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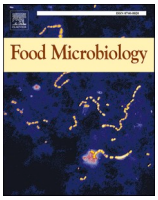

The stressing life of *Lactobacillus delbrueckii* subsp. *bulgaricus* in soy milk

Gwénaël Jan [*], Florian Tarnaud, Fillipe Luiz Rosa do Carmo, Nassima Illikoud, Fanny Canon, Julien Jardin, Valérie Briard-Bion, Fanny Guyomarc'h [1], Valérie Gagnaire [1]

STLO, INRAE, Institut Agro, F-35000, Rennes, France

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ABSTRACT

Lactobacillus delbrueckii subsp. *bulgaricus* is a beneficial lactic acid bacterium and constitutes one of the most used, and thus consumed, dairy starters, worldwide. This homofermentative bacterium was the first lactobacillus described and is involved in the fermentation of yogurt and of diverse other fermented products, including cheeses. It has a long history of safe use, as well as documented probiotic lato sensu effects, including alleviation of lactose intolerance. Plant-based fermented products presently experience a considerable development, as a result of evolution of consumers' habits, in a general context of food transition. This requires research and development, and thus scientific knowledge, to allow such transition, including the development of fermented soy milks. These last indeed offer an alternative source of live and active bacteria. The yogurt starters *L. delbrueckii* subsp. *bulgaricus*, together with *Streptococcus thermophilus*, have been implemented to generate yogurt-type fermented soy milks worldwide. While the adaptation of these starters to the dairy environment has been extensively studied, little is known about *L. delbrueckii* adaptation to the soy environment. We therefore investigated its adaptation to soy milk and compared it to cow's milk. Surprisingly, it did not grow in soy milk, neither alone, nor in co-culture with *S. thermophilus*. Acidification of soy milk was however faster in the presence of both species. In order to deepen such adaptation, we then compared *L. delbrueckii* growth and survival in soy milk ultrafiltrate (SUF, the aqueous phase of soy milk) and compared it to cow's milk ultrafiltrate (MUF, the aqueous phase of cow milk). This comparison revealed major differences in terms of cell morphology and proteome composition. Lactobacilli appeared deformed and segmented in soy. Major differences in both the surface and the cellular proteome indicated upregulation of stress proteins, yet downregulation of cell cycle and division machinery. Altogether, these results suggest that soy milk may be a stressing environment for the yogurt starter *L. delbrueckii* subsp. *bulgaricus*.

1. Introduction

In developed countries, the average consumption of fermented foods, and mainly fermented dairy products (including fermented milks and cheeses), supplies a daily dose of 10^{10} bacteria, mainly lactic acid bacteria (LAB), per person and per day (Rezac et al., 2018). Fermented foods have been a major part of human diet for thousands of year, mainly because fermentation of milk, meat and plant foods allows better preservation (Ross et al., 2002; Tamang et al., 2020). They also provide humans with a great variety, and amount, of bacteria involved in fermentation. A growing interest is paid to these bacteria and to their impact on physiology and health, and beneficial effects have long been suggested for fermented foods and for the corresponding bacteria. Indeed, emerging epidemiological and clinical evidence indicate that these microorganisms, either responsible for fermentation, or added for their probiotic properties, may contribute to gastrointestinal and systemic homeostasis, improve health status or reduce disease risk (Marco et al., 2017; Rezac et al., 2018).

As a result of the present food transition, less animal-sourced products, and more plant-based ones, are used. This aims towards sustainable and healthy food products, decreased environmental impact, use of new protein sources, modification of food habits, while keeping food savoury and safety. LAB starters can play a central role in such a transition, generating proper organoleptic, nutritional, and safety properties of new fermented foods. However, these new substrates contain compounds that need other types of enzymes. Carbohydrates present in legumes, responsible for intestinal discomfort, i.e. galactooligosaccharides as raffinose and stachyose, can only be hydrolyzed by α-galactosidases that

* Corresponding author. STLO, INRAE, Agrocampus Ouest, 65 rue de Saint Brieuc, F-35000, Rennes, France.
E-mail address: gwenael.jan@inrae.fr (G. Jan).
[1] These authors shared senior authorship.

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are expressed by a few number of strains (Canon et al., 2020a; Harlé et al., 2020). Starters should also reduce anti-nutritional and off-flavour compounds (Tangyu et al., 2019; Canon et al., 2020b).

The most recognized probiotic effect is that of the yogurt starters *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which alleviate the symptoms of lactose intolerance (EFSA Panel on Dietetic Products, Nutrition, and Allergies, 2010). They provide humans with β-galactosidase (lactase), helping digestion of lactose (Rezac et al., 2018) and healing the symptoms of lactose intolerance (Morelli, 2014). It is furthermore reported that consumption of yogurt is associated with reduced weight gain (Mozaffarian et al., 2011), as well as to a reduced risk of metabolic syndrome (Babio et al., 2015), of bladder cancer (Larsson et al., 2008). Interactions between these starters are known in milk (Sieuwerts et al., 2008, 2010): *L. delbrueckii* subsp. *bulgaricus* provides peptides and free amino acids, while *S. thermophilus* provides metabolites such as formic acid, pyruvic acid, folic acid and carbon dioxide. These starters were also used to produce yogurt-type fermented vegetable products. Indeed, *S. thermophilus* was used in numerous fermented plant-based products (Schaffner and Beuchat, 1986; Tangyu et al., 2019), due to its ability to use either the dairy carbohydrate lactose or the pant-derived sucrose and fructose, and to produce amino acids (Iyer et al., 2010). *S. thermophilus* strains have been shown to acidify soy milk down to pH values below 5.0 (Harlé et al., 2020) and one strain displayed similar growth and acidification dynamics in soy milk and cow's milk (Boulay et al., 2020). Growth and adaptation of *L. delbrueckii* subsp. *bulgaricus* in soy milk was contrastingly little explored. It is usually cultured together with *S. thermophilus*, as in milk, but interactions between these starters in soy milk are unknown. *S. thermophilus* was shown to be predominant and to produce excess diacetyl in fermented soy milk, while *L. delbrueckii* subsp. *bulgaricus* barely grew (Kaneko et al., 2014). Such a lack of lactobacilli can be overcome by the addition of glucose and yeast extract to soy milk (Chumchuere and Robinson, 1999).

New questions thus arise from these new technologies. Which bacterium, among *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, does what, in soy milk? How do they adapt, or not, to soy milk conditions? Does the culture media, cow milk and soy milk, modulate the cellular composition of the starters? In order to address such questions, we thus investigated the impact of soy milk conditions on *L. delbrueckii* physiology. We focused on growth and acidification, morphology, as well as on modulation of the proteome.

2. Materials and methods

2.1. Strains and pre-cultures

Lactobacillus delbrueckii subsp. *bulgaricus* CIRM-BIA1592 and *Streptococcus thermophilus* CIRM-BIA1345 were collected, stored and maintained by the CIRM-BIA International Center for Microbial Resources dedicated to bacteria of food interest, (INRAE Rennes, France, https://collection-cirmbia.fr/). *L. delbrueckii* was routinely cultured at 42 °C without agitation and under microaerophilic conditions in De Man, Rogosa, Sharpe (MRS) medium (De Man et al., 1960). *S. thermophilus* was routinely cultured in M17 medium (Terzaghi and Sandine, 1975) in the same conditions. As an alternative, these microbial strains were cultured in ultrafiltrate media, or in soy milk, as described below. For enumerations, thermophilic lactobacilli and streptococci were subjected to CFU counting by serial dilutions in physiological peptone water, prior to spreading into MRS, or M17, respectively, supplemented with 10 $g.L^{-1}$ agar.

2.2. Culture in cow's milk or soy milk

The LAB strains were inoculated (10^5 CFU/mL) into soy milk without added sugar (Soja nature sans sucre, Sojasun, Triballat, Noyal-sur-Vilaine, France) and incubated at 42 °C without agitation. The overall composition of soy milk, per 100 mL, was as follows: Energy 133 kJ; fat 1.8 g, of which saturates 0.3 g; carbohydrates 0.5 g; proteins 3.3 g; salt 0.07 g. Acidification was followed throughout incubation using a pH-meter equipped with a penetration electrode (Mettler Toledo, France). As an alternative, cow milk UHT milk (half-skimmed milk, UHT, Agrilait, Cesson-Sévigné, France) was used as a culture medium. *L. delbrueckii* population was monitored by serial dilutions followed by CFU counting in MRS-agar. *S. thermophilus* population was monitored by serial dilutions followed by CFU counting in M17-agar.

2.3. Culture in cow's milk or soy milk ultrafiltrate

Cow's milk ultrafiltrate (MUF) was prepared as previously described at the INRAE STLO dairy platform (Michalski et al., 2006; Cousin et al., 2012). Briefly, raw cow's milk was skimmed prior to ultrafiltration using a UF pilot equipment equipped with a ceramic membrane, with a molecular weight cut-off point of 8 kDa. The overall composition of MUF was as follows: carbohydrate 5%; non-protein nitrogen 0.28%, minerals 0.75% and dry matter 6.14%. MUF was supplemented with 5 $g.L^{-1}$ food grade casein hydrolysate (Casein Peptone Plus, Organotechnie, La Courneuve, France), brought to pH 7 using NaOH, sterilized by 0.2 μm filtration (Nalgene, Roskilde, Denmark) and stored at 4 °C.

A local company, Sojasun Technologies Triballat Noyal (Noyal-sur-Vilaine, France) supplied the soy milk ultrafiltrate (SUF) which was prepared according to patent N° EP 1,983,844 B1 (Efstathiou and Driss, 2010) and is commercially available under the designation BASOSOY (Triballat ingredients Triballat, Noyal-sur-Vilaine, France). Briefly, soybeans were dehulled prior to grinding in water, cooking under alkali conditions and eliminating the okara residue using a separator. The resulting soy juice was subjected to ultrafiltration to generate a retentate and a soy ultrafiltrate (SUF). The overall composition of the SUF was: carbohydrates 2.5%, protein 0.55%, non-protein nitrogen 0.20%, mineral 1%, dry matter 5%. The SUF was supplemented with 5 $g.L^{-1}$ food grade soy hydrolysate (Bacto Soytone, BD Bioscience), brought to pH 7 using NaOH and autoclaved (110 °C, 10 min). It was then centrifuged (28,000×*g*, 30 min), filtered on Whatman paper (from 30 to 8 μm) and then on Nylon Net Filters (from 10 to 0.4 μm, Millipore), in order to remove insoluble compounds. It was finally sterilized by 0.2 μm filtration (Nalgene, Roskilde, Denmark) prior to storage at 4 °C.

2.4. Phase contrast, fluorescence, atomic force and transmission electronic microscopies

The LAB strains were routinely examined as wet-mount fresh cultures using an immersion phase contrast × 100 objective on an Olympus B×51 optical microscope. In addition, polyphosphate granules were visualized by DAPI (4′,6′-diamidino-2-phenylindole) staining as previously described (Günther et al., 2009) and later adapted to dairy bacteria (Huang et al., 2016). Briefly, bacteria were washed in McIlvaine's buffer, fixed in 4% formaldehyde, permeabilized in 0.3% Triton-X100 and stained in 20 μg/mL DAPI in the same buffer. Stained cultures were observed on an epifluorescence BX51 Olympus microscope equipped with a U-MWU2 fluorescence filter cube (excitation filter 330–385 nm, emission filter 480–800 nm) and an Olympus plan 100x/1.25 oil objective.

As an alternative, LAB cultures were dried on a mica slide prior to analysis using AFM (Atomic Force Microscopy, as previously described (Deutsch et al., 2012). Once in the stationary phase (72 h), the fresh cultures were washed 3 times by centrifugation at 7000*g* × 5 min in HEPES-NaCl buffer (2 mM *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 50 mM NaCl buffer, pH 6.7) and at room temperature. Two μl of the suspension were immediately smeared over a freshly cleaved 1-cm mica disk glued onto a glass slide, then left to dry overnight in a desiccator. The samples were imaged by atomic force microscopy (AFM) in intermittent contact mode using silicon OMCL-AC240TS probes (nominal radius ~7 nm, nominal spring

constant 2 N m^{-1} – Olympus, Tokyo, Japan).

Transmission Electron Microscopy (TEM) was performed as described previously (Deutsch et al., 2010). Briefly, bacteria were washed in PBS, fixed using glutaraldehyde, postfixed using osmium tetroxide/potassium cyanoferrate/uranyl acetate and dehydrated in ethanol (30%–100%) prior to embedding in Epon. Thin sections (70 nm) were collected on 200-mesh copper grids and counterstained with lead citrate before examination using a Philips CM12 transmission electron microscope.

2.5. Proteolysis quantification

During incubation of cultures, proteolysis was monitored by measuring the content in free NH_2 groups, i.e., peptides and free amino acids present in fermented product. This was measured in triplicates using the *o*-phthalaldehyde (OPA) method of Church et al. (1983) adapted to microplate. The proteins were precipitated prior to the assay by half-diluting samples with 2% (w/w) trichloroacetic acid final concentration for allowing the free NH_2 groups present at the *N*-terminal extremity of the peptides and amino acids to be preferentially detected by the OPA. The results were expressed as mM equivalent methionine, used as a standard.

2.6. Electrophoresis

Whole-cell SDS protein extracts were prepared by disrupting bacterial pellets in Sodium Dodecylsulfate (SDS) lysis buffer, prior to centrifugation to discard debris, as previously described (Jan et al., 2001). Surface extractible Guanidine extracts were prepared as previously described (Le Marechal et al., 2015). Bacterial pellets were re-suspended in 5 M guanidine hydrochloride to reach a final OD_{650} of 20. After centrifugation (21,000×*g*, 20 min, 30 °C) to eliminate cells, the supernatant was dialyzed against 0.1% SDS in distilled water. The extracts were diluted in SDS sample buffer (Laemmli, 1970) prior to heat denaturation (10 min, 95 °C). One-dimensional Polyacrylamide Gel Electrophoresis (12.5%) was conducted according to Laemmli's procedure on a Protean II xi Cell (Bio-Rad, Hercules, USA) prior to Coomassie Blue-staining using Bio-Safe reagent (Bio-Rad, Marne La Vallée, France).

2.7. Label-free proteomics

Label-free proteomics was performed as previously described (Gaucher et al., 2019). Briefly, lactobacilli were cultivated in milk and soy ultrafiltrate until the start of stationary phase and 10 mL aliquots harvested by centrifugation (8000×*g*, 10 min, 20 °C). The cells were washed twice with 10 mL PBS buffer (NaCl 8 $g.L^{-1}$, KCl 2 $g.L^{-1}$ KH_2PO_4 2 $g.L^{-1}$, Na_2HPO_4 $12H_2O$ 35.8 $g.L^{-1}$) and resuspended in 1 mL lysis solution (50 mM Tris-HCl [pH 7.5], 0.3% SDS, 200 mM dithiothreitol (DTT), 0.4 mM phenyl methyl sulfonyl fluoride, PMSF), and then sonicated immediately using a Vibra Cell sonicator (Bioblock Scientific, Illkirch, France). The cells were broken down using 0.1 mm zirconium beads (1 mL suspension of 10^{10} lactobacilli, 0.1 g of beads) in a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). Cell debris were removed by centrifugation (20,000×*g*, 10 min, 20 °C) and the proteins extracts harvested. The proteins were further cleaned using the 2-D Clean-Up kit (GE Healthcare) and quantified with the 2-D Quant Kit. Tryptic digestion was performed on 100 μg whole-cell proteins from each sample for 15 h at 37 °C using Sequencing Grade Modified Trypsin (Promega, Madison, USA) according to the manufacturer's instructions and as described previously (Huang et al., 2018a). Spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma-Aldrich, USA) was added in order to stop tryptic digestion at pH 2.

2.8. Nano-LC-MS/MS

Liquid chromatography and mass spectrometry were conducted as previously described (Huang et al., 2018a). Briefly, experiments were performed using a nano RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA). The spectra of eluted peptides were recorded in full MS mode and selected within a mass range of 250–2000 m/z for MS spectra and a resolution of 70,000 at m/z 200. For each scan, the ten most intense ions were selected for fragmentation.

2.9. Protein identification

Protein identification was performed as previously described (Huang et al., 2018a). Peptides were identified from the MS/MS spectra using X! Tandem pipeline software (Langella et al., 2017). LU The search was performed against the proteome of *L. delbrueckii* subsp. *bulgaricus*. For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation, and a minimum of two peptides were required for protein identification.

2.10. Protein quantification

Protein quantification was performed as previously described (Huang et al., 2018a). Each peptide identified by tandem mass spectrometry was quantified using the free MassChroQ software before data treatment and statistical analysis under R software (R 3.2.2, Project for statistical computing). A specific R package called 'MassChroqR' was used to automatically filter dubious peptides for which the standard deviation of their retention time was longer than 30 s and to regroup peptide quantification data into proteins. For peak counting analysis, variance analysis was performed on proteins with a minimum peak ratio of 1.5 between the two culture conditions. Proteins with an adjusted p-value <0.05 were considered to be significantly different.

For XIC based quantification, normalization was performed to take account of possible global quantitative variations between LC-MS runs. Peptides shared between different proteins were excluded automatically from the data set as well as peptides present in fewer than 85% of samples. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein (Blein-Nicolas et al., 2015). Analysis of variance was used to determine proteins with significantly different abundances between our two culture conditions.

Proteins were considered to be differentially expressed if there was a significant ($p < 0.05$, ANOVA) change in expression of $\geq$ 2-fold (log2 ratio $\geq$1.5). A volcano plot was generated to visualize differentially expressed proteins in the core proteome of *L. delbrueckii* subsp. *bulgaricus* when cultivated in soy milk compared to cow's milk. Functional annotation and Clusters of Orthologous Groups (COGs) were obtained using the eggNOG-mapper v2 web tool (Huerta-Cepas et al., 2017; 2019).

2.11. Statistical analysis

The data were obtained from triplicate samples. All the results are presented as mean values with standard deviations. Statistical significance was set at $p < 0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows).

3. Results

3.1. Growth of thermophilic starters in yogurt-type fermented soy milk

Thermophilic streptococci and lactobacilli were first enumerated in commercial yogurt-type fermented soy milks purchased from a local supermarket. As shown in Table 1, thermophilic streptococci were numerated with a population comprised between 6.14×10^7 and 6.2×10^8 CFU per gram of product. By contrast, concentrations of thermophilic lactobacilli were very low in yogurt-type fermented soy milks, i.e. under the limit of detection or between 8.0×10^4 and 8.9×10^6 CFU per gram of product.

Table 1
Numeration of lactic 1 acid bacteria in commercial fermented soy milks.

Product	Time to EDC[a]	Thermophilic streptococci, CFU/g [b]	SD	Thermophilic lactobacilli CFU/g [c]	SD
Fermented soy milk A	EDC - 21	1.51×10^8	1.83×10^7	3.37×10^6	5.20×10^6
Fermented soy milk B	EDC - 16	2.13×10^8	4.37×10^7	8.90×10^6	5.37×10^6
Fermented soy milk C	EDC- 26	6.14×10^7	9.99×10^6	8.00×10^4	4.00×10^4
Fermented soy milk D	EDC- 13	6.20×10^8	1.57×10^8	ND	ND

ND: not detected, even at the lowest dilution tested (10^{-1}.
[a] EDC, Expiry Date for Consumption.
[b] Thermophilic streptococci were enumerated by serial dilutions followed by CFU counting in M17-agar.
[c] Thermophilic lactobacilli were enumerated by serial dilutions followed by CFU counting in MRS-agar.

We then investigated growth of two strains, i.e. *L. delbrueckii* subsp. *bulgaricus* CIRM-BIA1592 and *S. thermophilus* CIRM-BIA1345 in commercially available cow milk and soy milk (Fig. 1). As shown in Fig. 1A, lactobacilli grew in cow milk to reach a final concentration close to 10^8 CFU per gram, either alone or in coculture with streptococci. Interestingly, growth was however faster and led to a higher final concentration in the presence of streptococci. Growth of streptococci was more pronounced and led to final concentrations close to 10^9 CFU per gram, whatever the presence or not of lactobacilli. They then remained constant during the 24 h of culture in pure culture, while a drop in viability was observed after 20 h in coculture. As shown in Fig. 1C, acidification was faster in the case of cocultures, while single cultures of lactobacilli led to a slower acidification. The final pH, whatever the culture, was close to 4.

Results were different in soy milk, where only streptococci grew, to reach a final population close to 10^7 CFU per gram, either alone or in coculture. By contrast, a limited growth of lactobacilli in coculture was observed in soy milk, while a pronounced loss in viability was recorded in single cultures of lactobacilli. Concerning pH, similar kinetics of acidification was observed (Fig. 1D) for streptococci single cultures and cocultures, with a final pH close to 4.5. By contrast, lactobacilli in single cultures failed to acidify soy milk, in accordance with their absence of growth.

Wet mount microscopic examination of cow milk fermentations revealed the typical morphologies of streptococci (spherical coccoids arranged in chains) and of lactobacilli (long and straight rods which appear separate), either in single or in cocultures (Fig. 1E). Examination of soy milk fermentations revealed a similar aspect for streptococci (Fig. 1F). However, the morphology of lactobacilli in these conditions were clearly distinct. Long and curve structures were observed in single cultures, while lactobacilli were hardly observed in cocultures after 24 h.

3.2. Proteolysis of cow milk and soy milk proteins by L. delbrueckii subsp. bulgaricus

We then monitored proteolysis which is a key process of yogurt making (Fig. 2), by the measure of the free NH_2 groups that correspond to the overall production of peptides and free amino acids in the cow milk during fermentation. As shown in Fig. 2A, *L. delbrueckii* subsp. *bulgaricus* CIRM-BIA1592 was responsible for proteolysis of cow milk, as indicated by the increase in free NH_2 groups from 2 to 5 mM eq Methionine, in contrast to *S. thermophilus* CIRM-BIA1345, for which the free NH_2 groups remain unchanged at 2 mM. The increase of the free NH_2 group was lower in co-culture than for *L. delbrueckii* subsp. *bulgaricus* alone, suggesting that *S. thermophilus* used the peptides and amino acids produced by *L. delbrueckii*, as already observed in yogurt (Settachaimongkon et al., 2014). Accordingly, caseins appeared hydrolyzed only by the *L. delbrueckii* strain, with the presence of a new protein band that migrates around 20 kDa as soon as 8 h of single culture in milk and weakly in the coculture (Fig. 1C). By contrast, in soy milk, the initial content in peptides and free amino acids was higher than in milk, close to 5 mM. In soymilk, *L. delbrueckii* alone had no effect on the concentration of NH_2 groups. Contrastingly, S. *thermophilus* alone caused a drop in this concentration in soy milk, and so did the coculture. This is in agreement with the absence of growth of *L. delbrueckii* and the use of the NH_2 groups by at least *S. thermophilus*. Accordingly, no proteolytic fragment of soy proteins was detected by electrophoreses whatever the strains and the cultures used.

3.3. Cell morphology of L. delbrueckii subsp. bulgaricus in cow milk and in soy milk ultrafiltrates

To go deeper into the understanding of *L. delbrueckii* subsp. *bulgaricus* adaptation to cow milk versus soy milk, we then cultivated the yogurt starters in ultrafiltration permeates of these products. In order to compensate the absence of proteins as a nitrogen source, casein peptone and soy peptone were added to cow milk ultrafiltrate (MUF) and to soy milk ultrafiltrate (SUF), respectively. As shown in Fig. 3, *L. delbrueckii* subsp. *bulgaricus* grew in MUF (Fig. 3A), while limited growth was observed in SUF (Fig. 3B). Cultures were then examined after 20 h of incubation. DAPI staining and fluorescence microscopy revealed the typical morphology of dairy lactobacilli in MUF. Straight rods were observed, with a homogeneous blue fluorescence indicating the presence of DNA throughout the cell. Moreover, dots of intense yellow fluorescence indicated the presence of polyphosphate under the form of granules that seemed to be located at both ends of the lactobacilli cells (Fig. 3C). Accordingly, transmission electron microscopy revealed long and straight rods, with electron-dense inclusions close to lactobacilli ends (Fig. 3E). By contrast, DAPI staining revealed different morphologies in SUF. Long and curved rods appeared segmented in shorter segments (Fig. 3D). No polyphosphate was observed, while the DNA blue fluorescence was observed regularly distributed and compartmented within the long chains. Electron microscopy confirmed the presence of short bacilli comprised within long and curved chains (Fig. 3F). The amplitude AFM images of the cultures further confirmed that *L. delbrueckii* subsp. *bulgaricus*, when cultivated on MUF (Fig. 3G), produced longer cells than on SUF (Fig. 3H). In MUF, individuals could reach over 20 μm. Meanwhile, individuals were about 4–6 μm in soy ultrafiltrate and appeared to be linked to each other by capsular material that covered the gaps (up to ~1 μm) between two divided but unseparated neighbors.

3.4. Analysis of the cellular proteome

Such differences in the lactobacilli morphologies suggested that different environments may lead to differential modulation of the cellular composition. We therefore investigated modulation of the proteome. Electrophoretic analyses revealed major changes in the *L. delbrueckii* subsp. *bulgaricus* cellular proteome, depending on the growth medium, MUF or SUF. In particular, several major proteins were evidenced in MUF, yet not in SUF (Fig. 4A). In line with this, an analysis of lithium extracts confirmed major differences in terms of surface extractable proteins. In order to obtain further insight into proteomic readjustments induced by such media, whole-cell protein extracts were then analyzed by label-free proteomics using nano-LC-MS/MS. All the cellular proteins exhibiting a significantly different level ($p < 0.05$, ANOVA), depending on the growth medium, i.e. with a change in expression of $\geq$ 2-fold (log2 ratio $\geq$1.5), are listed in Table 2. As illustrated in Fig. 4B, a volcano plot was generated to visualize the whole set of differentially expressed proteins. A total of 185 proteins were found to be differentially expressed. More precisely, 75 were induced and 110 were repressed. The 185 affected proteins were found to belong to

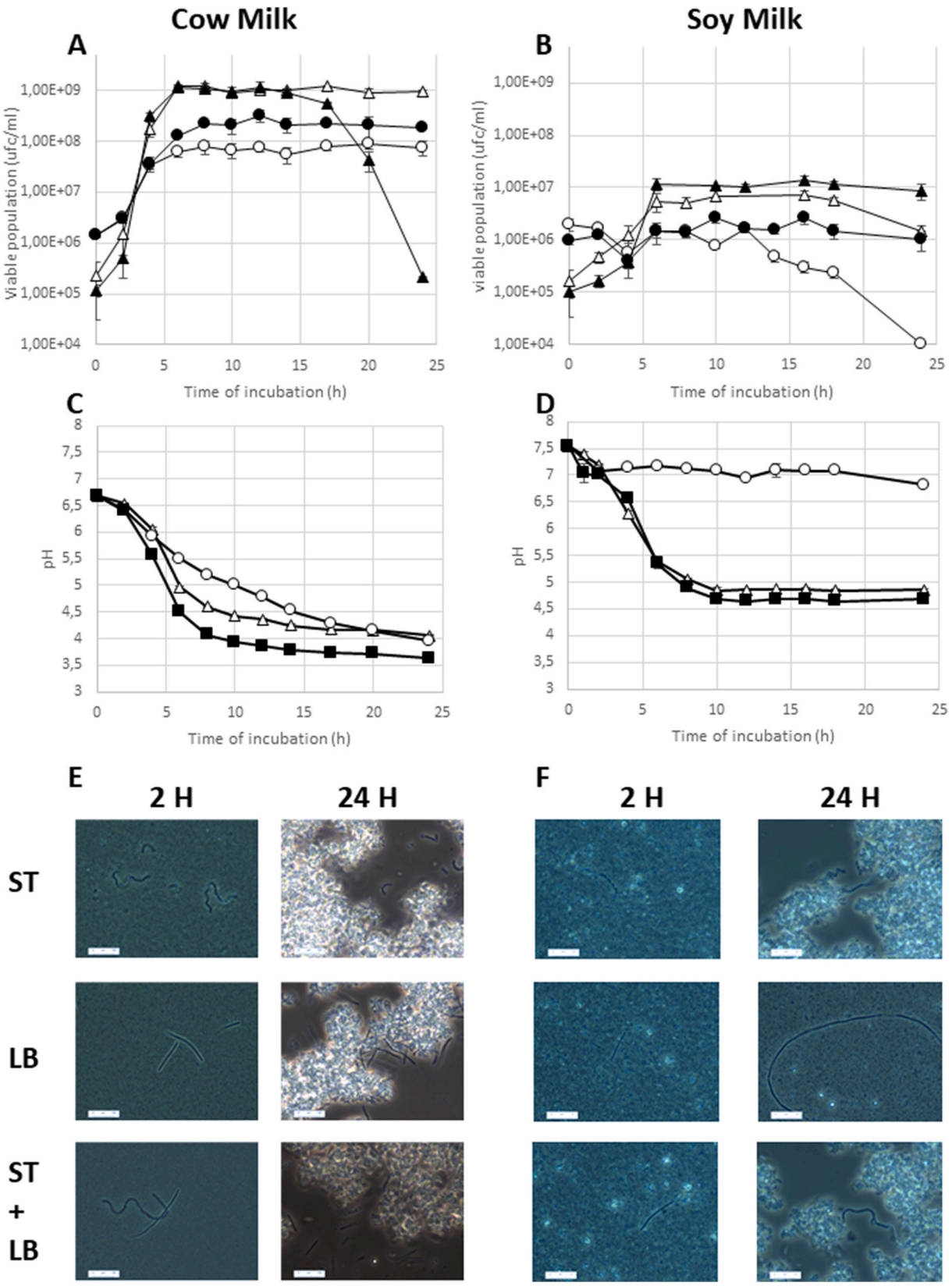

Fig. 1. Growth of yogurt starters *Streptococcus thermophilus* CIRMBIA1345 and *Lactobacillus delbrueckii* subsp. *bulgaricus* CIRMBIA1592 in cow milk and in soy milk. *L. bulgaricus* was cultivated in cow milk (A) or in soy milk (B), in pure culture (○), or in co-culture with *S. thermophilus* (●). *S. thermophilus* was cultivated in cow milk (A) or in soy milk (B), in pure culture (△), or in co-culture with *L. bulgaricus* (▲). Growth of lactobacilli and of streptococci was monitored by CFU counting on MRS and M17, respectively. Results shown are means (+/− SD) of 3 independent cultures. Acidification was monitored throughout incubation in *L. bulgaricus* cultures (○), in *S. thermophilus* cultures (△) or in co-cultures (■). E and F: during the course of incubation, wet-mount samples were examined using phase-contrast microscopy.

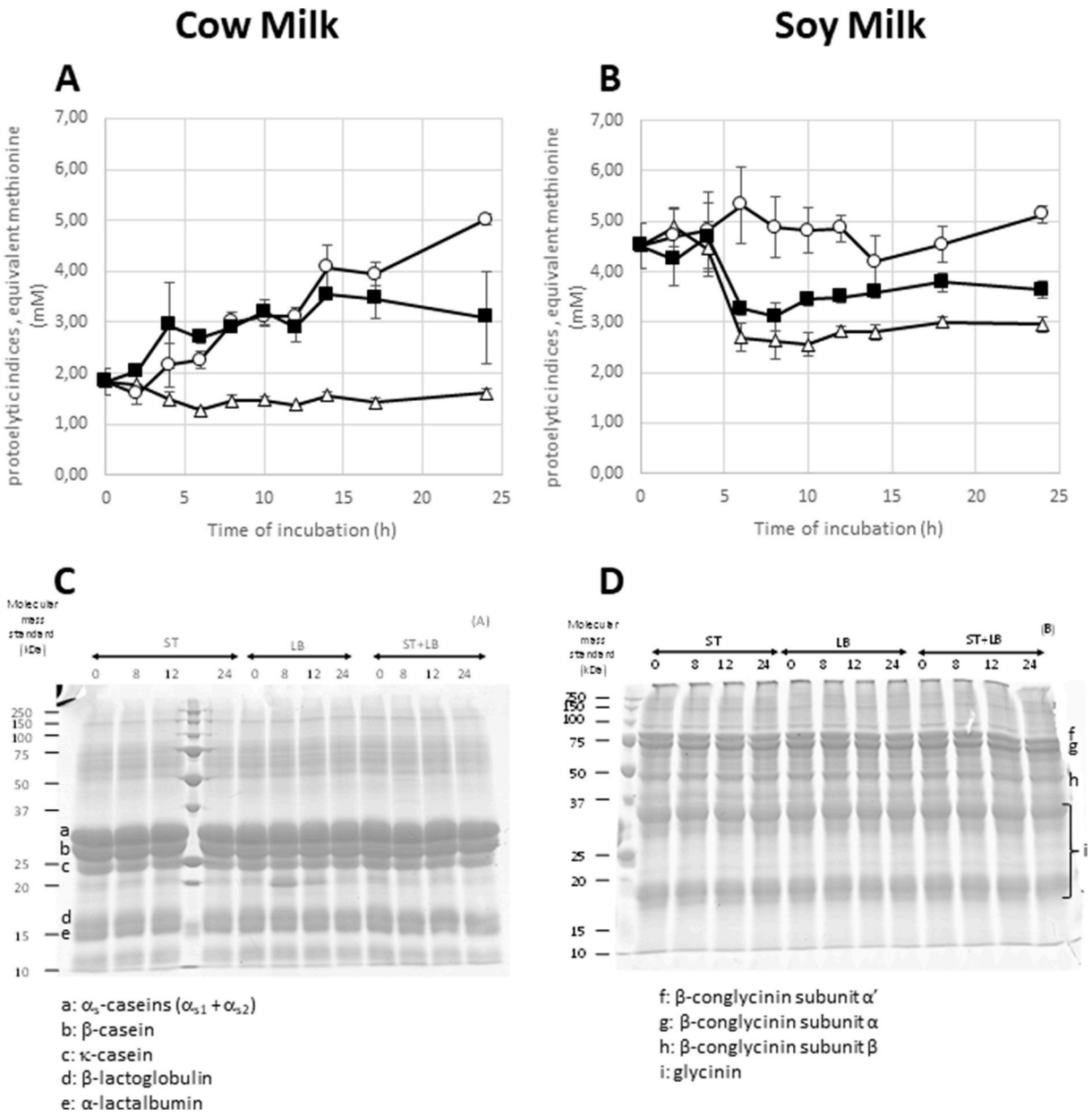

Fig. 2. Proteolysis of cow milk or of soy milk proteins by yogurt starters *Streptococcus thermophilus* CIRMBIA1345 and *Lactobacillus delbrueckii* subsp. *Bulgaricus* CIRMBIA1592. *L. bulgaricus* (○), or *S. thermophilus* cultures (△), or the combination thereof (■) were cultivated in cow milk (A) or in soy milk (B). Proteolysis was monitored using the OPA quantification of free amines (A, B). Results shown are means (+/− SD) of 3 independent cultures. Hydrolysis of proteins was further studied using SDS-PAGE electrophoresis (C, D).

various COG functional categories (Fig. 5).

Many affected proteins corresponded to metabolism categories (C, E, F, G) (Table 2). In the C category, energy production and conversion, 4 proteins were down-regulated, an inorganic pyrophosphatase, an ATP synthase, a phosphoenolpyruvate carboxylase, and a D-lactate dehydrogenase. In the E category, amino acid metabolism and transport, 6 proteins were down-regulated, Aminopeptidase G, His dipeptidase, Glutamine ABC transporter, Cysteine synthase, Aminotransferase and Threonine synthase. Down-regulated proteins also included 17 in F category, nucleotide metabolism and transport and 8 in G category, carbohydrate metabolism and transport. However, different proteins were also up-regulated in all these categories. Most striking modifications occurred in the translation J category, with 19 up-regulated and 33 down-regulated proteins. Moreover, proteins involved in cell cycle (D), in nucleotide metabolism (F), in replication and repair (L) and in envelop biogenesis (M) were also affected.

4. Discussion

Adaptation of *S. thermophilus* to the soy milk medium was recently investigated (Boulay et al., 2020). This study revealed that *S. thermophilus* can adapt and grow in soy milk, consuming sucrose, hydrolysing soy proteins and acidifying the medium down to pHs close to those reached in cow milk yogurt. However, to our knowledge, little is known about the adaptation of the other yogurt starter, *L. delbrueckii* subsp. *bulgaricus*, to soy milk environment. We therefore investigated such adaptation, either in the presence or in the absence of *S. thermophilus*.

In commercially available yogurt-type fermented soy milks, *S. thermophilus* was close to 10^8 CFU per gram of product, against significantly lower values down to 10^5 CFU/g for *L. delbrueckii* subsp. *bulgaricus*. Such a difference may be linked to a limited growth of *L. delbrueckii* subsp. *bulgaricus* in soy milk, or to a severe drop in its

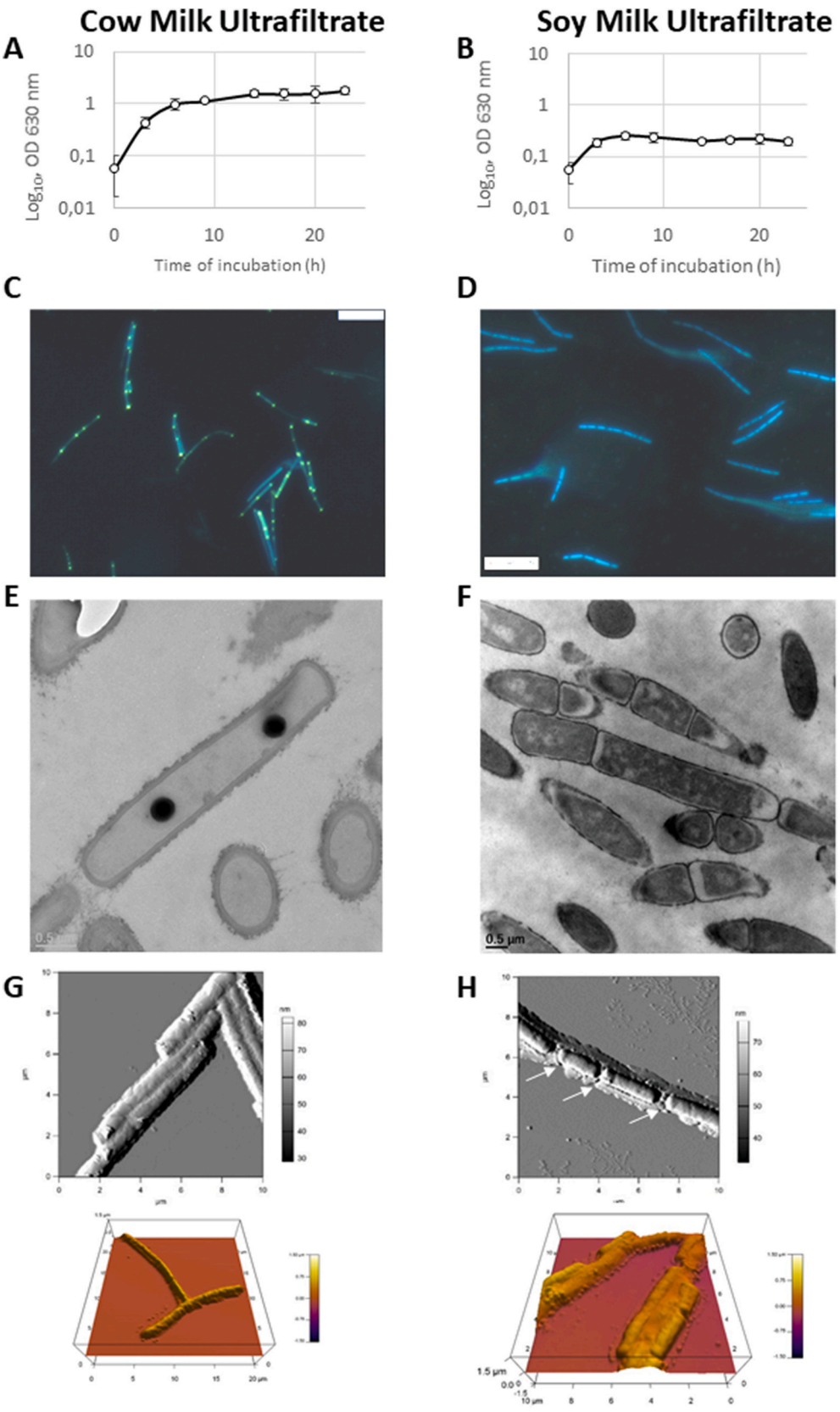

Fig. 3. Growth and morphology of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIRMBIA1592 in cow milk ultrafiltrate and in soy milk ultrafiltrate. *L. bulgaricus* was cultivated in the indicated ultrafiltrates. A and B: Growth was monitored by following the turbidity (OD at 650 nm). C and D: the presence of DNA (blue fluorescence, 475 nm) and of polyphosphate (green fluorescence, 525 nM) was assessed by epifluorescence microscopy after DAPI staining. E and F: lactobacilli were fixed, stained, and ultrathin sections observed using transmission electron microscopy. G and H: amplitude AFM images of lactobacilli grown on milk or soy ultrafiltrate, respectively, washed in HEPES-NaCl buffer then deposited onto mica and dried. Arrows indicate divided but unseparated cells connected with capsular material. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

viability after fermentation has occurred. We therefore investigated growth of both starters, either in single culture, or in cocultures, comparing both substrates, cow milk and soy milk. As expected, both starters grew in cow milk and acidified it to values usually found in yogurts, both in pure cultures and in cocultures. In this last case, growth of both starters in cow milk even led to acidification down to pH values close to 3.5, and to *S. thermophilus* cell death. In soy milk, growth of streptococci and acidification were favoured by the presence of

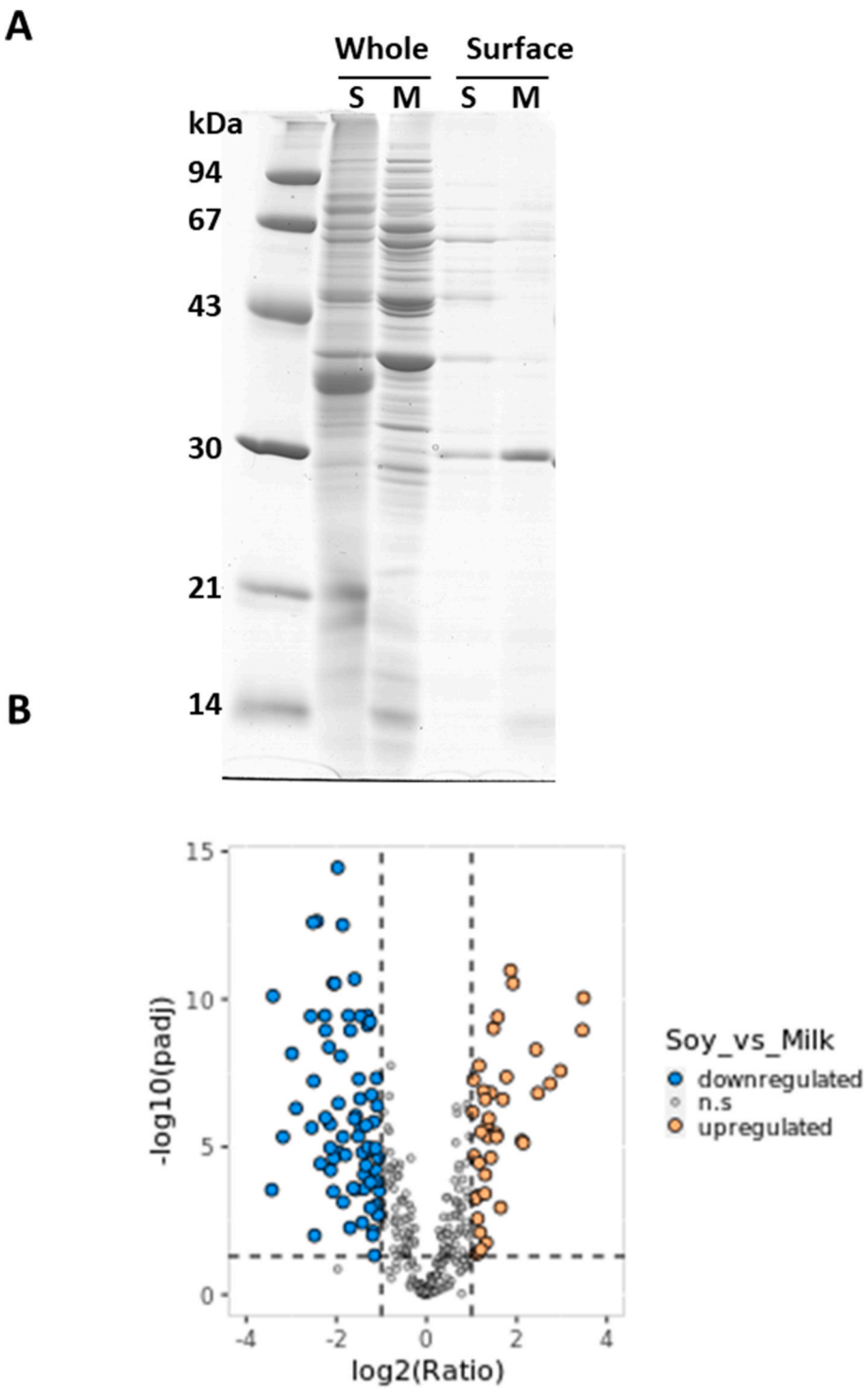

Fig. 4. Cellular proteome of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIRMBIA1592 in cow milk ultrafiltrate and in soy milk ultrafiltrate. *L. bulgaricus* was cultivated in the indicated ultrafiltrates (S: soy, M: milk). A: Whole-cell proteins SDS extracts and surface extractable proteins Lithium extracts were separated using SDS-PAGE. (B): Global cellular proteomes were analyzed by label-free proteomics using trypsinolysis and nano-LC-MS/MS. The Volcano plot shows a Log (2) fold change of the differentially expressed proteins of *L. bulgaricus* cultivated in SUF vs MUF. Orange (up-regulated proteins) and blue (down-regulated proteins) circles indicate proteins that were statistically different ($p \leq 0.05$) in terms of their abundance in soy milk and cow's milk by 2-fold or more. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lactobacilli. In soy milk, the coculture led to a comparable acidification to that recorded in cow milk, down to pH values close to 4.5. However, by contrast with cow milk, *L. delbrueckii* subsp. *bulgaricus* alone was unable to grow in and to acidify soy milk. Accordingly, we showed that it was able to hydrolyze dairy proteins, while not soy ones. This was confirmed by a production of peptides and free amino acids in cow milk, that can sustain the growth of both LAB strains, while only a decrease was observed in soy milk, suggesting the use of the peptides and free amino acids already present in soy milk prior to fermentation. Inability of the lactobacillus to use soy proteins, by contrast with the streptococcus, may explain the absence of growth of the lactobacillus alone, yet its limited growth in coculture with the streptococcus. Microscopic examination revealed that both starters grew in yogurt and presented their typical morphologies, while *L. delbrueckii* subsp. *bulgaricus* exhibited a deformed and curved morphology in soy milk. The absence of growth in soy milk could thus be caused by a lack of useable nitrogen source and/or by the limited availability of useable carbon source. Indeed, *L. delbrueckii* subsp. *bulgaricus* is known to use glucose and fructose (Chervaux et al., 2000), which are present in soy. However, the main sugar found in soy is sucrose, which *L. delbrueckii* does not use, so that the carbon source may also constitute a limiting factor.

To investigate L. *delbrueckii* subsp. *bulgaricus* adaptation further, we

Table 2
Proteins of different COG categories identified as having a significantly different abundance when bacteria were grown on soy vs milk.

COG C: « Energy production and conversion »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1GAN7	Putative fumarate reductase (Flavoprotein)	2,9	3,5E-06
Q1G882	Fumarate hydratase class II	2,2	1,8E-08
Q1GAA2	D-isomer specific 2-hydroxyacid dehydrogenase	1,7	2,3E-03
Q1GAB5	Probable manganese-dependent inorganic pyrophosphatase	0,6	2,9E-06
Q1GBD4	Phosphoenolpyruvate carboxylase	0,5	1,9E-03
Q1GAW4	ATP synthase epsilon chain	0,4	1,0E-03
P26297	D-lactate dehydrogenase	0,3	1,1E-09

COG D:« Cell cycle control and mitosis »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1GAT2	Cell division protein FtsZ	0,6	9,2E-05
Q1GA97	Cell cycle protein GpsB	0,6	3,4E-03
Q1G7Z5	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	0,4	7,1E-03

COG E: « Amino Acid metabolis and transport »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G9S0	Branched-chain amino acid aminotransferase	2,9	4,6E-06
Q1GBV9	Oligopeptide ABC transporter, ATP-binding protein	2,7	1,5E-07
Q1G8V8	Aminopeptidase C	2,6	4,6E-06
Q1GAP4	Peptide binding protein	2,0*	8,5E-03*
Q1G9K5	Oligopeptide ABC transporter, substrate binding protein	1,8	3,1E-04
Q1G7Z6	Pyruvate oxidase	1,8	7,7E-03
Q1GBC5	Uncharacterized protein	1,6	7,7E-04
Q1GBV8	Aminopeptidase G	0,9	6,9E-01
Q1G8U4	X-His dipeptidase	0,7	3,8E-05
Q1GBC0	Glutamine ABC transporter, ATP-binding protein	0,6	6,2E-05
Q1G9E6	Cysteine synthase	0,5	6,3E-05
Q1G9V8	Aminotransferase	0,4	3,6E-10
Q1G8T6	Threonine synthase	0,4	8,7E-05

COG F: « Nucleotide metabolism and transport »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G7W2	Adenosylcobalamin-dependent ribonucleoside-triphosphate reductase	6,7	7,2E-08
Q1GBJ7	Adenylate kinase	3,6	1,1E-11
Q1GAK1	Cytidylate kinase	2,1	2,0E-05
Q1G916	Bifunctional protein PyrR	1,9*	4,9E-02*
Q1G954	dITP/XTP pyrophosphatase	1,6	8,0E-04
Q1GBA7	Purine nucleosidase	1,6*	4,7E-02*
Q1G9N8	Uridylate kinase	0,6	3,5E-05
Q1GBV4	Adenylosuccinate synthetase	0,4	6,1E-10
Q1G923	Thymidylate kinase	0,4	2,3E-07
Q1GBA2	Hypoxanthine phosphoribosyltransferase	0,4*	4,7E-02*
Q1G9G3	Phosphoribosylamine–glycine ligase	0,4*	3,7E-10*
Q1G9L8	Putative secreted 5′-nucleotidase	0,3	3,6E-10
Q1GA94	Formate–tetrahydrofolate ligase	0,3	3,1E-13
Q1G9G1	Phosphoribosylglycinamide formyltransferase	0,3*	2,8E-04*
Q1G9F8	Phosphoribosylformylglycinamidine synthase subunit PurL	0,3*	3,5E-15*
Q1GBU8	GMP reductase	0,2	2,9E-11
Q1G9F4	Adenylosuccinate lyase	0,2	2,9E-11
Q1G9F3	N5-carboxyaminoimidazole ribonucleotide synthase	0,2	3,3E-04
Q1G9H8	Guanylate kinase	0,2	6,1E-05
Q1G9F2	N5-carboxyaminoimidazole ribonucleotide mutase	0,2	2,3E-13
Q1G9F5	Phosphoribosylaminoimidazole-succinocarboxamide synthase	0,2*	2,5E-13*
Q1G9F9	Amidophosphoribosyltransferase	0,2*	2,2E-06*
Q1G9G2	Bifunctional purine biosynthesis protein PurH	0,1	6,9E-09

COG G: « Carbohydrate metabolism and transport »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G8B9	PTS system, fructose-specific enzyme IIABC component	11,0	1,1E-09
Q1G7U9	Putative fructose-2,6-bisphosphatase	7,8	2,7E-08
P22733	Lactose permease	4,3	6,2E-06
Q1G9V5	Putative mutarotase	2,2	4,5E-04
Q1GBA5	Phosphoketolase	2,1*	5,7E-04*
Q1G8B8	Tagatose-6-phosphate kinase	1,4	2,8E-02
Q1G983	Fructose-bisphosphate aldolase	0,6	1,0E-04
Q1GB25	Triosephosphate isomerase	0,6	5,4E-05
Q1GAL0	Pyruvate kinase	0,6	1,5E-05
Q1GBA8	Ribose-5-phosphate isomerase A	0,6	1,7E-04
Q1GC14	*N*-acetylglucosamine-6-phosphate deacetylase	0,5	2,5E-03
Q1GB12	Phosphoglucosamine mutase	0,5	1,3E-05
Q1GBY6	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	0,5	4,7E-08
Q1G8T1	6-phosphofructokinase	0,2	1,7E-06

COG H: « Coenzyme metabolism »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G850	D-alanine–D-alanyl carrier protein ligase	1,8	1,2E-04
Q1GBG7	NH(3)-dependent NAD (+) synthetase	0,5	2,0E-03

COG I: « Lipid metabolism »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1GAH7	Acetyl-CoA acetyltransferase	2,6*	1,6E-06*
Q1G8V5	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	2,5*	1,7E-02*
Q1GBF2	Putative kinase	1,6	1,9E-03
Q1GAH5	Hydroxymethylglutaryl-coenzyme A synthase	0,6	4,9E-05
Q1GAF7	3-oxoacyl-[acyl-carrier-protein] synthase 2	0,3	2,1E-11
Q1GAF1	Enoyl-[acyl-carrier-protein] reductase [NADH]	0,3	4,6E-06
Q1GAF8	3-oxoacyl-[acyl-carrier-protein] reductase	0,2	3,6E-10

COG J: « Translation »

Accession	Description	Ratio Soy/Milk	adjusted p-value	COG Functional Category
Q1GAS8	Isoleucine–tRNA ligase	4,4	7,3E-06	**J**
Q1GBK6	50 S ribosomal protein L5	3,8	2,9E-11	**J**
Q1GAU9	Valine–tRNA ligase	3,4	4,3E-08	**J**
Q1GBQ0	50 S ribosomal protein L31 type B	3,2	2,5E-07	**J**
Q1GAK0	30 S ribosomal protein S1	3,0	4,0E-10	**J**
Q1GBK4	30 S ribosomal protein S8	2,8	9,8E-10	**J**
P54262	Asparagine–tRNA ligase	2,7	2,3E-05	**J**
Q1G905	50 S ribosomal protein L7/L12	2,4	1,3E-07	**J**
Q1GAX3	Threonylcarbamoyl-AMP synthase	2,2	3,5E-05	**J**
Q1GAG7	Aspartate–tRNA ligase	2,1*	4,2E-02*	**J**
Q1G9N6	30 S ribosomal protein S2	1,9	4,3E-06	**J**
Q1G9N7	Elongation factor Ts	1,8	3,5E-05	**J**
Q1G9L7	50 S ribosomal protein L19	1,7	1,1E-03	**J**
Q1G971	Leucine–tRNA ligase	1,7	1,2E-03	**J**
Q1GB46	Ribosome hibernation promoting factor	1,6	4,1E-05	**J**
Q1G9N9	Ribosome-recycling factor	1,6	5,8E-05	**J**
Q1G947	Alanine–tRNA ligase	1,6	4,6E-07	**J**
Q1GAG8	Histidine–tRNA ligase	1,6	6,3E-04	**J**
Q1G9A8	Threonine–tRNA ligase	1,6	2,0E-02	**J**
Q1GBL9	30 S ribosomal protein S10	0,7	2,9E-02	**J**
Q1GBR4	Methionine–tRNA ligase	0,6	5,4E-05	**J**
Q1GAV4	30 S ribosomal protein S4	0,6	1,1E-02	**J**
Q1G9V1	Methylenetetrahydrofolate–tRNA-(uracil-5-)-methyltransferase TrmFO	0,6	4,5E-02	**J**
Q1GBK1	30 S ribosomal protein S5	0,6	2,4E-03	**J**
Q1GC37	30 S ribosomal protein S6	0,6	7,3E-03	**J**
Q1GBJ1	50 S ribosomal protein L17	0,6	2,9E-02	**J**
Q1G9P9	Translation initiation factor IF-2	0,6	1,8E-08	**J**
Q1GBK7	50 S ribosomal protein L24	0,5	2,4E-04	**J**
Q1G8N2	Serine–tRNA ligase	0,5	6,7E-07	**J**
Q1GBJ4	30 S ribosomal protein S13	0,5	4,7E-03	**J**
Q1G8K9	Elongation factor P	0,5	3,1E-04	**J**
Q1G999	Phenylalanine–tRNA ligase beta subunit	0,5	1,4E-04	**J**
Q1GBJ9	50 S ribosomal protein L15	0,5	8,8E-04	**J**
Q1GBL3	50 S ribosomal protein L22	0,5	2,0E-03	**J**
Q1GAN1	Arginine–tRNA ligase	0,5	1,6E-04	J
Q1GAX5	Peptide chain release factor 1	0,4	1,7E-07	**J**
Q1GBL7	50 S ribosomal protein L4	0,4	1,2E-03	**J**
Q1GBI5	30 S ribosomal protein S9	0,4	1,4E-05	**J**
Q1GBL0	50 S ribosomal protein L29	0,4	4,2E-05	**J**
Q1G8Z0	Cysteine–tRNA ligase	0,4	2,6E-04	**J**
Q1G9W2	30 S ribosomal protein S21	0,4	3,7E-03	**J**
Q1G9L2	30 S ribosomal protein S16	0,4	2,2E-06	**J**
Q1GBK9	30 S ribosomal protein S17	0,4	4,4E-06	**J**
Q1G7Z1	50 S ribosomal protein L34	0,4*	9,7E-03*	**J**
Q1G9D2	50 S ribosomal protein L33 1	0,3	8,7E-07	**J**
Q1G9G6	50 S ribosomal protein L27	0,3	1,1E-06	**J**
Q1GBK0	50 S ribosomal protein L30	0,3	2,5E-04	**J**
Q1GC35	30 S ribosomal protein S18	0,3	1,9E-05	**J**
Q1GBM2	30 S ribosomal protein S12	0,3	1,5E-05	**J**
Q1GAQ4	30 S ribosomal protein S20	0,2	1,0E-06	**J**
Q1G9J5	50 S ribosomal protein L28	0,2	3,6E-05	**J**
Q1G9B3	50 S ribosomal protein L35	0,1	5,0E-07	**J**

COG K: « Transcription »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G960	Catabolite control protein A (CcpA)	2,5	8,7E-05
Q1G9C9	Transcription elongation factor GreA	2,2	2,7E-03
Q1G9P7	Uncharacterized protein	2,2*	3,7E-02*

(*continued on next page*)

Table 2 (*continued*)

COG K: « Transcription »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1GBM5	DNA-directed RNA polymerase subunit beta	2,1	5,3E-08
Q1G9A5	Transcriptional repressor NrdR	1,9	5,4E-07
Q1GBJ2	DNA-directed RNA polymerase subunit alpha	1,9	3,6E-07
Q1G8Z7	Transcription termination/antitermination protein NusG	1,8	3,4E-03
Q1GBD6	Transcriptional regulator (AsnC family)	0,6	1,2E-04
Q1GBH3	Transcriptional regulator (GntR family)	0,6	1,9E-07
Q1G9P6	Transcription termination/antitermination protein NusA	0,5	7,4E-04
Q1GAZ3	Probable transcriptional regulatory protein Ldb0677	0,5	4,0E-07
Q1GAJ5	Transcriptional regulator (LysR family)	0,4	1,6E-04
Q1GBV5	Transcription elongation factor GreA	0,1*	2,8E-04*

COG L: « Replication and repair »

Accession	Description	Ratio Soy/Milk	adjusted p-value	COG Functional Category
Q1GC38	DNA gyrase subunit A	0,5	2,3E-05	**L**
Q1GC32	Replicative DNA helicase	0,2*	9,9E-03*	**L**

COG M: « Cell wall/membrane/envelop biogenesis »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1GBD5	Glutamine–fructose-6-phosphate aminotransferase [isomerizing]	11,2	9,0E-11
Q1G7Y4	Uncharacterized protein	5,5	1,5E-07
Q1G8D8	Uncharacterized protein	3,1*	1,1E-03
Q1GBC7	Glutamate racemase	2,6	1,1E-06
Q1GB91	UDP-*N*-acetylglucosamine 2-epimerase	1,7*	3,4E-02
Q1G7Q5	D-alanine–D-alanine ligase	1,6	9,0E-03
Q1GBP6	Alanine racemase	1,3	4,5E-02
Q1G8D6	Uncharacterized protein	0,8	3,4E-01
Q1G8B5	UDP-*N*-acetylmuramyl-tripeptide synthetase	0,4	1,5E-05

COG NA: « Not found »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G855	Uncharacterized protein	1,5	2,1E-03
Q1G870	Uncharacterized protein	0,3*	5,5E-03
Q1G7Y6	Uncharacterized protein	0,2	2,4E-05
Q1GC03	Uncharacterized protein	0,2	5,9E-08
Q1G8Z4	Uncharacterized protein	0,1	4,6E-06

COG O: « Post-translational modification, protein turnover, chaperone functions »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G989	Foldase protein PrsA	2,5	3,8E-04
Q1GBM8	ATP-dependent Clp protease, ATP-binding subunit	2,3*	7,9E-03
Q1GB31	ATP-dependent Clp protease proteolytic subunit	2,0	6,7E-07
Q1G933	tRNA N6-adenosine threonylcarbamoyltransferase	1,7	6,7E-03
Q1G9R3	Chaperone protein DnaJ	1,6	2,5E-04
Q1GA52	Peptidyl-prolyl *cis*-trans isomerase	1,6	7,1E-06
Q1G998	Thioredoxin	0,6	2,6E-04
Q1G936	10 kDa chaperonin	0,4	1,4E-06
Q1G937	60 kDa chaperonin	0,4	1,0E-05
Q1GB39	Thioredoxin reductase	0,4	7,7E-10
Q1G931	Putative peptidase	0,3	3,3E-07
Q1G9R1	Protein GrpE	0,2	4,2E-09

COG P:« Inorganic ion transport and metabolism »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1GBG6	Cation transporting P-type ATPase (Probable Ca2+ transporter)	2,3*	2,9E-02*
Q1GC09	Phosphonate ABC transporter, substrate binding protein	0,5	2,2E-06
Q1G9X4	Cation transporting P-type ATPase (Probable copper transporter)	0,5	8,4E-05

COG S: « Function Unknown »

Accession	Description	Ratio Soy/Milk	adjusted p-value
Q1G920	Nucleoid-associated protein Ldb1634	5,4	5,0E-09
Q1GAP7	Probable GTP-binding protein EngB	2,3	3,1E-06
Q1G832	Putative lipoprotein	1,6	7,2E-07
Q1G9J6	Uncharacterized protein	1,5	1,3E-04
Q1GB33	Putative gluconeogenesis factor	0,6	3,1E-03
Q8KH12	GTPase Der	0,6	1,2E-03
Q1GBW8	Uncharacterized protein	0,5	1,1E-05
Q1G838	Putative lipoprotein	0,4	5,8E-10
Q1G978	Uncharacterized protein	0,4	1,9E-06
Q1G9J7	Putative kinase	0,4	5,0E-08
Q1GB58	Uncharacterized protein	0,3	7,3E-04

(*continued on next page*)

Table 2 (*continued*)

COG S: « Function Unknown »			
Accession	**Description**	**Ratio Soy/Milk**	**adjusted p-value**
Q1G824	Putative lipoprotein	0,3	8,4E-09
Q1GAR5	UPF0356 protein Ldb0761	0,2	1,1E-05
Q1G7U2	4-oxalocrotonate tautomerase	0,2	3,8E-10
Q1GA88	UPF0210 protein Ldb1026	0,1	7,8E-11
COG T			
Accession	**Description**	**Ratio Soy/Milk**	**adjusted p-value**
Q1GAV8	Universal stress protein	2,5	2,5E-07
Q1G7T1	Two-component system, response regulator	1,9	1,0E-02
Q1GAR3	GTP-binding protein TypA	1,9	5,2E-04
Q1GC51	S-ribosylhomocysteine lyase	0,2	1,1E-09
COG U: « Intracellular trafficing and secretion »			
Accession	**Description**	**Ratio Soy/Milk**	**adjusted p-value**
Q1G9K9	Signal recognition particle receptor FtsY	1,7	5,3E-03

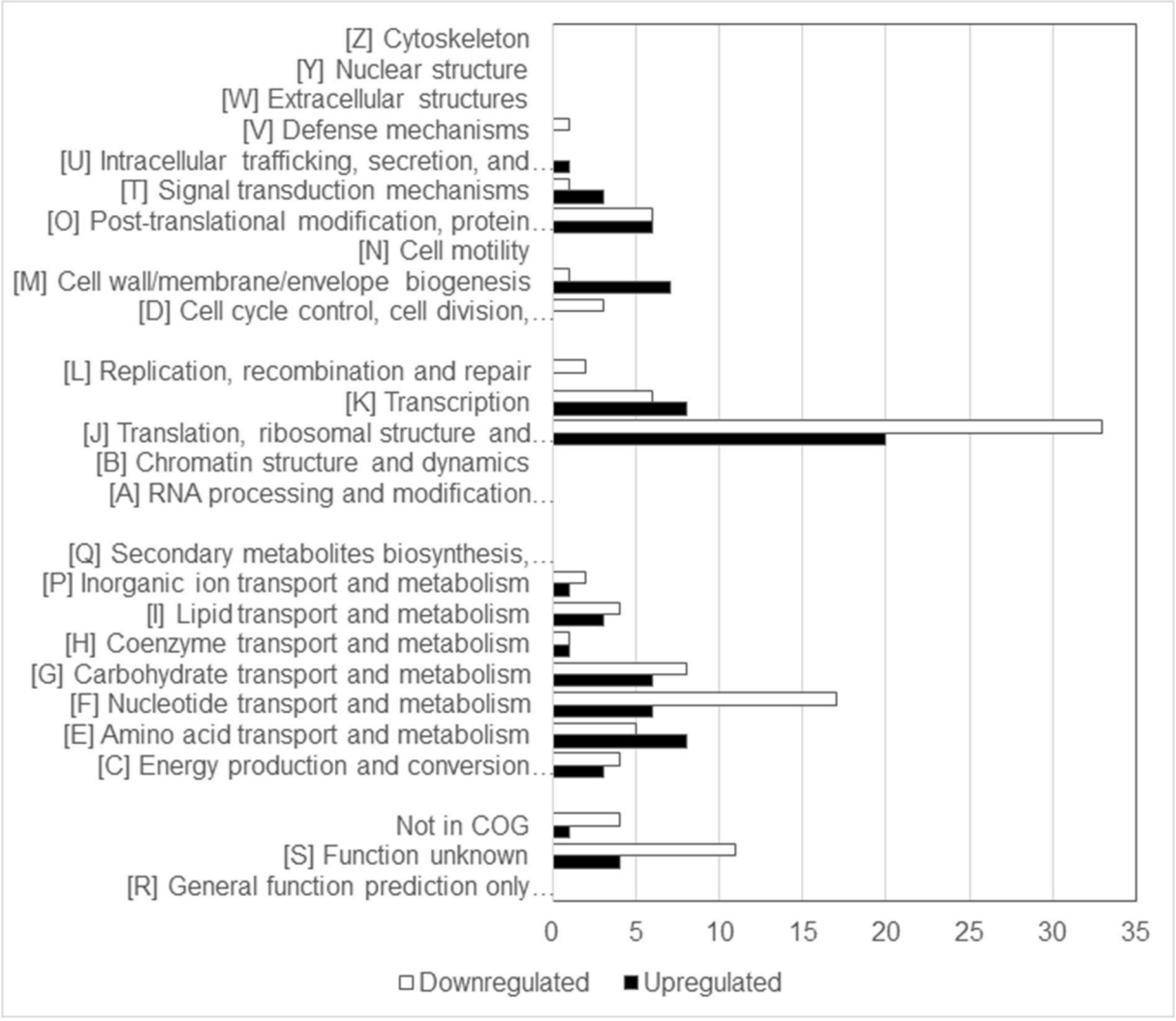

Fig. 5. Breakdown of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIRMBIA1592 differential proteins in biological processes. *L. bulgaricus* was cultivated in both ultrafiltrates (soy, milk). Proteins upregulated in soy are presented in black, proteins downregulated in soy in white. The functional distribution and biological processes were predicted based on the functional classifications of the COG database.

used cow milk (MUF) and soy milk (SUF) ultrafiltrates, which we supplemented with casein peptone and soy peptone, respectively. Growth occurred in MUF, while very limited growth was observed in SUF, although nitrogen was provided under the form of peptone. Fluorescence microscopy revealed the typical morphology of *L. bulgaricus*, with long and straight rods, in MUF. By contrast, the morphology appeared more segmented in SUF with smaller rods, but assembled in chains, suggesting an uncompleted cell division process.

DAPI staining also revealed the typical yellow fluorescence indicating the presence of accumulated polyphosphate inclusion in MUF (Alcantara et al., 2014), but not in SUF. This was confirmed by transmission electron microscopy, which revealed long rods with electron-dense inclusions in MUF cultures, yet segmented rods, without such inclusions, in SUF cultures. Polyphosphate accumulation under the form of granules has been reported for several lactobacilli (Alcantara et al., 2014). This accumulation depends on the availability of inorganic phosphate, and on the presence of the polyphosphate kinase (*ppk*) gene. It was shown in different media, including in cheese whey, for another lactobacillus species, *L. casei* (Huang et al., 2018b). It reflects adaptation to the medium and it is involved in enhanced stress tolerance as shown in *L. rhamnosus* (Correa Correa Deza et al., 2017), while disruption of the *ppk* gene leads to reduced survival (Brown and Kornberg, 2008). It recently gained further interest as lactobacilli polyphosphates were shown to participate in the maintenance of the gut barrier function (Segawa et al., 2011; Tanaka et al., 2015; Saiki et al., 2016) and in the prevention of inflammatory response (Isozaki et al., 2021). To our knowledge, this is the first report of polyphosphate accumulation in *L. delbrueckii* subsp. *bulgaricus*, although this bacterium is known to possess the *ppx* and *ppk* genes that are involved in the turnover of polyphosphate (van de Guchte et al., 2006). Atomic force microscopy further confirmed the unusual morphology of *L. delbrueckii* subsp. *bulgaricus* in soy environment, with segmented rods connected together at both their ends, as if uncompleted cellular division failed to split extracellular material and left remnant junctions between the daughter bacteria.

These observations suggest that *L. delbrueckii* subsp. *bulgaricus* adapted well to the dairy environment, while it was subjected to major stress in the soy one. To investigate this further, we performed a differential proteomic investigation of the bacterium cultivated in both media. An electrophoretic analysis evidenced major differences in the whole proteome between soy and cow milk as well as in the surface proteome extracted by lithium for which higher numbers of bands in soy was observed. This was confirmed when we further identified modulated proteins using a whole-cell proteomic mass spectrometry analysis. Changing the growth medium, i.e. MUF versus SUF, actually caused major changes in proteins involved in energy production and conversion, in amino acid transport and metabolism, in carbohydrate transport and metabolism, and in cell cycle and division. As an example, growth in soy induced proteins involved in fumarate metabolism (fumarate reductase, fumarate hydratase), as well as proteins involved in amino acid transport and metabolism; such as branched-chain amino acid aminotransferase, oligopeptide transporter and a peptide binding protein. This may reflect the different nitrogen sources present in both media and the need of the lactobacilli of specific amino acids to sustain its growth due to its high auxotrophy, and notably on branched chain amino acids (Hébert et al., 2004). In accordance, proteins involved in amino acids transport and synthesis were more expressed in the dairy medium. Moreover, major modulations also occurred in proteins involved in carbohydrate transport and metabolism. In soy, a fructose-specific phospho-transferase system was induced, and so was fructose-2,6-bisphosphatase, in accordance with the presence of fructose in soy. By contrast, 6-phosphofructokinase, a key actor of the glycolytic pathway, was more abundant in the dairy medium culture.

Major changes concerned proteins involved in translation, including ribosomal proteins and aminoacid-tRNA ligases. A series of ribosomal proteins was induced in soy. The induced ribosomal protein L5, encoded by *rplE*, is essential for cell viability and L31 was identified as a heat-shock protein in *L. plantarum* (De Angelis et al., 2004). S1 was described as a RNA chaperone (Duval et al., 2013), while S8, encoded by *rpsH*, was involved in quinoxaline resistance in *Escherichia coli* (Guo et al., 2012), and L7/L12 in antibiotic resistance and in cold acclimation (Wu et al., 2008; Nabu et al., 2014). Induced 30 S ribosomal protein S2 was also identified as a multi-stress response protein in *L. kefiranofaciens* (Chen et al., 2017).

Accordingly, several proteins involved in stress response were upregulated in the soy environment. This includes the catabolite control protein CcpA, involved in stress response in *L. plantarum* (Chen et al., 2021) and in *L. bulgaricus* (Zhang et al., 2020). The GreA transcription factor aids adaptation to stressful environments in various bacteria (Feng et al., 2020). In this COG category K, by contrast, several proteins involved in transcription regulation and elongation were more expressed in the dairy environment, and so were proteins of the L category involved in replication, in accordance with better growth. Stressing soy conditions are further evidenced by overexpression of typical stress proteins involved in protein turnover, such as foldase prsA, ATP-dependent Clp protease and Chaperone DnaJ. This is consistent with overexpression of actors of stress response, such as universal stress protein, of a two-component system response regulator and of GTP-binding protein TypA in the signal transduction U category.

Interestingly, proteins involved in cell cycle and divison, such as cell division protein, FtsZ, cell cycle proteins GpsB and MnmG were repressed in soy. So was the UDP-*N*-acetylmuramyl-tripeptide synthetase MurE involved in peptidoglycan biosynthesis and cell wall biogenesis. This is consistent with the drastic modifications of bacterial morphology, with segmented rods and with remnant extracellular junctions between individual neighbors, which suggests uncompleted cell division.

As a conclusion, the soy environment was shown here to be far from optimal for the growth of the yogurt starter *L. delbrueckii* subsp. *bulgaricus*. Limited growth was observed and morphological and proteomic symptoms indicate that a severe stress took place. This is consistent with the limited population of lactobacilli encountered in yogurt-type fermented soy milks. The development of new fermented products, based on soy milk, may thus require different microbial starters more adapted to vegetable matrices. Different strains of *L. delbrueckii*, or of other lactobacilli species, may open new perspectives in this aim, and so may stress adaptation prior to use of these starters in soy fermentation.

Authors contribution statement

Florian Tarnaud, Fanny Canon, Fanny Guyomar'h, Julien Jardin and Valérie Briard -Bion performed the experiments. Gwénaël Jan and Valérie Gagnaire designed the experiments, performed some experiments and supervised the work. Fillipe Rosa do Carmo and Nassima Illikoud processed the data and designed the figures and tables. All authors participated in writing the paper.

Conflict of interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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