometer measuring the absorbance at 412 and 600 nm, respectively. The mean values are based on three sample replicates. The error bars indicate standard deviations.



**Figure 2.** Intracellular GSH concentration of *L. lactis* LVA. 2 in M17 medium supplemented and unsupplemented with GSH. The cells were incubated statically or in a rotary shaker (200 rpm) at 30°C for 7 h. The cells were also seeded in anaerobic M17 medium statically incubated at 30°C for 7 h. The intracellular GSH concentration was assessed using a spectrophotometer measuring the absorbance at 412 nm. The mean values are based on three sample replicates. The error bars indicate standard deviations.



**Figure 3.** Normalized relative expression of the *gshR* gene of *L. lactis* LVA. 2 under different growth conditions in M17 medium supplemented with GSH. The inoculated medium was incubated at 30°C for 7 h. The error bars represent the standard deviations.

M17 medium supplemented with GSH. The growth of *L. lactis* LVA. 2 under the different growth conditions caused different variations in the relative expression of the *gshR* gene (Figure 3). The gene expression under continuously stirred aerobic growth conditions increased 2-fold as compared to the growth in static aerobic conditions (control), and its expression increased 8-fold as compared to the growth in the anaerobic medium.

### Viability assessment

The PI and syto9 dyes were used simultaneously to assess the viability of *L. lactis* LVA. 2 prior to and following lyophilization (Figure 4). Differences in the distribution of the cell population were observed among the cell populations prior to (Figure 4a) and following lyophilization (Figure 4b). Most of the cell population following the lyophilization process was damaged.

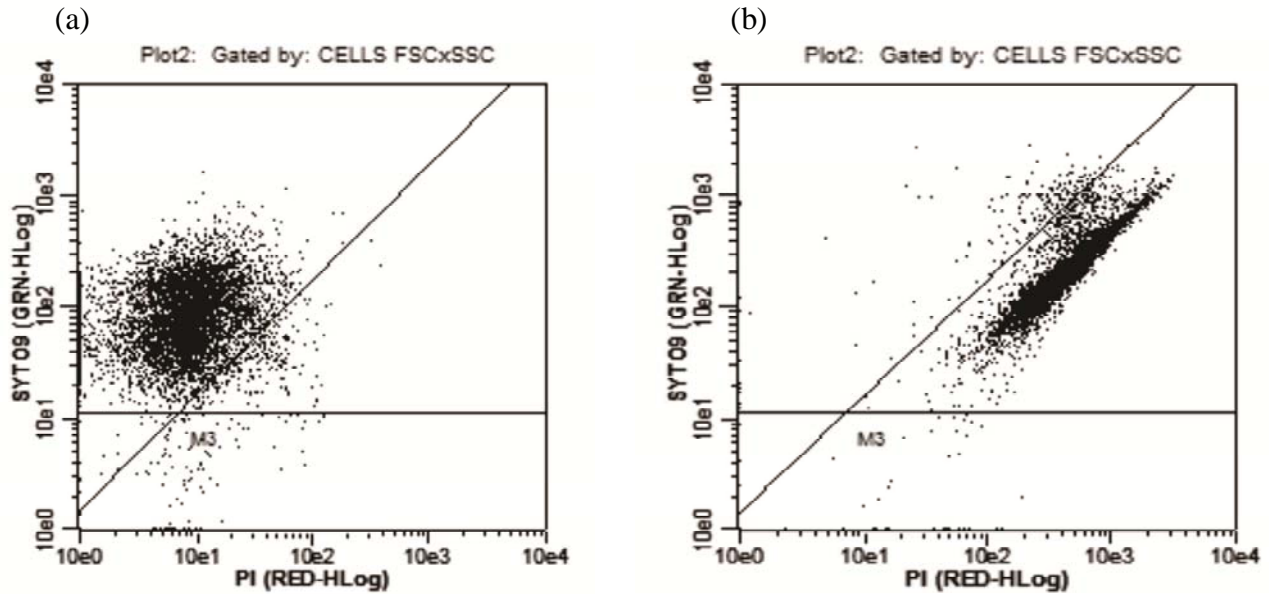
The growth in CDM with and without GSH was used to examine whether the GSH accumulation in *L. lactis* LVA. 2 had a protective effect on the cells subjected to the lyophilization process. The GSH accumulation in *L. lactis* LVA. 2 provided no apparent protection when the cells were exposed to the lyophilization process (Figure 5a). The cells grown in the anaerobic medium were apparently more sensitive to the lyophilization process than the cells grown in the static aerobic and

continuously stirred aerobic conditions when *L. lactis* was grown in M17 medium supplemented with GSH under different growth conditions (Figure 5b).

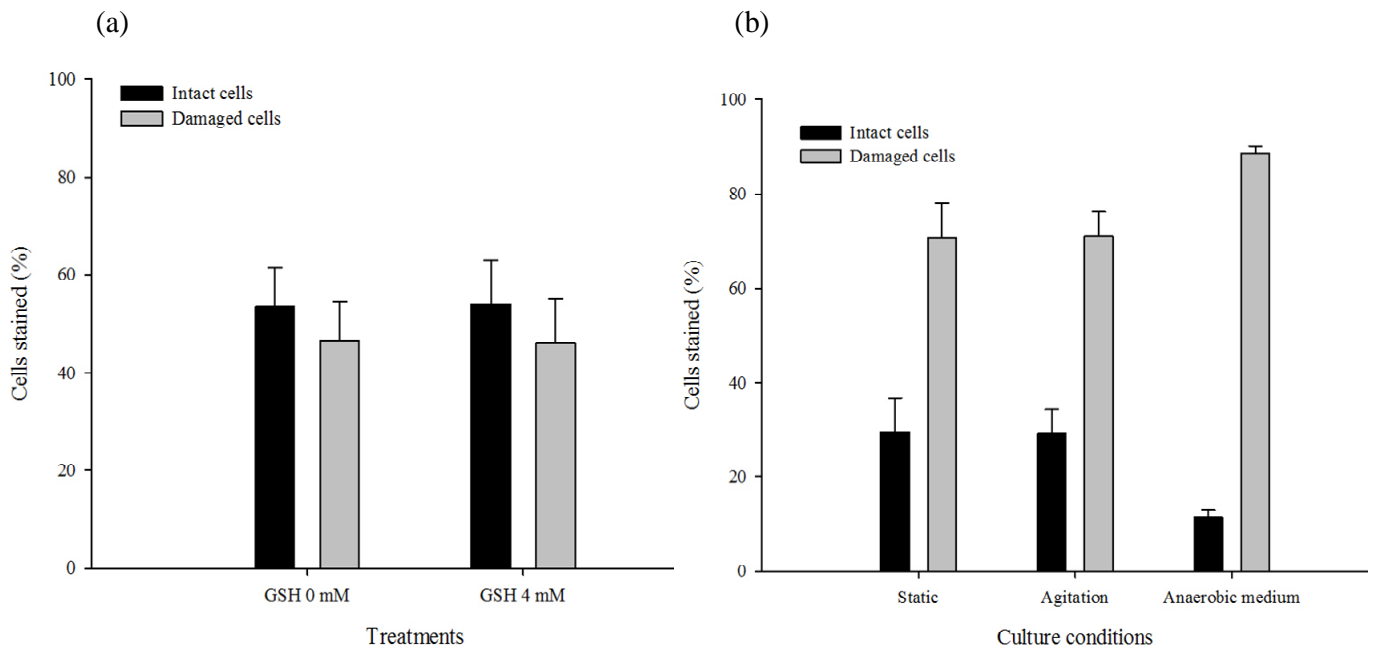
### DISCUSSION

The selection of *L. lactis* strains with the ability to transport GSH from the growth medium into the intracellular environment has been regarded as an alternative to prepare starter cultures with more stable viability under stress conditions (Pophaly et al., 2012). The existence of *L. lactis* strains with the ability to biosynthesize GSH has not been reported in the literature, and there are only reports of strains with the ability to transport GSH into the intracellular medium (Li et al., 2003). Given the functional role that GSH has in cells, it is interesting that GSH must be accumulated in large amounts in cells to play its protective role. Thus, the use of strains using GSH as a nutrient source is not recommended. GSH is noticeably consumed as a cysteine source during growth in *Streptococcus mutans* (Sherrill and Fahey, 1998) and *Leuconostoc mesenteroides* (Kim et al., 2012).

The microorganism growth conditions must be optimized to prepare a freeze-drying or spray-drying culture or a frozen culture with a higher proportion of viable cells, to provide physiological changes favorable to



**Figure 4.** Multiparametric dot plot obtained after the double staining with syto9/PI in the labeling of *L. lactis* LVA. 2 (a) cells collected prior to and (b) following lyophilization.



**Figure 5.** Percentage of undamaged and damaged cells of *L. lactis* LVA. 2 following the lyophilization process, analyzed using flow cytometry. The cells were grown in CDM (supplemented and unsupplemented with GSH) (a) and in M17 medium supplemented with GSH (static, continuously stirred or in anaerobic medium) (b). The data are mean values based on three replicates.

the cells, making them more resistant to those conditions. The increased accumulation of GSH in *L. lactis* LVA. 2 under the continuously stirred aerobic and NaCl-supplemented aerobic growth conditions appears to act as a cellular regulatory defense mechanism when the

cells are exposed to stress conditions. It has already been found that the accumulation of GSH in *Escherichia coli* is high when the cells are exposed to NaCl-induced hypertonic conditions (McLaggan et al., 1990). A similar result was not found when *L. lactis* LVA. 2 was grown in

static aerobic conditions because the regulatory mechanism most likely did not need to be very active given the lower oxygen incorporation. However, we believe that the large accumulation of GSH noted in the anaerobic-medium condition resulted from the low cellular consumption of GSH to maintain intracellular homeostasis.

The physiological status of *L. lactis* LVA. 2 cells can be monitored by the assessment of *gshR* gene expression. It has already been shown that the presence or absence of GSH has no effect on the activity of the GshR enzyme (Li et al., 2003). Thus, the factor that most likely led to an alteration in the *gshR* gene expression in the present study was the oxygen concentration of the culture medium. Therefore, the expression of the gene is expected to increase in continuously stirred aerobic growth conditions, and its expression would be reduced in anaerobic medium. Conversely, the *gshR* gene may have been repressed in the *L. lactis* LVA. 2 cells grown under osmotic stress conditions upon medium-level supplementation with NaCl.

GSH appears not to trigger any protective mechanism in the *L. lactis* LVA. 2 plasma membrane during lyophilization. There is no reference of any study in the literature that evaluated the protective effect of GSH in *L. lactis* following lyophilization, and only the protective effect of GSH on the *Lactobacillus sanfranciscensis* strain has been evaluated and verified (Zhang et al., 2010a). The detection of the protective effect provided by GSH might have been favored by the fact that the *L. sanfranciscensis* strain is very sensitive to the lyophilization process. However, we believe that GSH may have provided a type of protection by physiological regulation, as found in *L. sanfranciscensis* (Zhang et al., 2010b), and that protection cannot be found in *L. lactis* LVA. 2 immediately following lyophilization. The use of flow cytometry, a very sensitive technique that evaluates the integrity of the cell membrane in combination with dyes, showed that GSH had no protective effect on the membrane regarding the damage caused by ice-crystal formation and osmotic stress. The same result was also found using the plate-count method (data not shown).

Although the anaerobic medium provided better growth conditions to *L. lactis* LVA. 2, the absence of oxygen during growth may have inactivated some mechanisms involved in protection against the lyophilization process.

## Conclusion

Although GSH failed to provide an apparent protection to *L. lactis* LVA. 2 following the lyophilization process, the fact that this strain accumulates GSH and expresses the *gshR* gene makes it a promising strain to cope with other stress situations, and GSH functionality has already been confirmed in other *L. lactis* strains.

## Conflict of Interests

The authors have not declared any conflict of interest.

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