Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Characterization of the response to low pH of *Lactobacillus casei* ΔRR12, a mutant strain with low D-alanylation activity and sensitivity to low pH

A. Revilla-Guarinos[1], C. Alcántara[1], N. Rozès[2], B. Voigt[3] and M. Zúñiga[1]

1 Dpto. Biotecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Instituto de Agroquímica y Tecnología de Alimentos (IATA), Paterna, Valencia, Spain
2 Dpt. Bioquímica i Biotecnología, Facultat d'Enologia, Universitat Rovira i Virgili, Tarragona, Spain
3 Institute for Microbiology, University of Greifswald, Greifswald, Germany

Keywords
acid sensitivity, acid tolerance response, D-alanylation of teichoic acids, fatty acids, *Lactobacillus casei*.

Correspondence
Manuel Zúñiga, Dpto. Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC) Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain.
E-mail: btcman@iata.csic.es

2013/1566: received 2 August 2013, revised 8 November 2013 and accepted 6 January 2014

doi:10.1111/jam.12442

Abstract

Aims: To identify the differences that account for the acid sensitivity of *Lactobacillus casei* ΔRR12. RR12 controls the expression of the *dlt* operon, and its inactivation leads to a diminished teichoic acid D-alanylation activity. To this end, a comparison of its response of ΔRR12 to low pH with the parental strain *Lact. casei* BL23 was carried out.
Methods and Results: The ability to induce an acid tolerance response (ATR), fatty acid (FA) composition and proteome changes induced in both strains in response to acid were investigated. Results obtained showed that both strains induce a growth-phase-dependent ATR. However, significant differences in the content of FAs and membrane-associated proteins were detected.
Conclusions: The greater abundance of cytoplasmic proteins in the membrane fraction of the mutant strain ΔRR12 suggests an increased permeability of the cell membrane in this strain.
Significance and Impact of the Study: The analysis of the response to low pH of strain ΔRR12 indicated that the inactivation of TCS12 affected the content of FAs and proteins associated to the cell envelope. Increased abundance of cytoplasmic proteins suggested that low alanylation of teichoic acids affected the permeability of the cell membrane and possibly accounts for the acid sensitivity of strain ΔRR12.

Introduction

Lactic acid bacteria (LAB) are Gram-positive, nonsporulating fermentative bacteria that produce lactic acid as the major end product of carbohydrate metabolism so that proliferation of LAB is generally accompanied by acidification of the surrounding medium. Bacteria maintain a pH gradient (ΔpH) across the membrane so that the cytoplasmic pH (pH_{in}) remains more alkaline than the medium pH (pH_{out}) (Booth 1985; Kashket 1987). As the medium pH decreases, the ratio of undissociated forms of organic acids increases. Undissociated acids can enter the cell, resulting in the acidification of the cytoplasm. If the cell cannot compensate this proton influx, ΔpH cannot be maintained and cellular functions are impaired (Booth 1985; Kashket 1987; Hutkins and Nannen 1993). To survive in an acidic environment, several mechanisms contribute to regulate the homeostasis of pH_{in} in LAB and the response to acid stress. These mechanisms include proton pumps, mainly the F_0F_1-ATPase, decarboxylases acting on amino acids such as glutamic acid or organic acids (e.g., malic acid), deiminases acting on amino acids or amines such as arginine or putrescine and urease (for reviews see Hutkins and Nannen 1993; van de Guchte *et al.* 2002; Cotter and Hill 2003; De Angelis and Gobbetti 2004; Corcoran *et al.* 2008). Response to acid stress in LAB has also been associated to upregulation of chaperones and other stress responsive proteins (Hamilton and Svensäter 1998; Lim *et al.* 2000; De Angelis *et al.* 2001; Frees *et al.* 2003).

However, the analysis of the transcriptional response of *Lactobacillus casei* to acid stress showed that acid adaptation in this organism involved the triggering of the stringent response with concomitant downregulation of components of the translational apparatus as well as a number of chaperones and other stress-related proteins (Broadbent *et al.* 2010). Involvement of stringent response in acid resistance had also been suggested for *Lactococcus lactis* (Rallu *et al.* 2000). In addition, a number of studies have shown that LAB change the fatty acid (FA) composition of their membranes in response to acidification of the medium (Fozo and Quivey 2004; Fozo *et al.* 2004; Broadbent *et al.* 2010). Modulation of the surface cell properties via D-alanylation of teichoic acids also plays a major role in acid resistance in LAB as evidenced by the increased acid sensitivity observed in Dlt-defective mutants (Boyd *et al.* 2000; Vélez *et al.* 2007; Revilla-Guarinos *et al.* 2013).

A number of regulatory mechanisms involved in acid resistance have been characterized in LAB (van de Guchte *et al.* 2002; Cotter and Hill 2003; Corcoran *et al.* 2008). Among them, signal transduction two-component systems (TCSs) have been shown to play a major role. For example, inactivation of two response regulators of *Lactobacillus sakei* led to mutants that were more acid sensitive than the parental strain (Morel-Deville *et al.* 1998). Inactivation of a TCS possibly involved in the regulation of the proteolytic apparatus of *Lactobacillus acidophilus* also resulted in an acid-sensitive phenotype (Azcárate-Peril *et al.* 2005). A survey of the phenotypic effect of TCS inactivation in *Lact. casei* identified six TCS whose inactivation led to growth defects at acidic pH (Alcántara *et al.* 2011). Growth defects in acid media associated to TCS inactivation have also been described in *Lc. lactis* (O'Connell-Motherway *et al.* 2000), *Streptococcus pyogenes* (Ichikawa *et al.* 2011) and *Streptococcus suis* (Han *et al.* 2012) among others. These results indicate that TCSs modulate the acid resistance ability of many LAB although the mechanisms by which they exert their effect remain to be determined in many cases.

Lactobacillus casei is a facultative heterofermentative LAB naturally found in food (dairy and meat products, fermented vegetables, etc.) and the oral cavity, gastrointestinal and genital tracts of humans and other animals. *Lactobacillus casei* is an organism of industrial interest for its use as a starter culture for some dairy products and because some strains are considered as probiotics (de Vrese and Schrezenmeir 2008; Kleerebezem and Vaughan 2009). Probiotic micro-organisms are currently the focus of an intense research effort that aims to determine their possible health benefits. A number of these studies have concluded that probiotics must survive the transit through the gastrointestinal tract. Survival in this environment depends, among other factors, on its ability to survive to acid (Hutkins and Nannen 1993; Walter and Ley 2011). We previously reported that strain ΔRR12, a *Lact. casei* BL23 mutant lacking the response regulator of TCS12, displayed a premature arrest of growth, and it was very sensitive to low pH (Alcántara *et al.* 2011). A subsequent study showed that this defect was mainly due to low expression of the *dlt* operon, and it could be alleviated by increasing the buffering capacity of the growth medium (Revilla-Guarinos *et al.* 2013). These results suggested that TCS12 could be involved in the mechanisms that allow *Lact. casei* BL23 to adapt to acidic environments. To further investigate this hypothesis, the acid tolerance and acid stress responses of *Lact. casei* BL23 and ΔRR12 were compared.

Material and methods

Bacterial strains and growth conditions

Lactobacillus casei BL23 and the derivative strain ΔRR12 (Alcántara *et al.* 2011) were routinely grown in deMan Rogosa Sharpe (MRS) broth (Oxoid) at 37°C without shaking. Agar was added at 1·8% for plates. Cells were stored at −80°C in MRS medium supplemented with 15% (v/v) glycerol.

Acid tolerance response (ATR) assays were carried out as follows: cells from the stock cultures were inoculated on MRS agar plates. For the growth curves at 37°C and ATR induction assays, single colonies of each strain were used to inoculate three 5-ml aliquots of MRS medium, and the cultures were incubated overnight at 37°C. Each culture was used to inoculate 50 ml batches of prewarmed MRS medium at optical density of 0·05 at 595 nm (OD_{595}). Incubation was continued at 37°C, and two samples of 1 ml were withdrawn at selected time points. Both sample cells were washed with one volume of 0·05% peptone water. Subsequently, they were resuspended in either one volume of peptone water (control) or one volume of MRS adjusted to pH 2·5 with HCl (acid challenge). Incubation of acid-challenged cells was continued at 37°C for 45 min. Survival after acid challenge was determined by comparing viable cell countings in the control samples and acid-challenged samples. Significant differences were estimated using Student's *t*-test (2-tail unpaired with significance measured at a probability level of $P < 0{\cdot}05$) as implemented in Graphpad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Determination of cell membrane fatty acid composition

Three independent 5 ml overnight cultures of *Lact. casei* BL23 and derivative strain ΔRR12 were used to inoculate

six batches of 200 ml of MRS broth at OD_{595} 0·1. Cultures were incubated at 37°C, and changes in OD_{595} and pH were monitored. When the cultures media reached pH values of 4·7, 4·3 and 4, two samples of each culture corresponding to 20 OD units were taken, washed twice with water and kept frozen at −80°C until use. The extraction of FAs was carried out as previously described (Rozès *et al.* 1993). Briefly, methanolysis of cell materials was carried out with 500 μl of 1 mol l^{-1} sodium methoxide in methanol. Heptanoic acid methyl ester (C7:0) and heptadecanoic acid methyl ester (C17:0) were added to the samples as internal standards. The preparations were mixed for 1 min, and then, FA methyl esters were extracted by shaking with 100 μl hexane for 30 seconds. Samples were pelleted, and the upper phase was recovered for analysis on a Hewlett-Packard 6850 gas chromatograph (Agilent Technologies, Agilent Technologies España S.L., Las Rozas, Madrid, Spain). Two microlitres were injected (splitless, 0·75 min) into an HP-FFAP column (Agilent Technologies) with an HP 6850 automatic injector. The initial temperature was set at 100°C and increased by 3·5°C min^{-1} up to 240°C during 10 min. The injector and detector temperatures were set at 220 and 260°C, respectively. The carrier gas was helium at a flow rate of 1 ml min^{-1}. Relative amounts of FAs were calculated from their respective chromatographic peak areas. These values were related to the dry weight of cells and expressed as a percentage of the total FA extracted.

Two-dimensional SDS-PAGE analysis of the response of *Lact. casei* BL23 and ΔRR12 to acid challenge

Single colonies of *Lact. casei* BL23 and ΔRR12 were used to inoculate three 5-ml aliquots of MRS medium, and the cultures were incubated overnight at 37°C. Each culture was used to inoculate two 250-ml batches of prewarmed MRS medium at OD_{595} 0·06 for the treatment or control assays. Incubation was continued to OD_{595} 0·6. At this point, 250 ml of prewarmed MRS was added to the control samples and 250 ml of MRS adjusted to pH 3·6 with HCl was added to the treated cultures until a final pH of 4·4 was reached. Incubation was continued for 45 min at 37°C. At this point, the cultures were collected and the proteins associated to membrane fractions were purified as previously described (Alcántara and Zúñiga 2012). Two biological replicates and three technical replicates of each biological replicate were analysed for each growth condition. 2D gel electrophoresis and analysis were performed as described previously (Rivas-Sendra *et al.* 2011). A protein was considered to be under- or overproduced when, after image analysis and subsequent computing of the normalized spot volumes, the means from at least four gels were 1·5-fold different among the conditions tested at a significance level of $P < 0{\cdot}05$ (Student's *t*-test for paired samples).

Selected spots were excised from 2D gels and transferred to polypropylene tubes containing ultrapure water. Proteins were identified by MALDI-TOF/TOF after trypsinolysis at the Institute of Microbiology (University of Greifswald) according to Wolff *et al.* (2008). MALDI-MS and MS/MS data were searched using the Mascot search engine (version 2.1.0.4) with the Uniprot database (http://www.uniprot.org/uniprot/?query=Lactobacillus+casei+BL+23&sort=score&format=*).

Results

The mutant strain ΔRR12 can induce a growth-phase-dependent acid tolerance response (ATR)

One of the mechanisms that protect bacteria from acid killing is the adaptive ATR: a brief exposure of the cells to a sublethal extracellular pH induces physiological changes that allow the bacteria to survive to the subsequent exposure to a lethal extracellular pH (Goodson and Rowbury 1989). To determine whether the higher sensitivity of ΔRR12 strain to acid was due to a defective ATR response, survival after an acid challenge of *Lact. casei* BL23 and ΔRR12 was determined. Previous studies of the laboratory showed that an acid challenge at pH 2·5 for 45 min was lethal for *Lact. casei* BL23 at early stages of growth; however, the cells were able to survive to this acid challenge in the final stages of growth (data not shown). Thus, pH 2·5 was chosen for the ATR induction studies. Cells from cultures in MRS at 37°C of *Lact. casei* BL23 and ΔRR12 were withdrawn as the growth medium reached predetermined pH values. They were transferred to MRS medium adjusted to pH 2·5 with HCl and incubated at 37°C for 45 min. Survival after the acid challenge was determined by viable counting in MRS agar plates. Results obtained showed that both strains elicit a growth-phase-dependent ATR as evidenced by their increased survival after the acid challenge (Table 1). Induction of ATR occurred at the end of the exponential growth phase in the wild-type strain (Fig. 1). However, induction of ATR was delayed in strain ΔRR12 compared with the wild-type strain BL23 (Fig. 1 and Table 1). In this experiment, a similar trend was observed in the evolution of the pH for the mutant strain and BL23, and the same final pH was reached by both strains at the end of the assay. This observation indicated that ΔRR12-impaired growth was not due to loss of metabolic activity. Taken together, these observations suggested that inactivation of TCS12 did not impair ATR induction but it could be interfering cellular processes involved in the adaptation to grow at low pH of *Lact. casei*.

Table 1 Growth-phase-dependent ATR induction in *Lactobacillus casei* BL23 and ΔRR12*

BL23			ΔRR12		
OD_{595}	pH	% survival	OD_{595}	pH	% survival
0·82 ± 0·02	5·31 ± 0·03	$1·52 \times 10^{-5} \pm 2·6 \times 10^{-5}$	0·91 ± 0·03	5·08 ± 0·04	$7·4 \times 10^{-6} \pm 4 \times 10^{-6}$
2·51 ± 0·14	4·76 ± 0·02	$4·52 \times 10^{-4} \pm 5·3 \times 10^{-4}$	2·42 ± 0·05	4·66 ± 0·03	$5·1 \times 10^{-5} \pm 0·8 \times 10^{-5}$
3·72 ± 0·04	4·44 ± 0·02	2·17 ± 0·85	3·23 ± 0·07	4·36 ± 0·02	$4·4 \times 10^{-3} \pm 2·4 \times 10^{-3}$
6·77 ± 0·03	3·71 ± 0·01	65·29 ± 1·00	4·61 ± 0·21	3·90 ± 0·01	72·80 ± 4·8

ATR is represented by the percentage of survival of the acid-challenged cells (incubation for 45 min at 37°C in MRS adjusted to pH 2·5 with HCl) relative to the control nonacid-challenged cells. OD_{595} and pH of the cultures at the time of acid challenge are indicated.
ATR, acid tolerance response; MRS, deMan Rogosa Sharpe.
*Means and standard deviations of three independent determinations.

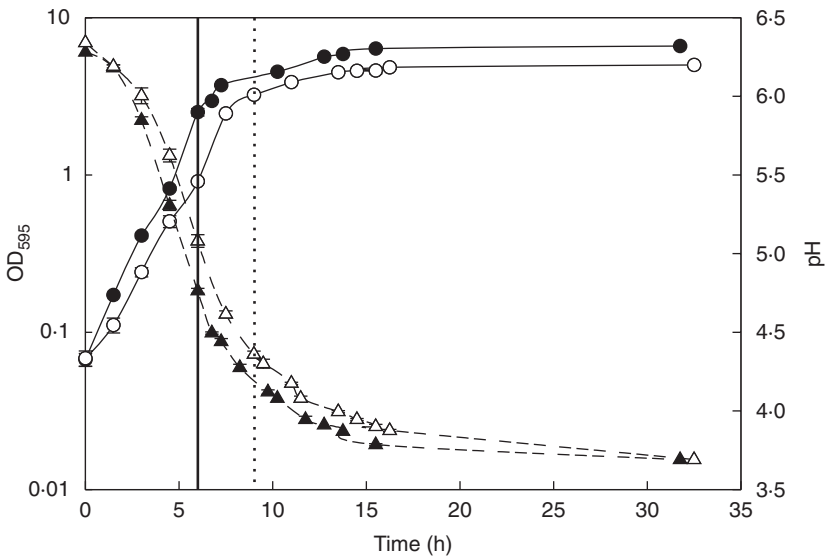

Figure 1 Variation of OD_{595} and medium pH during growth of *Lactobacillus casei* BL23 and its derivative strain ΔRR12 in deMan Rogosa Sharpe at 37°C. The vertical solid and dotted lines indicate the sampling points at which acid tolerance response induction was detected for strains BL23 and ΔRR12, respectively. Values represent the means of three independent experiments; error bars represent standard deviations. (–●–) OD BL23; (–○–) OD ΔRR12; (---▲---) pH BL23; (---△---) pH ΔRR12.

Variation of the cell membrane fatty acid composition

To determine whether the FA composition of the membrane of *Lact. casei* BL23 changes during growth and whether this change is dependent on TCS12, the FA composition of BL23 and ΔRR12 strains was analysed. For this purpose, cells were grown in MRS at 37°C and samples were withdrawn for FA determination when the pH of the culture media reached 4·7, 4·3 and 4. Results obtained are shown in Table 2. In both strains, the major FAs were palmitic (C16 30–38% of total FA approximately), oleic (C18:1 c9; 13–24%), vaccenic (C18:1 c11; 15–16%) and dihydrosterculic (C19 cyc9; 9–17%) acids. Significant differences between strains were observed at the three pH assayed for palmitic, oleic, dihydrosterculic and lactobacillic (C19 cyc11) acids (Table 2). No significant differences were observed at any pH value only for cis-11-hexadecenoic acid (C16 c11; Table 2). The FA content varied with external pH in both strains. As the medium pH decreased, the content of unsaturated FAs decreased, whereas the content of cyclic FAs increased in both strains (Fig. 2). However, whereas the content of unsaturated FAs always was higher than that of saturated FAs in the wild-type strain, as pH decreased, the content of saturated FAs exceeded that of unsaturated FAs in the mutant strain ΔRR12 (Fig. 2). Considering the individual FAs, a significant increase in the ratios of dihydrosterculic acid and lactobacillic acid was observed in both strains ($P < 0·05$), whereas the oleic acid ratio significantly decreased ($P < 0·05$; Table 2). The evolution of other FA varied depending on the strain considered. For example, a significant increase in the palmitic acid content and a decrease in the myristic and myristoleic acids content as the pH decreased were observed in strain ΔRR12, whereas no significant changes were observed in the parental strain (Table 2). In contrast, the content of palmitoleic acid increased in strain BL23, whereas no significant changes were observed in strain ΔRR12. These results show that the FA content of strain ΔRR12 differs significantly of that of the parental strain BL23, and the

Table 2 Variation of fatty acid composition of *Lactobacillus casei* BL23 and ΔRR12 during growth*. Fatty acids: myristic (C14), myristoleic (C14 c9), palmitic (C16), palmitoleic (C16 c9), cis-11-hexadecenoic (C16 c11), stearic (C18), oleic (C18 c9), vaccenic (C18 c11) and the cyclopropanes dihydrosterculic (C19 cyc9) and lactobacillic acid (C19 cyc11)

	pH 4·7			pH 4·3			pH 4·0		
FA	BL23	ΔRR12	*P*-value†	BL23	ΔRR12	*P*-value	BL23	ΔRR12	*P*-value
C14	6·70 ± 0·14	6·37 ± 0·67	0·262	6·38 ± 0·42	5·80 ± 0·62	0·089	7·34 ± 1·02	5·78 ± 0·43	**0·006**
C14 c9	0·82 ± 0·03	0·74 ± 0·07	**0·045**	0·74 ± 0·05	0·75 ± 0·25	0·433	0·83 ± 0·06	0·58 ± 0·10	**4.57×10^{-4}**
C16	31·01 ± 0·39	34·27 ± 1·14	**5.86×10^{-5}**	32·70 ± 2·10	37·31 ± 0·92	**6.05×10^{-4}**	30·18 ± 0·89	37·60 ± 2·79	**9.96×10^{-5}**
C16 c9	6·97 ± 0·09	7·34 ± 0·17	**0·001**	7·37 ± 0·10	7·42 ± 0·22	0·594	7·87 ± 0·28	6·98 ± 0·31	**4.24×10^{-4}**
C16 c11	0·73 ± 0·02	0·78 ± 0·02	0·574	0·84 ± 0·05	0·86 ± 0·08	0·681	0·87 ± 0·06	0·81 ± 0·02	0·055
C18	0·95 ± 0·02	1·13 ± 0·14	**0·022**	0·91 ± 0·06	1·04 ± 0·03	**0·001**	0·99 ± 0·19	1·02 ± 0·08	0·679
C18 c9	24·46 ± 0·48	21·30 ± 0·67	**3.98×10^{-5}**	18·25 ± 1·47	15·01 ± 0·97	**0·001**	16·05 ± 1·21	13·07 ± 2·88	**0·041**
C18 c11	15·11 ± 0·26	16·49 ± 0·99	**0·013**	16·37 ± 0·43	16·22 ± 1·36	0·796	15·57 ± 1·03	14·74 ± 0·82	0·155
C19 cyc9	11·00 ± 0·41	9·03 ± 0·79	**1.37×10^{-4}**	13·28 ± 0·76	11·86 ± 0·30	**0·002**	16·67 ± 1·71	14·29 ± 0·58	**0·009**
C19 cyc11	1·46 ± 0·12	1·68 ± 0·12	**0·013**	2·62 ± 0·36	3·45 ± 0·32	**0·002**	3·22 ± 0·31	4·54 ± 1·03	**0·013**

FA, fatty acid.
*Results are expressed as the mean percentage of each FA species over the total FA pool ± standard deviation. Six independent samples of each strain were used.
†*T*-test. Statistically significant differences ($P < 0.05$) are highlighted in bold characters.

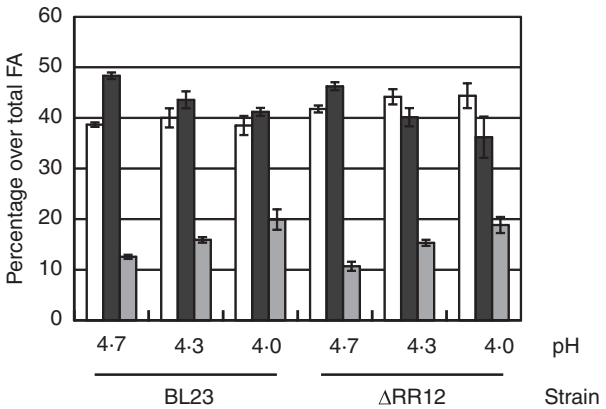

Figure 2 Variation of the percentage of fatty acid (FA) classes of strains BL23 and ΔRR12 during growth. Values represent the means of six independent determinations; error bars represent standard deviations. (■) saturated fatty acids; (▨) unsaturated FA; (□), cyclic FA.

changes in the content of some FA in response to decreasing medium pH also differed between strains.

Proteomic analysis of the response of *Lact. casei* BL23 and ΔRR12 to sublethal acidic pH exposition

To gain insight into the possible role of TCS12 in the control of the ATR of *Lact. casei*, a proteomic analysis was carried out. Figure 3 shows representative gels resulting from the analysis of the membrane-associated proteins. When the samples subjected to acid pH challenge were compared with the reference samples, 15 and 17 spots were significantly more abundant in *Lact. casei* BL23 and ΔRR12, respectively, whereas one and two spots were less abundant in *Lact. casei* BL23 and ΔRR12, respectively. Furthermore, comparison of ΔRR12 with the wild-type strain BL23 showed that six proteins were more abundant and two less abundant in the mutant strain relative to the wild-type strain in reference conditions. In response to acid, nine proteins were more abundant and four less abundant in the mutant relative to the wild-type strain. Thirty-two spots could be identified by MS; four of them (spots LC07, LC08, LC30, LC31) had a double identification. Another four spots did not render reliable mass spectra. Calculated fold changes are listed in Table 3, and the quantitative data of the spots identified are shown in Table S1.

Identified proteins were putatively involved in a wide variety of cellular functions, thus indicating that acid challenge induced global changes in *Lact. casei* BL23 physiology. The changes in the abundance of some proteins in both strains suggest that these proteins are involved in the response to acid of *Lact. casei* BL23. On the other hand, the abundance of some proteins varied when both strains were compared, indicating changes

brought about by the inactivation of TCS12. These results are discussed below.

Discussion

In the present work, we have performed a comparative analysis of the response to acid stress of *Lact. casei* BL23 and the derivative strain ΔRR12. Previous studies showed that the pleiotropic phenotype of strain ΔRR12 was mostly due to low expression of *dlt* (Revilla-Guarinos *et al.* 2013). Notwithstanding, we decided to focus our study on strain ΔRR12 as a Dlt-defective mutant displayed poor growth and poor data reproducibility in some assays (results not shown).

First, the ability to induce a growth-phase-dependent ATR of strains BL23 and ΔRR12 was determined. This growth-phase-dependent ATR is naturally induced as pH of the media decreases as a consequence of the accumulation of acid end products of metabolism (Buchanan and Edelson 1996). The results obtained showed that *Lact. casei* BL23 is able to induce a growth-phase-dependent ATR and that ΔRR12 also induces an ATR although induction was delayed compared with the parental strain (Table 1 and Fig. 1). Furthermore, this experiment showed that strain ΔRR12 reached the same final pH value than strain BL23. This observation indicates that ΔRR12 remains metabolically active, but it is not able to increase the biomass of the culture. The ATR in both strains was induced when the pH of the media decreased below 4·5. At this pH, a substantial part of lactic acid is undissociated (23% in pure water) and can enter the cell by diffusion (Lambert and Stratford 1999). LAB maintain a cytoplasm that is more alkaline than the medium, but whose pH decreases as the medium is acidified during growth (Kashket 1987). We suggest that ATR in *Lact. casei* is triggered when pH_{in} reaches a critical value as previously proposed for *Lc. lactis* (O'Sullivan and Condon 1997). These results indicated that inactivation of TCS12 did not impair induction of ATR but led to a premature entry into stationary phase.

Significant differences in the FA content of both strains and their variation in response to pH were observed. Major FAs were palmitic, oleic, vaccenic and dihydrosterculic acids. This result contrasts with values previously reported by Broadbent *et al.* (2010), but the use of a different experimental design and a different strain may account for these discrepancies. In response to acid stress, bacteria change the membrane FA composition to decrease the proton permeability (Fozo and Quivey 2004; Fozo *et al.* 2004; Broadbent *et al.* 2010). These changes usually involve an increase in the concentration of saturated FAs and cyclopropane FAs (Cotter and Hill 2003; Fozo *et al.* 2004; Broadbent *et al.* 2010) although Wu *et al.* (2012b) observed an increase in unsaturated FA and cyclopropane FAs in *Lact. casei* Zhang in response to acid stress. In the present study, an increase in cyclopropane FAs and a concomitant decrease in the unsaturated FA content were observed in both strains as pH decreased. A similar trend had been observed in *Lact. casei* ATCC 334 (Broadbent *et al.* 2010). However, whereas it was observed a dramatic increase in the content of myristic acid of strain ATCC 344 in response to acid, no significant differences in the content of myristic acid were observed in either strain BL23 or its derivative ΔRR12. Again, different experimental conditions and strain may explain this difference. On the other hand, significant differences in FA content were observed between strains BL23 and ΔRR12 and changes in the FA content in response to decreasing pH also varied (Table 2). This observation correlated with an increase in the abundance of FabZ in ΔRR12 compared with BL23 (Table 3). *Lactobacillus casei* FabZ is homologous to the *Enterococcus faecalis* V583 FabZ1 protein, which has been shown to be a dual enzyme that converts *β*-hydroxyacyl-ACPs to *trans*-2 unsaturated acyl-ACPs and isomerizes them into *cis*-3-decenoyl-ACPs, the key step of the classical anaerobic unsaturated FA biosynthetic pathway (Wang and Cronan 2004). These results indicate that the inactivation of TCS12 affected the membrane FA content although additional data are required to explain the consequences of this fact.

To further investigate the response of *Lact. casei* BL23 and ΔRR12 to acidic pH, a proteomic approach was used to identify differentially produced membrane-associated proteins from cells exposed to pH 4·4 for 45 min. This pH was chosen because ΔRR12 mutant prematurely enters in stationary phase around this value (Fig. 1), and significant differences in ATR induction between the wild-type and the mutant were observed when the pH of the media was between 4·5 and 4 (Fig 1 and Table 1). An exposition time of 45 min was chosen. Hamilton and Svensäter (1998) reported that most of the proteins induced in response to acid shock in *Lact. casei* 151 were expressed between 30 and 60 min after shock. As our previous data suggested that TCS12 inactivation mostly affected the cell envelope (Alcántara *et al.* 2011; Revilla-Guarinos *et al.* 2013) and changes in cytoplasmic proteins in response to low pH in *Lact. casei* had already been characterized (Wu *et al.* 2011), we decided to analyse in detail proteins associated to the cell membrane.

Acid stress led to an increased abundance of two stress proteins in both strains, GroES and UspA (Table 3). Induction of stress proteins was also observed in *Lact. casei* Zhang (Wu *et al.* 2011, 2012a). In contrast, Broadbent *et al.* (2010) observed a decrease in the expression of stress response proteins after acid adaptation. Furthermore, the

Table 3 Differential spots detected in the proteomic analysis of the membrane protein fractions of *Lactobacillus casei* BL23 and ΔRR12 mutant cells after an acid challenge at pH 4·4 for 45 min

			Ratio†			
Spot no	Putative function*	Locus tag (gene name)	BL23 pH/BL23 MRS	ΔRR12 pH/ΔRR12 MRS	ΔRR12 MRS/BL23 MRS	ΔRR12 pH/BL23 pH
Stress response						
LC03	10 kDa chaperonin (groES protein)	LCABL_24210 (*groS*)	**2·68**	**4·11**	1·00	1·54
LC22	Universal stress protein, UspA family	LCABL_14120 (*usp6*)	**4·44**	**4·23**	0·64	**0·61**
LC32	Trypsin-like serine protease	LCABL_30080 (*htrA*)	0·74	**4·02**	0·52	**2·81**
Energy metabolism						
LC12	ATP synthase gamma chain	LCABL_13870 (*atpG*)	0	0	0·82	NC‡
LC13	Pyruvate dehydrogenase complex, E1 component, alpha subunit	LCABL_15360 (*pdhA*)	**1·57**	**1·55**	1·02	1·01
LC14	ATP synthase gamma chain	LCABL_13870 (*atpG*)	**2·11**	**1·78**	1·01	0·86
LC15	L-lactate dehydrogenase	LCABL_27160 (*ldh*)	**0·69**	**0·52**	1·20	0·92
LC16	Glyceraldehyde 3-phosphate dehydrogenase	LCABL_11300 (*gap-1*)	1·30	**4·18**	0·61	1·97
LC19	Dihydrolipoyl dehydrogenase	LCABL_15390 (*pdhD*)	**2·73**	**3·01**	1·12	1·23
Carbohydrate transport						
LC20	EIIB phosphotransferase system	LCABL_04730 (*PTS-EIIB*)	0·49	NC	0	0
LC25	EIIAB: phosphotransferase system; glucose transport	LCABL_30340 (*manL*)	**2·46**	1·16	1·24	0·58
Lipid metabolism						
LC04	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase	LCABL_23020 (*fabZ*)	0·72	1·46	**2·10**	**4·24**
Amino acid and peptide transport and metabolism						
LC11	Oligopeptide ABC transporter, substrate-binding lipoprotein	LCABL_22460 (*oppA*)	**2·06**	**1·99**	0·97	0·93
LC26	Oligopeptide ABC transporter, ATP-binding subunit	LCABL_22430 (*oppD*)	**2·86**	**2·23**	**2·11**	**1·65**
LC30	Oligopeptide ABC transporter (ATP-binding protein)	LCABL_18260 (*oppF*)	**4·57**	**6·32**	0·71	0·98
	Oligopeptide ABC transporter, ATP-binding subunit	LCABL_22420 (*oppF*)				
LC31	Oligopeptide ABC transporter (ATP-binding protein)	LCABL_18260 (*oppF*)	NC	**3·67**	NC	0·90
	Oligopeptide ABC transporter, ATP-binding subunit	LCABL_22420 (*oppF*)				
LC33	Oligopeptide ABC transporter, ATP-binding protein	LCABL_21090 (*oppD*)	0·72	1·28	0·95	**1·70**
LC17	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	LCABL_00660 (*opuA*)	2·19	**3·12**	0·73	1·04
LC08	Glutamine ABC transporter, ATP-binding protein	LCABL_14910 (*glnQ4*)	**5·83**	**5·15**	0·65	0·58
	Proline dipeptidase	LCABL_18590 (*pepQ-1*)				
LC27	Proline dipeptidase	LCABL_18590 (*pepQ-1*)	**2·57**	3·92	0·91	1·39
LC18	Glutamine synthetase	LCABL_18680 (*glnA*)	1·41	**3·84**	0·55	**1·51**
LC28	Glutamyl-tRNA synthetase	LCABL_24920 (*gltX*)	**1·73**	1·78	1·55	**1·60**

Table 3 (*Continued*)

Spot no	Putative function*	Locus tag (gene name)	Ratio†			
			BL23 pH/BL23 MRS	ΔRR12 pH/ΔRR12 MRS	ΔRR12 MRS/BL23 MRS	ΔRR12 pH/BL23 pH
Uncharacterized transporters						
LC09	ABC-type antimicrobial peptide transport system, ATPase component	LCABL_21680	1·58	1·53	**0·25**	**0·24**
LC35	ABC-type uncharacterized transport system, periplasmic component	LCABL_07680	0·75	1·36	1·90	**3·46**
Transcription and translation						
LC05	DNA-directed RNA polymerase subunit alpha	LCABL_26450 (*rpoA*)	0·86	1·04	1·75	**2·11**
LC01	50S ribosomal protein L4	LCABL_26690 (*rplD*)	1·45	0·76	**2·41**	1·26
LC06	30S Ribosomal protein S1	LCABL_15990 (*rpsA*)	0·70	0·76	**1·97**	**2·13**
LC29	30S Ribosomal protein S5	LCABL_26540 (*rpsE*)	**2·04**	**2·21**	1·04	1·12
Two possible identification with different function						
LC07	Glutamine ABC transporter, ATP-binding protein	LCABL_15660 (*glnQ3*)	1·36	**1·88**	0·73	1·01
	DNA-directed RNA polymerase subunit alpha	LCABL_26450 (*rpoA*)				
Uncharacterized or unidentified proteins						
LC36		LCABL_22340 (*wze*)	3·34	0·66	**3·66**	0·73
LC02		LCABL_08670	0·85	**0·44**	**1·94**	1·02
LC21	Not identified		2·01	0·66	6·31	2·08
LC23	Not identified		**2·63**	1·94	0·54	**0·40**
LC24	Not identified		0·67	NC	NC	NC
LC34	Not identified		**2·21**	**1·99**	1·23	1·11

MRS, deMan Rogosa Sharpe.
Bold numbers: statistically significant difference ($P < 0·05$).
*Hypothetical function based on the *Lact. casei* BL23 genome annotation.
†Normalized volume ratios for each protein at the indicated conditions.
‡Not calculable.

trypsin-like serine protease HtrA was specifically induced in response to pH in the mutant strain ΔRR12 (Table 3). HtrA is predicted to be located at the cell membrane, and it has been shown to participate in the degradation of abnormal exported proteins in *Lc. lactis* (Poquet *et al.* 2000) and streptococci (Tsui *et al.* 2011; Cassone *et al.* 2012). The induction of this protein had been previously observed in *Lact. casei* BL23 in response to p-coumaric acid (Rivas-Sendra *et al.* 2011) where it was proposed that p-coumaric acid disturbed protein structure at the cell envelope. This result suggests that low pH damaged cell envelope proteins to a greater extent in strain ΔRR12 than in the parental strain.

Changes were also observed in both strains in the abundance of proteins participating in energy production. An increase in the α subunit and the dihydrolipoyl dehydrogenase of the pyruvate dehydrogenase (PDH) complex and a decrease in L-lactate dehydrogenase (Ldh) were observed. The increase in PDH and decrease in Ldh abundances after acid exposure may suggest that in *Lact. casei* BL23 there is an increase in the metabolic pathway from pyruvate to acetyl-CoA by means of the PDH complex, and a reduction in the flux from pyruvate to L-lactate, in response to acid. Similar results had been previously described in *Lactobacillus rhamnosus* GG where Ldh was found to be less abundant and PDH complex E2 component (PdhC) more abundant when grown at pH 4·8 relative to pH 5·8, suggesting a shift of pyruvate metabolism towards the formation of acetyl-CoA (Koponen *et al.* 2012). Furthermore, the same change in pyruvate metabolism was observed in *Lactobacillus bulgaricus* during adaptation to pH 4·9 (Fernández *et al.* 2008). However, it must be considered that the result presented here was obtained from the analysis of membrane-associ-

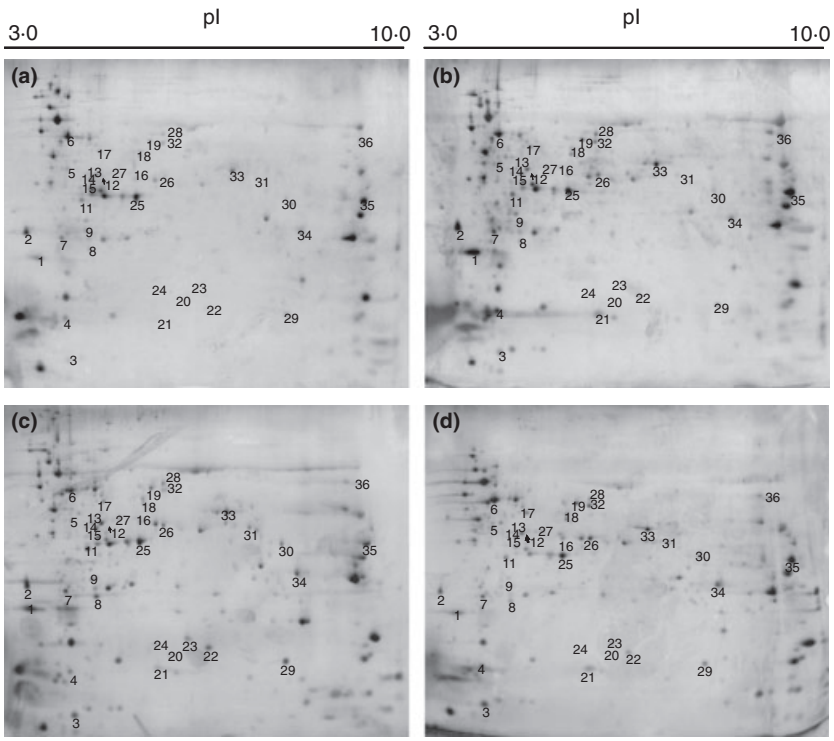

Figure 3 Silver-stained 2D electrophoresis gels of cell membrane fraction proteins extracted from *Lactobacillus casei* BL23 or ΔRR12 cells diluted in deMan Rogosa Sharpe (MRS) (pH 6·8) or acid MRS (pH 4·4). (a) BL23 pH 6·8; (b) ΔRR12 pH 6·8; (c) BL23 pH 4·4; (d) ΔRR12 pH 4·4. The figure shows one representative gel for each sample. Spot numbers indicate differentially expressed proteins.

ated proteins, and Ldh has been previously reported in the cell envelope of *Lact. casei* (Nezhad *et al.* 2012), and therefore, it may reflect a redistribution of Ldh between cytoplasm and cell envelope rather than a net decrease in the Ldh content of the cell.

A 4·18-fold increased abundance of glyceraldehyde 3-phosphate dehydrogenase (Gap) was observed in strain ΔRR12 in response to acid (Table 3). Association of Gap to the cell envelope has been previously reported in other bacteria (Pancholi and Fischetti 1992; Ruiz *et al.* 2009) including lactobacilli (Antikainen *et al.* 2007; Nezhad *et al.* 2012; Kinoshita *et al.* 2013). Interestingly, Antikainen *et al.* (2007) observed that Gap is associated to the cell envelope in *Lactobacillus crispatus* at acidic pH, whereas at neutral pH is rapidly released to the media and that Gap interacts with negatively charged LTAs. The increased abundance of Gap in strain ΔRR12 may therefore relate to low D-alanylation of LTAs in this strain compared with the parental strain BL23 (Revilla-Guarinos *et al.* 2013). However, an increased association of Gap with the membrane within the cell cannot be ruled out with the data currently available.

A controversial result was obtained for the subunit γ of the F_0F_1-ATPase after exposure to acid as it was identified in two spots. While spot LC12 disappeared after pH exposure in both strains, spot LC14 showed a significant increase in response to pH in both strains. This result may indicate a post-translational modification of this subunit in response to pH. The F_0F_1-ATPase plays a crucial role in acid tolerance in lactobacilli (Corcoran *et al.* 2008), but regulation of its activity in these organisms is still poorly understood. Regulation of the activity of chloroplast ATPase by the redox state of two Cys residues in subunit γ has been reported (Arana and Vallejos 1982) although the regulatory domain encompassing these cysteines is absent in bacterial γ subunits. As far as we know, post-translational modifications of γ subunit have not been reported in bacterial F_0F_1-ATPases.

Adaptation to low pH also brought about changes in the abundance of proteins involved in peptide and amino acid metabolism as well as putative amino acid and peptide transporters (Table 3). In particular, our results showed that several subunits of oligopeptide ABC transporters (Opp) were more abundant after acid exposure, both in the wild-type and the mutant strain (Table 3). *Lactobacillus casei* BL23 encodes three putative Opp systems (Mazé *et al.* 2010), but most spots identified belonged to the Opp system encoded by the gene cluster LCABL_22420-LCABL_22460. This result agrees with that reported by Broadbent *et al.* (Broadbent *et al.* 2010) who observed an increased expression of the homologous Opp system of *Lact. casei* ATCC334 (LSEI_2061-LSEI_2065) after 20 minutes of acid adaptation at pH 4·5. In contrast, a decrease in the homologous OppA subunit was observed in *Lacto. rhamnosus* GG (Koponen *et al.* 2012).

Glutamine synthetase (*glnA*) was significantly more abundant in the mutant strain in response to acid exposure relative to reference conditions (ratio 3·84), and it

was also more abundant in the mutant relative to the wild type in response to acid (ratio 1·51). Location of GlnA on the cell envelope has been previously reported in bifidobacteria (Candela *et al.* 2007; Ruiz *et al.* 2009) and *Lact. crispatus* (Kainulainen *et al.* 2012). As previously described for Gap (see above), binding of GlnA to the cell envelope preferentially occurred at low pH (Kainulainen *et al.* 2012), thus suggesting that GlnA also binds to LTAs. However, and as indicated for Gap, an increased association with the membrane inside the cell cannot be ruled out.

The ATPase component of a putative ABC antimicrobial peptide transport system (LCABL_21680) was less abundant in the mutant relative to the wild type both in reference conditions and in response to acid. This ABC transporter is the Orphan ABC transporter (OrABC) that we previously described to be under transcriptional control of TCS12 (Revilla-Guarinos *et al.* 2013) although its functional role has not been ascertained. Interestingly, Wu *et al.* reported that this ABC transporter was 3·3-fold induced in the *Lact. casei* Zhang acid-resistant strain relative to the wild type, after acid challenge (Wu *et al.* 2012a). A subunit of a putative sugar ABC transporter was more abundant in ΔRR12 in acidic conditions (Table 3) although its functional role has not been established.

A number of ribosomal proteins also showed significant differences in abundance in response to acid (Table 3). Association of ribosomal proteins with the cell envelope has been previously observed in a number of studies (Wilkins *et al.* 2003; Ruiz *et al.* 2009; Sánchez *et al.* 2009; Koskenniemi *et al.* 2011) although their role at this location has not been determined. An increase in the abundance of the ribosomal protein S5 (RpsE) was detected in both strains (Table 3). Interestingly, increased abundance of RpsE in the cell envelope of *Lacto. rhamnosus* in response to bile had been previously observed (Koskenniemi *et al.* 2011). These results may suggest that RpsE plays a role in the response against cell envelope stress in these organisms.

In summary, the results reported here showed that *Lact. casei* BL23 and ΔRR12 induce a growth-phase-dependent ATR response although the latter prematurely enters into stationary phase whereas the wild-type strain keeps growing. The analysis of CMFAs and protein content of the cell envelope revealed significant differences between both strains that correlate with the differences in sensitivity to cationic antimicrobial peptides previously reported (Alcántara *et al.* 2011; Revilla-Guarinos *et al.* 2013). In particular, the greater abundance of cytoplasmic proteins such as Gap and GlnA in the cell envelope of the mutant strain may indicate a greater permeability of the cell membrane in ΔRR12. The mechanism by which these proteins are exported to the cell wall has not been clearly established. However, evidence has been reported pointing to a link between increased cell membrane permeability and efflux of cytoplasmic proteins in *Lactobacillus plantarum* (Saad *et al.* 2009), and recently, a similar result was reported for *Lact. crispatus* (Kainulainen *et al.* 2012). Therefore, the results presented here suggests that D-alanylation of TAs has a remarkable influence on the functioning of the cell membrane of *Lact. casei* although further research will be required to elucidate this point. Furthermore, differences in acid tolerance between *Lact. casei* BL23 and its derivative strain ΔRR12 are possibly related to differences in surface physiology.

Acknowledgements

This work was financed by funds from the former Spanish Ministry of Science and Innovation (AGL2007-60975 and AGL2010-15679) and Generalitat Valenciana (ACOMP2012/137). A. Revilla-Guarinos was the recipient of a research fellowship from the Instituto Danone. We thank Amalia Blasco for technical assistance.

Conflict of Interest

No conflict of interest declared.

References

Alcántara, C. and Zúñiga, M. (2012) Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23. *Microbiology* **158**, 1206–1218.

Alcántara, C., Revilla-Guarinos, A. and Zúñiga, M. (2011) Influence of two-component signal transduction systems of *Lactobacillus casei* BL23 on tolerance to stress conditions. *Appl Environ Microbiol* **77**, 1516–1519.

Antikainen, J., Kuparinen, V., Lähteenmäki, K. and Korhonen, T.K. (2007) pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids. *J Bacteriol* **189**, 4539–4543.

Arana, J.L. and Vallejos, R.H. (1982) Involvement of sulfhydryl-groups in the activation mechanism of the ATPase activity of chloroplast coupling factor-I. *J Biol Chem* **257**, 1125–1127.

Azcárate-Peril, M.A., McAuliffe, O., Altermann, E., Lick, S., Russell, W.M. and Klaenhammer, T.R. (2005) Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. *Appl Environ Microbiol* **71**, 5794–5804.

Booth, I.R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol Rev* **49**, 359–378.

Boyd, D.A., Cvitkovitch, D.G., Bleiweis, A.S., Kiriukhin, M.Y., Debabov, D.V., Neuhaus, F.C. and Hamilton, I.R. (2000)

Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *J Bacteriol* **182**, 6055–6065.

Broadbent, J.R., Larsen, R.L., Deibel, V. and Steele, J.L. (2010) Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. *J Bacteriol* **192**, 2445–2458.

Buchanan, R.L. and Edelson, S.G. (1996) Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. *Appl Environ Microbiol* **62**, 4009–4013.

Candela, M., Bergmann, S., Vici, M., Vitali, B., Turroni, S., Eikmanns, B.J., Hammerschmidt, S. and Brigidi, P. (2007) Binding of human plasminogen to *Bifidobacterium*. *J Bacteriol* **189**, 5929–5936.

Cassone, M., Gagne, A.L., Spruce, L.A., Seeholzer, S.H. and Sebert, M.E. (2012) The HtrA protease from *Streptococcus pneumoniae* digests both denatured proteins and the competence-stimulating peptide. *J Biol Chem* **287**, 38449–38459.

Corcoran, B.M., Stanton, C., Fitzgerald, G. and Ross, R.P. (2008) Life under stress: the probiotic stress response and how it may be manipulated. *Curr Pharm Des* **14**, 1382–1399.

Cotter, P.D. and Hill, C. (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol Mol Biol Rev* **67**, 429–453.

De Angelis, M. and Gobbetti, M. (2004) Environmental stress responses in *Lactobacillus*: a review. *Proteomics* **4**, 106–122.

De Angelis, M., Bini, L., Pallini, V., Cocconcelli, P.S. and Gobbetti, M. (2001) The acid-stress response in *Lactobacillus sanfranciscensis* CB1. *Microbiology* **147**, 1863–1873.

Fernández, A., Ogawa, J., Penaud, S., Boudebbouze, S., Ehrlich, D., van de Guchte, M. and Maguin, E. (2008) Rerouting of pyruvate metabolism during acid adaptation in *Lactobacillus bulgaricus*. *Proteomics* **8**, 3154–3163.

Fozo, E.M. and Quivey, R.G. Jr (2004) Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. *Appl Environ Microbiol* **70**, 929–936.

Fozo, E.M., Kajfasz, J.K. and Quivey, R.G. Jr (2004) Low pH-induced membrane fatty acid alterations in oral bacteria. *FEMS Microbiol Lett* **238**, 291–295.

Frees, D., Vogensen, F.K. and Ingmer, H. (2003) Identification of proteins induced at low pH in *Lactococcus lactis*. *Int J Food Microbiol* **87**, 293–300.

Goodson, M. and Rowbury, R.J. (1989) Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Lett Appl Microbiol* **8**, 77–79.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D. and Maguin, E. (2002) Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **82**, 187–216.

Hamilton, I.R. and Svensäter, G. (1998) Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock. *Oral Microbiol Immunol* **13**, 292–300.

Han, H., Liu, C., Wang, Q., Xuan, C., Zheng, B., Tang, J., Yan, J., Zhang, J. *et al.* (2012) The two-component system Ihk/Irr contributes to the virulence of *Streptococcus suis* serotype 2 strain 05ZYH33 through alteration of the bacterial cell metabolism. *Microbiology* **158**, 1852–1866.

Hutkins, R.W. and Nannen, N.L. (1993) pH homeostasis in lactic acid bacteria. *J Dairy Sci* **76**, 2354–2365.

Ichikawa, M., Minami, M., Isaka, M., Tatsuno, I. and Hasegawa, T. (2011) Analysis of two-component sensor proteins involved in the response to acid stimuli in *Streptococcus pyogenes*. *Microbiology* **157**, 3187–3194.

Kainulainen, V., Loimaranta, V., Pekkala, A., Edelman, S., Antikainen, J., Kylväjä, R., Laaksonen, M., Laakkonen, L. *et al.* (2012) Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37. *J Bacteriol* **194**, 2509–2519.

Kashket, E.R. (1987) Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiol Rev* **46**, 233–244.

Kinoshita, H., Imoto, S., Suda, Y., Ishida, M., Watanabe, M., Kawai, Y., Kitazawa, H., Miura, K. *et al.* (2013) Proposal of screening method for intestinal mucus adhesive lactobacilli using the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Anim Sci J* **84**, 150–158.

Kleerebezem, M. and Vaughan, E.E. (2009) Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. *Annu Rev Microbiol* **63**, 269–290.

Koponen, J., Laakso, K., Koskenniemi, K., Kankainen, M., Savijoki, K., Nyman, T.A., de Vos, W.M., Tynkkynen, S. *et al.* (2012) Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG. *J Proteomics* **75**, 1357–1374.

Koskenniemi, K., Laakso, K., Koponen, J., Kankainen, M., Greco, D., Auvinen, P., Savijoki, K., Nyman, T.A. *et al.* (2011) Proteomics and transcriptomics characterization of bile stress response in probiotic *Lactobacillus rhamnosus* GG. *Mol Cell Proteomics* **10**, M110.002741.

Lambert, R.J. and Stratford, M. (1999) Weak-acid preservatives: modelling microbial inhibition and response. *J Appl Microbiol* **86**, 157–164.

Lim, E.M., Ehrlich, S.D. and Maguin, E. (2000) Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Electrophoresis* **21**, 2557–2561.

Mazé, A., Böel, G., Zúñiga, M., Bourand, A., Loux, V., Yebra, M.J., Monedero, V., Correia, K. *et al.* (2010) Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. *J Bacteriol* **192**, 2647–2648.

Morel-Deville, F., Fauvel, F. and Morel, P. (1998) Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*. *Microbiology* **144**, 2873–2883.

Nezhad, M.H., Knight, M. and Britz, M.L. (2012) Evidence of changes in cell surface proteins during growth of *Lactobacillus casei* under acidic conditions. *Food Sci Biotechnol* **21**, 253–260.

O'Connell-Motherway, M., van Sinderen, D., Morel-Deville, F., Fitzgerald, G.F., Ehrlich, S.D. and Morel, P. (2000) Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363. *Microbiology* **146**, 935–947.

O'Sullivan, E. and Condon, S. (1997) Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. *Appl Environ Microbiol* **63**, 4210–4215.

Pancholi, V. and Fischetti, V.A. (1992) A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *J Exp Med* **176**, 415–426.

Poquet, I., Saint, V., Seznec, E., Simoes, N., Bolotin, A. and Gruss, A. (2000) HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. *Mol Microbiol* **35**, 1042–1051.

Rallu, F., Gruss, A., Ehrlich, S.D. and Maguin, E. (2000) Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. *Mol Microbiol* **35**, 517–528.

Revilla-Guarinos, A., Gebhard, S., Alcántara, C., Staroń, A., Mascher, T. and Zúñiga, M. (2013) Characterization of a regulatory network of peptide antibiotic detoxification modules in *Lactobacillus casei* BL23. *Appl Environ Microbiol* **79**, 3160–3170.

Rivas-Sendra, A., Landete, J.M., Alcántara, C. and Zúñiga, M. (2011) Response of *Lactobacillus casei* BL23 to phenolic compounds. *J Appl Microbiol* **111**, 1473–1481.

Rozès, N., Garbay, S., Denayrolles, M. and Lonvaud-Funel, A. (1993) A rapid method for the determination of bacterial fatty acid composition. *Lett Appl Microbiol* **17**, 126–131.

Ruiz, L., Couté, Y., Sánchez, B., de los Reyes-Gavilán, C.G., Sanchez, J.C. and Margolles, A. (2009) The cell-envelope proteome of *Bifidobacterium longum* in an in vitro bile environment. *Microbiology* **155**, 957–967.

Saad, N., Urdaci, M., Vignoles, C., Chaignepain, S., Tallon, R., Schmitter, J.M. and Bressollier, P. (2009) *Lactobacillus plantarum* 299v surface-bound GAPDH: a new insight into enzyme cell walls location. *J Microbiol Biotechnol* **19**, 1635–1643.

Sánchez, B., Bressollier, P., Chaignepain, S., Schmitter, J.M. and Urdaci, M.C. (2009) Identification of surface-associated proteins in the probiotic bacterium *Lactobacillus rhamnosus* GG. *Int Dairy J* **19**, 85–88.

Tsui, H.-C.T., Keen, S.K., Sham, L.-T., Wayne, K.J. and Winkler, M.E. (2011) Dynamic distribution of the SecA and SecY translocase subunits and septal localization of the HtrA surface chaperone/protease during *Streptococcus pneumoniae* D39 cell division. *Mbio* **2**, e0020211.

Vélez, M.P., Verhoeven, T.L.A., Draing, C., Von Aulock, S., Pfitzenmaier, M., Geyer, A., Lambrichts, I., Grangette, C. *et al.* (2007) Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG. *Appl Environ Microbiol* **73**, 3595–3604.

de Vrese, M. and Schrezenmeir, J. (2008) Probiotics, prebiotics, and synbiotics. *Adv Biochem Eng Biotechnol* **111**, 1–66.

Walter, J. and Ley, R. (2011) The human gut microbiome: ecology and recent evolutionary changes. *Annu Rev Microbiol* **65**, 411–429.

Wang, H. and Cronan, J.E. (2004) Functional replacement of the FabA and FabB proteins of *Escherichia coli* fatty acid synthesis by *Enterococcus faecalis* FabZ and FabF homologues. *J Biol Chem* **279**, 34489–34495.

Wilkins, J.C., Beighton, D. and Homer, K.A. (2003) Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*. *Appl Environ Microbiol* **69**, 5290–5296.

Wolf, C., Hochgräfe, F., Kusch, H., Albrecht, D., Hecker, M. and Engelmann, S. (2008) Proteomic analysis of antioxidant strategies of *Staphylococcus aureus*: diverse responses to different oxidants. *Proteomics* **8**, 3139–3153.

Wu, R., Zhang, W., Sun, T., Wu, J., Yue, X., Meng, H. and Zhang, H. (2011) Proteomic analysis of responses of a new probiotic bacterium *Lactobacillus casei* Zhang to low acid stress. *Int J Food Microbiol* **147**, 181–187.

Wu, C., Zhang, J., Chen, W., Wang, M., Du, G. and Chen, J. (2012a) A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance. *Appl Microbiol Biotechnol* **93**, 707–722.

Wu, C., Zhang, J., Wang, M., Du, G. and Chen, J. (2012b) *Lactobacillus casei* combats acid stress by maintaining cell membrane functionality. *J Ind Microbiol Biotechnol* **39**, 1031–1039.

Supporting Information

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

Table S1 Characteristics of the identified protein spots differentially expressed of *Lactobacillus casei* BL23 and ΔRR12 mutant cells after an acid challenge at pH 4·4 for 45 min.