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Role of Curing Agents in the Adaptive Response of the Bioprotective *Latilactobacillus curvatus* CRL 705 from a Physiologic and Proteomic Perspective

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Abstract: During meat processing, lactic acid bacteria (LAB) have to competitively adapt to the hostile environment produced by curing additives (CA). The objective of this study was to investigate the ability of *Latilactobacillus curvatus* CRL 705, a bioprotective strain of meat origin, to adapt to CA. A physiological and proteomic approach was performed. CRL 705 was grown in a chemically defined medium (CDM) containing specific concentrations of CA (NaCl, nitrite, sucrose, and ascorbic acid). The results showed minor differences in growth kinetics in the presence of CA. Glucose consumption, present in the CDM, and production of lactic acid and bacteriocins were not significantly affected. Proteomic analyses indicated that most of the identified proteins (36 out of 39) mainly related to carbohydrate metabolism (18%), posttranslational modifications (15.6%), energy production and conversion (11.1%), translation (11.1%), and nucleotide metabolism (8.9%) were underexpressed. In response to the studied CA, CRL 705 slowed down its general metabolism, achieving slight changes in physiological and proteomic parameters. The observed performance is another characteristic that extends the well-known competitive profile of CRL 705 as a meat starter and bioprotective culture. This is the first report dealing with the impact of CA on LAB proteomics.

Keywords: lactic acid bacteria; bioprotective cultures; meat products; meat processing conditions; curing agents; adaptive response; proteomics; differential protein expression

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1. Introduction

Lactic acid bacteria (LAB) contribute to the hygienic and sensory quality of fermented meat products primarily through their carbohydrate and protein catabolism, resulting in sugar depletion, pH reduction, production of antimicrobial agents, and the generation of flavor compounds [1]. Based on these characteristics, LAB are the preferred bacteria for the formulation of starter cultures. Appropriate cultures of indigenous microorganisms must often be selected to be more competitive. This means that they are well adapted to a particular substrate and have high metabolic capacities to beneficially affect quality and safety while preserving product typicity [2]. Therefore, competitive, functional cultures have gained increasing attention to naturally control the shelf-life and safety of meat products [3]. The existence of a sequence of hurdles either specifically included, such as preservative compounds generally known as curing additives (CA), or indirectly created in the stuffed mixture—lower redox potential (Eh), pH, water activity (a_w), and LAB bacteriocins—will regulate bacterial growth in this ecosystem [2].

In spontaneously fermented sausages, the facultative heterofermentative lactobacilli *Latilactobacillus sakei*, *Latilactobacillus* (*L.*) *curvatus*, and *Lactiplantibacillus (L.) plantarum* constitute the predominant microbiota throughout ripening. *L. curvatus* has shown ubiquity and characteristics to deal with meat environment [4,5]. The *L. curvatus* CRL 705 strain, isolated from an Argentinean artisanal fermented sausage [6], produces the bacteriocins

Lac705 and AL705 which show antibacterial activity against *Brochothrix thermosphacta* and *Listeria* spp, respectively. Indeed, strain CRL 705 has great technological and bioprotective potential and, thus, constitutes our study model, having been the subject of detailed biochemical, molecular, and technological studies [7–10]. In addition, it was the first strain of the *L. curvatus* species whose genome was sequenced and deposited in the GenBank [11]. The adaptation of the CRL 705 strain to the meat environment may be related to the presence in its genome of the *rbsUDKR* gene cluster for ribose catabolism that encodes a *rbsU* ribose transporter [4,5,12] similar to that of *L. sakei* strains [13,14]. Another adaptive feature of CRL 705 related to meat competitiveness is the presence of the catabolic cluster of N-acetylglucosamine, which allows the use of this compound, present in the muscle, as an energy source. In addition, as in other strains of *L. curvatus*, CRL 705 has an additional gene that encodes D-lactyl ether N-acetylmuramic 6-phosphate acid etherase for the catabolism of N-acetylmurein, also present in meat [5].

As mentioned above, CA are included in meat products to ensure better preservation and color/flavor development. The CA most used in the processing of dry fermented sausages are (i) sodium chloride (NaCl), which enhances the flavor, favors drying, and hinders microbial growth, (ii) sodium nitrite ($NaNO_2$), which acts as a preservative and, when it is reduced to nitric oxide, participates in color development, (iii) ascorbic acid, used as an antioxidant, and (iv) sucrose, which acts as an additional energy source to ensure dominance of the starter culture [1,15]. Thus, during the processing, drying, and ripening of fermented meat products, a hostile environment develops that selectively limits the microbiota present and to which the lactic acid strains manage to competitively adapt.

In this context, the objective of this work was to evaluate the response of *L. curvatus* CRL 705 during its growth in the presence of curing additives, through a physiological and proteomic approach. This approach would allow us to explore the metabolic routes mainly affected by the curing mixture and the strategies and culture conditions that this strain needs to improve its performance during the fermentation of meat subjected to curing conditions.

2. Results

2.1. Growth of L. curvatus CRL 705 in a CDM with and without Curing Additives

Although similar growth of *L. curvatus* CRL 705 cells was observed in both media (Figure 1A,C), the lag phase of CRL 705 was 2 h longer when cultured in the presence of the curing mixture (Figure 1A). After exponential growth started, both cultures showed similar growth rates and reached maximum cell density (4.44×10^7 and 5.07×10^7 CFU mL^{-1} in CDM + CA and CDM without CA (CDM^-), respectively) after 10–12 h of incubation. It was observed that the OD increased between 10 h and 12 h, while the viability values remained largely unaltered. This discrepancy can be explained by the density of dead cells, which contribute to the turbidity of the culture. After 16 h, a slight decrease in growth was observed in CA+, although the final CFU mL^{-1} values at 24 h of incubation were similar in both conditions (1.62×10^7 CFU mL^{-1} in CDM + CA and 2.64×10^7 CFU mL^{-1} in CDM^-). Cell growth was accompanied by a concomitant pH decrease, reaching minimal values of 4.23 after 10 h (Figure 1B). Nevertheless, the acidifying potential of *L. curvatus* CRL 705 was not affected by the curing additives, showing kinetics and pH values similar to those exhibited by the control during the 24 h (Figure 1A–C).

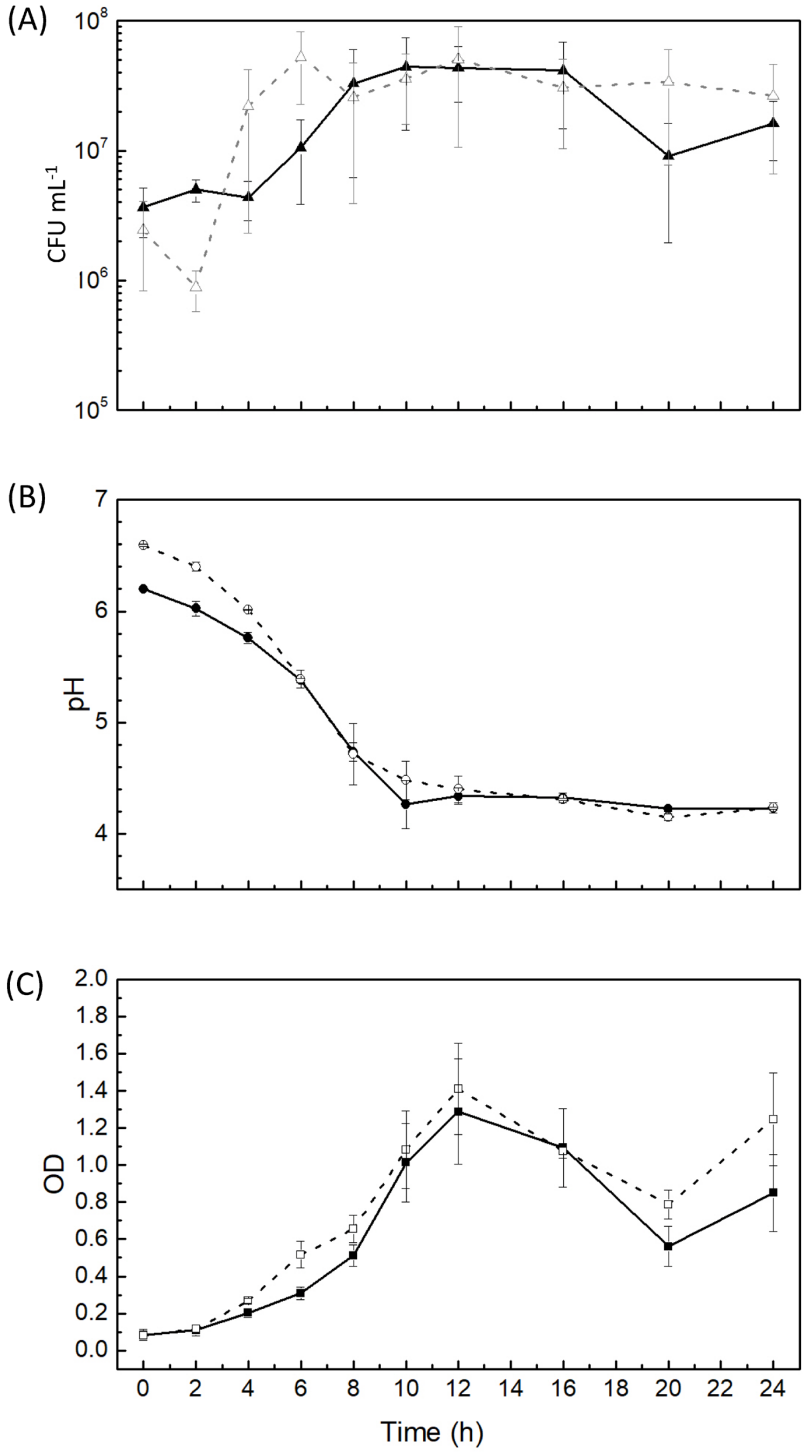


Figure 1. Growth kinetics. (**A**) Viability (CFU mL^{-1}), (**B**) pH, and (**C**) viability (OD600) of *L. curvatus* CRL 705 in CDM + CA (solid line) and CDM^{-} (dashed line) at 25 °C during 24 h.

2.2. Bacteriocin Activity

The production of the two bacteriocins, Lactocin 705 and AL705, by CRL 705 cells was evaluated using the corresponding sensitive strains, *L. plantarum* CRL 691 and *Listeria monocytogenes* FBUNT, respectively (Table 1). Both bacteriocins were produced during cell growth in the absence and presence of CA. Lactocin Lac705 was detected in the supernatants of both CDM cultures after 8 h of incubation, up to 24 h of growth, while the antilisteria bacteriocin AL705 was produced after two hours of growth. Similar production kinetics,

for both bacteriocins, was observed when the CRL 705 cells were grown in culture media rich in nutrients, such as MRS.

Table 1. *L. curvatus* CRL 705 bacteriocin activity in MRS, CMD$^-$ and CDM + CA during 24 h at 25 °C.

	Bacteriocin							
	Lactocin 705 *				**AL 705 ****			
Growth Medium/Time (h)	2	8	16	24	2	8	16	24
MRS	-	+	+++	++	+	++	+++	+++
CDM$^-$	-	+	+++	++	+	++	+++	+++
CDM + CA	-	++	+++	++	+	+++	+++	+++

MRS: MRS broth; CDM$^-$: Chemically defined medium without curing additive supplementation inoculated with CRL705 at 25 °C; CDM + CA: Chemically defined medium with curing additive supplementation inoculated with CRL705 at 25 °C; *: Lactocin 705 activity (AU mL^{-1}): - <100 UA; +: 100–200; ++: 201–400; +++: >400; **: AL 705 activity (AU mL^{-1}): - <5000; +: 6800–12,800; ++: 12,801–25,600; +++: >25,600. Indicator strains: *L. plantarum* CRL691 for Lactocin 705; *Listeria monocytogenes* FBUNT for AL705.

2.3. Consumption of Carbon Sources and Production of Acids

The effects of CA on the consumption of sugars and the concomitant production of acids by *L. curvatus* CRL 705 are shown in Figure 2. Initially, both media (CDM$^-$ and CDM + CA) contained glucose (0.75% equivalent to 7.5 g L^{-1}), while CDM + CA also contained 7.5 g L^{-1} sucrose. CRL 705 cells experienced higher glucose consumption in CDM without additives, especially after 8 h of growth, reaching approximately 4.7 g L^{-1} at 24 h, while in CDM + CA glucose consumption reached 2.9 g L^{-1} at the same time point. Sucrose added to CDM + CA, as a secondary carbon source, maintained the initial concentration throughout the incubation period, indicating the absence of sucrose consumption by CRL 705 in CDM + CA (results not shown). With respect to the production/yield of lactic acid, it was equivalent in both media during the first 8 h; thereafter, lactic acid production by CRL 705 became higher in the absence of CA. Yields of 2.5 g L^{-1} of this metabolite were observed in CDM-CA at 24 h, compared with 1.7 g L^{-1} in CDM + CA, such an outcome being related to the higher consumption of glucose. Finally, a similar content of acetic acid coming from the CDM composition was observed from T0 throughout the study (Figure 2).

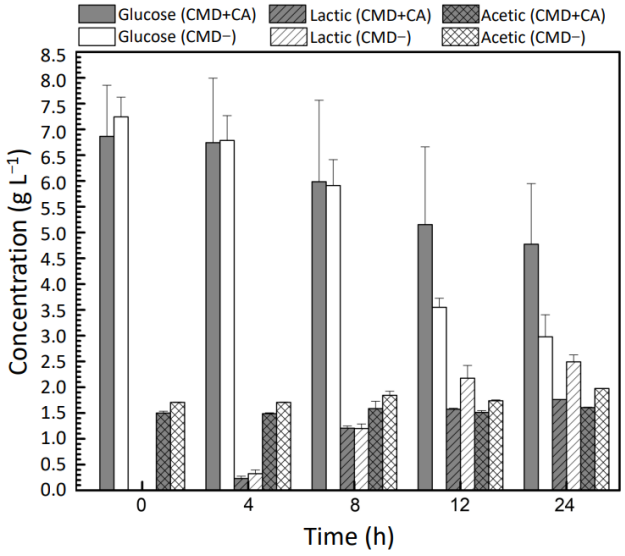
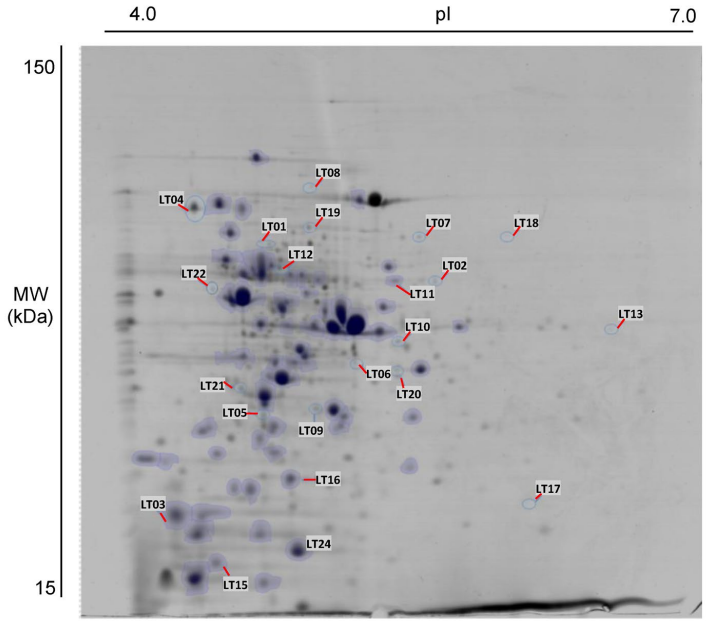


Figure 2. Consumption of sugars and production of acids by *L. curvatus* CRL 705 during its growth in medium CDM + CA (full bar) and CDM$^-$ (empty bar). The concentration (g L^{-1}) of glucose, lactic acid, and acetic acid was determined by HPLC.

2.4. Differential Protein Expression by L. curvatus CRL 705 in the Presence of Curing Additives

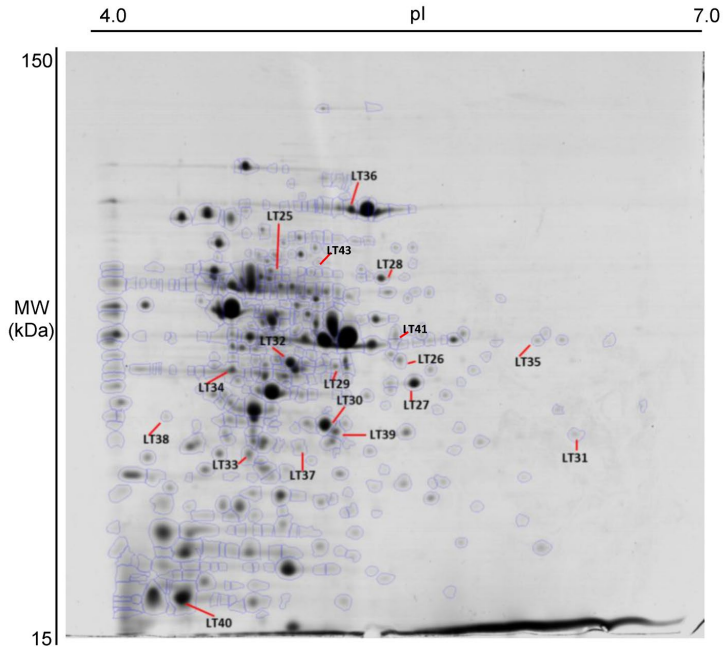
The differential protein expression of *L. curvatus* CRL 705 cultured in CDM with or without CA was evaluated by 2DE. Figure 3 shows representative 2DE proteome maps of CRL 705 cells grown under both conditions. The results of two-dimensional electrophoresis showed 44 differential protein spots ($p < 0.05$, fold > 1.2), which were subjected to MS/MS identification. Thirty-nine proteins were successfully identified (Table S1). All the identified proteins were assigned to different functional categories; five of them were included in more than one category. Most of the identified proteins (36 out of 39) showed lower expression levels in cells cultured in CDM with CA, with fold changes between 1.2 and 2.8, as shown in Table S1. Figure 4 illustrates the abundance (%) of the 36 proteins underexpressed in CDM + CA, grouped according to their functional COG category: 15% of the identified proteins were related to carbohydrate metabolism (G), 5% to amino acid metabolism (E), 10% to nucleotide metabolism (F), and 10% to energy production and conversion (C), with translation, ribosomal structure, and biogenesis (J) accounting for 10%. In addition, six proteins (15%) corresponded to posttranslational modification, protein turnover, and chaperones (O). Only three proteins were overexpressed in CDM + CA, including the spot LT10 that corresponds to the ATP-binding protein UgpC of the sn-glycerol-3-phosphate ABC transporter, spot LT19, identified as pyruvate oxidase, and spot LT41, corresponding to L-lactate oxidase. These proteins are related to carbohydrate metabolism, amino acid/coenzymes metabolism, and energy production/conversion, respectively (Table S1).

Furthermore, according to hypergeometric distribution analysis, certain COG categories could be enriched, such as cell wall/membrane/envelope biogenesis (M), posttranslational modification (O), transcription (K), nucleotide metabolism (F), carbohydrate metabolism (G), and energy conversion and production (C). This suggests that, among the differentially expressed proteins, there were more proteins from those specific categories than expected based on their coding relationship in the *L. curvatus* CRL 705 genome (Figure S1A,B). In fact, although four proteins related to carbohydrate metabolism and transport are most likely to be found in the genome, eight proteins were identified in our study. In contrast, for the translation (J), replication, recombination, and repair (L), and general function prediction (R) categories, more proteins are likely to be found than were identified in our analysis, so it could imply an impoverishment of these categories as seen in Figure S1B.



(**A**)

Figure 3. *Cont.*



(**B**)

Figure 3. Gels 2DE indicating the differentially expressed proteins (arrows) by *L. curvatus* CRL 705 during its growth in CDM. (**A**) Without curing agents (CA) or (**B**) with the mix of CA (44 spots). The differentially expressed and identified proteins (39 spots) are numbered according to the spot number shown in Table S1. For clarity, the 39 spots are numbered in two gels: (a) LT1 to LT22 and (b) LT23 to LT43.

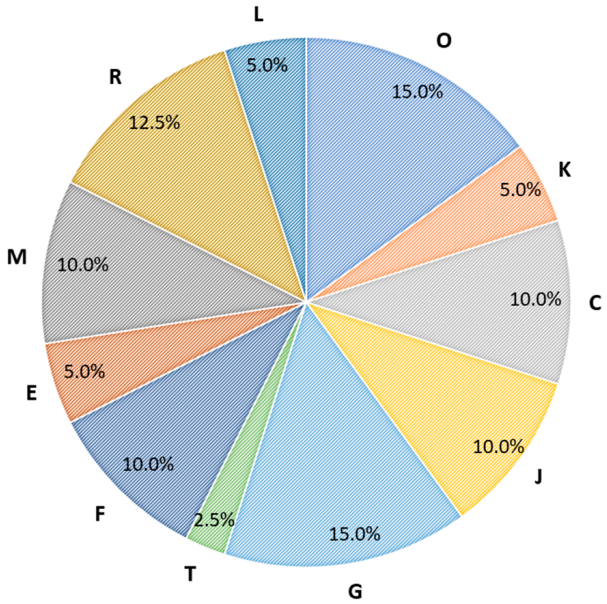


Figure 4. Relative abundance (%) of the 36 proteins underexpressed by *L. curvatus* CRL 705 in CDM + CA, grouped according to their functional COG category. The COG categories are represented by one letter, as follows: (O) molecular chaperones and related functions; (J) translation, including ribosome structure and biogenesis; (G) carbohydrate metabolism and transport; (C) energy production and conversion; (T) signal transduction mechanisms; (K) transcription; (E) amino acid transport and metabolism; (F) nucleotide transport and metabolism; (M) cell wall structure, biogenesis, and outer membrane; (L) replication, recombination, and repair; (R) general functional prediction only.

2.5. Functional Analysis and Protein Interaction

A protein–protein interaction network was constructed to find relationships with the performance of CRL 705 under the influence of CA from a proteomic perspective. As shown in Figure 5, 5 of the 39 identified proteins have no interactions with each other. The remaining 34 proteins interact through 71 edges, with different strengths which represent the magnitude of their interactions. Seven proteins belong to the metabolism of carbohydrates, one of the categories that could be more represented in the sample. Also, the translational and posttranslational modification of proteins is represented by seven proteins. Categories related to redox processes, cell wall biosynthesis, and nucleotide metabolism and transport are each represented by four proteins (Figure 5).

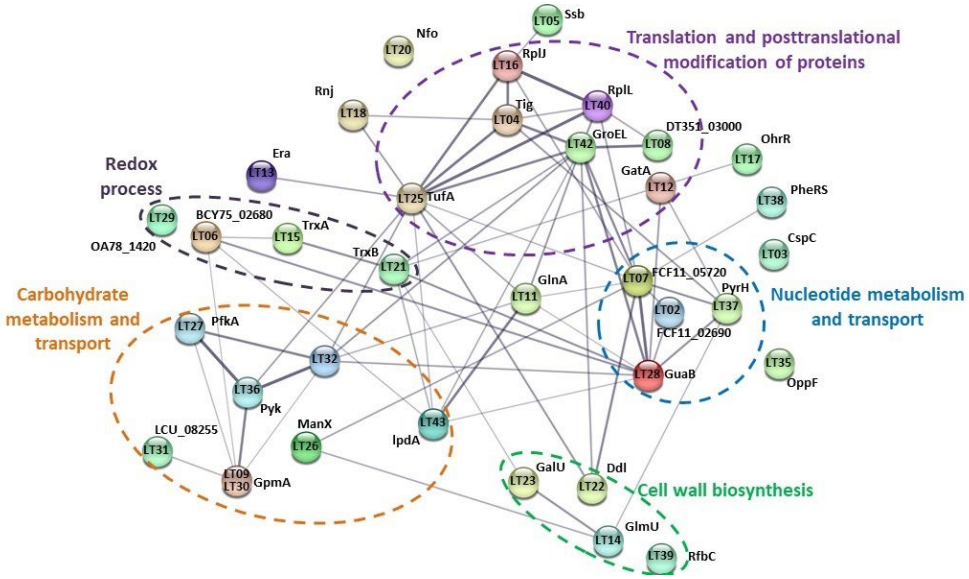


Figure 5. Protein–protein interaction network of the differentially expressed proteins of *L. curvatus* CRL 705 during growth in CDM with and without additives. The circles highlight the proteins related to carbohydrate metabolism, redox processes, cell wall biosynthesis, nucleotide metabolism, and translation and posttranslational modifications. Proteins are represented by nodes, while the interactions between them are represented by edges. The strength of the different interactions is represented by the thickness of the lines. The network was constructed with STRING v10.05. See code and protein name in Table S1.

3. Discussion

The ability of *L. curvatus* CRL 705 to grow in a CDM containing the most used CA in meat processing was investigated. This is the first study that evaluates the adaptation of LAB to the curing conditions used in the meat industry through a physiological and proteomic approach.

In the present work, the presence of CA produced a longer lag phase compared to the control, probably due to the more stressful environment established as a result of the curing mixture. Orihuel et al. (2018) [16] also reported optimal growth of a bioprotective LAB strain (*Enterococcus mundtii* CRL 35) under curing conditions in a meat-based medium. On the contrary, it is generally assumed that sodium chloride and sodium nitrite negatively affect the growth of bacterial cells when used in meat curing processes [17,18].

Other studies demonstrated the ability of *L. curvatus* CRL 705 to grow at 5.3% NaCl in MRS as well as the tolerance of *L. curvatus* and *L. sakei* to high concentrations of sodium chloride (10–18%) [19]. Furthermore, no marked variation in pH decrease or glucose consumption was recorded during CRL 705 growth as a result of CA presence, also indicating that CA did not highly affect metabolic activity. On the other hand, the ability to produce both types of bacteriocins was not affected by curing conditions. On the contrary,

Orihuel et al. [16] reported increases in Enterocin CRL35 production and activity when CRL 35 cells were grown under similar curing conditions, suggesting a stabilizing effect of curing additives, specifically attributed to ascorbic acid, on the antimicrobial peptide. A different impact of the curing conditions on the production of sakacin K by *L. sakei* CTC 494 was reported: a decrease in biomass due to NaCl resulted in a drop in bacteriocin production. In contrast, although sodium nitrite did not specifically affect bacteriocin production, this CA increased the toxic effect of lactic acid on bacterial growth [17].

During adaptation to different growth conditions, microorganisms react by modifying/regulating the expression of proteins involved in DNA replication and repair, metabolism, and protein biosynthesis. In fact, the effect of numerous environmental stresses on different LAB has been studied from a proteomic perspective [20]. In our study, CRL 705 cultured in the presence of a mixture containing NaCl, sodium nitrite, ascorbic acid, and sucrose in the concentrations usually used for the production of fermented sausages showed statistically significant differences in protein expression. Mainly underexpression of those involved in carbohydrate metabolism, posttranslational modifications, energy production, translation, and nucleotide metabolism were evidenced, with variations between 1.2 and 2.8-fold. Even if statistically significant, these differences reflected slight changes in the growth kinetics and acidification rates of the strain, showing the robust nature of CRL 705 able to withstand curing conditions.

Proteins involved in translation, such as the trigger factor, glutamyl-tRNA(Gln) amidotransferase subunit A, 50S ribosomal proteins L10 and L7/L12, the elongation factor Tu, and the molecular chaperone GroEL, were moderately repressed with 1.2–1.5-fold change in expression in the presence of CA. It is important to highlight that the high degree of interactions observed between differentially downregulated proteins mainly involved translation. This underexpression could be related to the elapsed growth observed during the first hours. In this sense, Fadda et al. [21] observed a slight degree of repression on an elongation factor (tuf) in *L. sakei* growing in a CDM supplemented with myofibrillar meat proteins.

As mentioned above, carbohydrate metabolism was downregulated and showed six proteins with close interactions with each other (STRING analysis). The underexpression of glycolytic enzymes was also reported in *L. sakei* cells growing under salt stressing conditions [22]. The underexpression of proteins involved in nucleotide synthesis when CRL 705 was grown in CDM + CA could indicate that the de novo nucleotide synthesis is repressed in the presence of CA [23]. On the other hand, the underexpression of proteins related to redox processes such as the ATP-binding subunit of the ATP-dependent protease Clp (spot LT08) or thioredoxin (LT15) could be explained by the presence of ascorbic acid in the curing mixture; this compound is a powerful antioxidant that participates in reduction–oxidation processes and could reduce the biological requirement of these enzymes [24].

4. Materials and Methods

4.1. Bacterial Strains and Culture Conditions

L. curvatus CRL 705 was isolated from an Argentinean dry fermented sausage [9] and belongs to the CERELA-CONICET culture collection (GenBank AGBU01000000). This strain was stored at −70 °C in a milk yeast extract medium (10% *w/v* skim milk, Ilolay, Buenos Aires, Argentina; 0.5% *w/v* yeast extract, Britania, Buenos Aires, Argentina, Britania, Buenos Aires, Argentina) containing 10% (*v*/*v*) glycerol (Merck, Buenos Aires, Argentina) as a cryoprotectant. *Lactiplantibacillus plantarum* CRL 691 (CERELA-CONICET culture collection), used as a sensitive target organism for the bacteriocin lactocin 705, was cultured using similar procedures to those used for CRL705; on the other hand, *Listeria monocytogenes* FBUNT from the National University of Tucumán (Tucumán, Argentina) was used as an indicator strain for the bacteriocin AL705. This indicator microorganism was stored in Brain Heart Infusion (BHI) medium (Britania, Buenos Aires, Argentina) and activated before use in the same medium.

4.2. Bacterial Growth

A preculture of *L. curvatus* CRL 705 was activated for 24 h in MRS broth (Britania, Buenos Aires, Argentina) at 30 °C, transferred to a chemically defined medium (CDM, all components were from Britania, Sigma Aldrich Co., St. Louis, MO, USA or Merck) [25] [16] and incubated for 16 h at 30 °C. This subculture was used to inoculate 150 mL CDM containing CA (CDM + CA) to an initial OD600 = 0.05–0.1. A culture grown in CDM without CA was used as a control. Glucose (Merck), as a carbon source, was added at a final concentration of 0.75% in both culture media. CDM + CA also contained 0.75% sucrose (Britania) (*w*:*v*), 3% sodium chloride (Merck) (*w*:*v*), 0.02% sodium nitrite (*w*:*v*) (Ciccarelli, Buenos Aires, Argentina), and 10 mg mL^{-1} ascorbic acid (Sigma Aldrich). Growth of *L. curvatus* CRL 705 was monitored during 24 h at 25 °C (temperature used for fermentation during dry fermented sausage processing) by measuring optical density (OD) at 600 nm and cell viability in samples taken at 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h of incubation. For bacterial enumeration, decimal dilutions were prepared, plated on MRS agar (Britania, Buenos Aires, Argentina), and incubated at 30 °C for 48 h. Results were expressed as colony forming units per milliliter (CFU mL^{-1}). The acidification potential of *L. curvatus* during growth on CDM was determined by measuring pH using an Altronix TPX 1 (New York, NY, USA) digital pH-meter. Three independent cultures were performed for each condition.

4.3. Determination of the Activity of Lac705 and AL705 Bacteriocins

To investigate antimicrobial activity, culture samples of *L. curvatus* CRL 705 were centrifuged (12,000× *g*, 10 min) and cell-free supernatants (CFS) were collected and heated at 80 °C for 20 min. The lactocin Lac705 activity was evaluated by the well diffusion assay, according to Salvucci et al. [26] although with some modifications. Briefly, 10 mL of MRS soft agar (0.7%) containing 70 µL of the culture of the indicator strain *L. plantarum* CRL 691 was poured on a Petri dish with 10 mL of MRS agar (1.2%). After solidification, 5 µL of a two-fold serial dilution of each CFS at different sampling times (from 0 to 72 h) were seeded. After incubation at 30 °C for 24 h, the presence or absence of a zone of growth inhibition (halo) around the seeded spot was observed. Results were expressed in arbitrary units per milliliter (AU mL^{-1}) (AU mL^{-1} = 1/seeded vol × dilution factor). To test the activity of AL705, the same protocol was adopted, although using *Listeria monocytogenes* FBUNT as the indicator strain in BHI medium.

4.4. Consumption of Carbon Sources and Production of Lactic Acid, Acetic Acid, and Ethanol

The sugars and the end-products were determined in the Special Analyses Laboratory of CERELA-CONICET, Tucumán, Argentina. Sugar consumption and metabolite production were assessed at 0, 4, 12, and 24 h of cell growth in CDM with or without additives, as well as at the time of cell harvest for proteomic analyses. Lactic and acetic acids were measured by HPLC, using an Aminex HPX-87H ion exclusion column (ISCO 2350 model, 300 × 7.8 mm^2, Bio-Rad Laboratories Inc., Hercules, CA, USA), as previously described by Gerez et al. (2010) [27]. End-products concentrations were expressed as g L^{-1}. Sugar consumption was also evaluated by HPLC, using an Aminex HPX-87P column (Bio-Rad), according to Ortiz et al. (2012) [28]. All data were analyzed using Eurochrom Basic Edition 3.5 software for Windows.

4.5. Differential Protein Expression Analysis

For proteomic analysis, two-dimensional electrophoresis (2DE) was applied [29]. Differential protein expression of *L. curvatus* cells growing on CDM with and without supplementation with curing additives was compared. To specifically study the effect of curing conditions, optimal growing conditions (25 °C, CDM^- rich medium and normal pH) of *L. curvatus* were applied. Furthermore, the late exponential growth phase was selected to ensure active metabolism of the strain during protein expression analysis.

4.5.1. *L. curvatus* CRL705 Cells Recovery

For the proteomic assay, cells in the late exponential growth phase in CDM + CA (6 h) or without CA (6 h) were collected by centrifugation at 8000× *g* (10 min, 20 °C), washed, and the pellets stored at −20 °C until lysis to obtain the CRL705 proteome [22]. The experiment was replicated independently three times for each condition.

4.5.2. Cell-Free Protein Extraction

Lysis of cells was carried out using glass beads (150 ± 212 µm in diameter, Sigma-Aldrich Co., St. Louis, MO, USA) resuspended in 0.1 mol L^{-1} Tris-HCl buffer pH 7.5 in a ratio 1:2:1 (cell:buffer:bead) as described by Orihuel [29]. After lysis, samples were centrifuged (14,500× *g*, 10 min, 15 °C) to recover extracts of cell-free supernatants containing the proteome of *L. curvatus* CRL 705. Protein concentration analysis (Bradford assay) was conducted. Aliquots of 600 µg of protein were finally stored at −80 °C until further analysis.

4.5.3. Two-Dimensional Gel Electrophoresis (2DE)

Sample preparation and 2DE gels were carried out according to Orihuel [20]. Isoelectrofocusing (IEF) was carried out in IPGphor (GE Healthcare, Uppsala, Sweden) at 53,500 Vh with immobilized pH gradient (IPG) strips (ImmobilineDryStrip Gels, linear pH 4–7, 18 cm; GE Healthcare, Uppsala, Sweden). After IEF, strips were equilibrated at room temperature in 6 mol L-1 urea, 2% (*w*/*v*) SDS, 30% (*w*/*v*) glycerol, 50 mmol L^{-1} Tris-HCl, pH 8.0, and the SDS-PAGE step was performed on homogeneous 12.5% (*w*/*v*) polyacrylamide gels (25 × 20.5 cm) at a constant current of 15 mA/gel at 15 °C (~16 h), using an Ettan DALTsix Large Vertical System (GE Healthcare). Colloidal Coomassie Blue Stain according to Candiano [30] was used for gel staining. The 2DE gels were digitalized using Image Scanner III LabScan 6.0 (GE Healthcare).

4.5.4. Image Acquisition and Data Analysis

Quantization and normalization of volume spots were performed on digitalized gel images (600 dpi) using Prodigy SameSpots software version 1.0.3400.25570 (Totallab, Newcastle, UK). The volume of each spot was calculated and normalized by referencing the values to the sum of the total spot volumes within each gel. The student's t-test was applied for unpaired samples. A protein was considered differentially abundant if the mean normalized spot volume varied at least 1.5-fold between the compared spots. The effect was confirmed by analysis of variance at a significance level of $p < 0.05$. Protein spots showing significant variations between the different conditions were manually excised from the gels using a scalpel blade and identified using mass spectrometry.

4.5.5. Mass Spectrometry Protein Identification

Mass spectrometry analysis was carried out at CEQUIBIEM QB-FCEN-UBA/IQUIBICEN-CONICET (Buenos Aires, Argentina) and at Institut Pasteur (Montevideo, Uruguay). Selected spots were excised from the gels and submitted to tryptic digestion and mass spectrometry analyses as previously [31]. The samples were processed at CEQUIBIEM and analyzed with an Ultraflex II Bruker Daltonics UV-MALDI-TOF-TOF mass spectrometer, equipped with a Nd:YAG laser (λem 355 nm). Flex Analysis 3.3 software was applied for visualization and comparison of the generated spectra. The peak list generated was based on signal-to-noise filtering and a contaminant exclusion list. Two high S/N MS peaks per sample were selected for MALDI TOF-TOF fragmentation. MS and MS/MS spectra for each spot were combined using BioTools 3.1 software (Bruker Daltonics). Peptide samples analyzed at Institut Pasteur (Montevideo, Uruguay) were subjected to analysis on an ABI 4800 (Sciex, Foster City, CA, USA) mass spectrometer. The resulting file was then searched using Mascot (Matrix Science, Boston, MA, USA) against the NCBInr database (20160618), taxonomy: *L. curvatus* CRL 705 (https://www.ncbi.nlm.nih.gov/nuccore/AGBU00000000.1) (URL accessed on 23 October 2023). All proteins were identified using BLASTp in the

NCBI database [32]. The database search parameters included a peptide mass tolerance of 100 ppm, fragment mass tolerance of 0.5 Da, one missed cleavage, methionine oxidation as variable modifications, and cysteine carbamidomethylation as fixed modification. Only matched proteins with significant scores ($p < 0.05$) were considered.

4.6. Functional Analysis and Interaction of the Differentially Expressed Proteins

Functional analysis of the identified proteins that were assigned to the different Clusters of Orthologous Groups (COGs) [33] was performed using COGNITOR. Interacting protein networks were obtained using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) version 10.05 [34]. Interacting proteins (represented by nodes) are linked by edges. Prediction methods for interactions available in STRING were used with a confidence level of 0.4 (medium) [34].

4.7. Statistical Analyses

Three biological replicates were performed for all experiments (growth kinetics, growth inhibition, and differential protein expression assays), and values and standard error were calculated. In the proteomic analysis, a one-way analysis of variance was performed alongside the t-test, and $p < 0.05$ was considered to indicate a statistically significant difference, as detailed in Section 2.5. The hypergeometric distribution was tested to evaluate the enrichment of COG categories that were determined with COGNITOR on the Operon Mapper web platform [33,35]. This allowed for the evaluation of the enrichment of the COG categories of the proteins encoded by *L. curvatus* CRL705 related to those differentially expressed by this strain in CDM^{-} and in CDM + CA.

5. Conclusions

L. curvatus is a ubiquitous species with a metabolism adapted to grow in different niches. Specifically, the performance of strain CRL 705 during growth in the presence of the curing mixture was slightly affected. This indicates that the additives exerted a mild depression on certain metabolic pathways, a phenomenon which was reflected in the growth delay during the first hours and in the moderate/mild repression of protein expression.

Taken together, the physiological and proteomic results could indicate that *L. curvatus* CRL 705, in response to the more stressful environment produced by the curing additives, slowed down its overall metabolism to adapt and maintain its viability, achieving only minor modifications in growth/metabolic parameters and protein expression. Considering the meat origin of the strain, these are positive results and constitute another characteristic that expands the well-known competitive profile of *L. curvatus* CRL705 as a starter/bioprotective culture for meat, definitively postulating the suitability of this strain to be used in fermentation of cured meat products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bacteria2040011/s1, Figure S1: (A) Hypergeometric distribution for the probabilities (Prob) of finding a certain COG functional category a certain number of times "x" of the differentially expressed proteins of L. curvatus CRL 705 while growing in CDM with and without additives at 25 °C. The highest value of the y axis (Prob) for each of the curves represents the higher probability of the times of occurrence of proteins from a certain COG. (B) Differences between measured and expected occurrence of the proteins of each COG category, where 0 represents no differences in occurrence and separates potentially enriched categories (positive values) from the potentially impoverished categories (negative values). Table S1: Differentially expressed proteins by L. curvatus CRL 705 during growth in CDM with (CDM + CA) and without (CDM^{-}) the presence of curing additives at 25 °C.

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