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Streptococcus thermophilus growth in soya milk: Sucrose consumption, nitrogen metabolism, soya protein hydrolysis and role of the cell-wall protease PrtS

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ABSTRACT

Societal demand for plant-based foods is increasing. In this context, soya products fermented using lactic acid bacteria (LAB) are appealing because of their potential health and nutritional benefits. The thermophilic LAB *Streptococcus thermophilus* is an essential starter species in the dairy industry. However, while its physiology is well characterized, little is known about its general metabolic activity or its techno-functional properties when it is grown in soya milk. In this study, *S. thermophilus* LMD-9 growth, sugar production, and lactic acid production in soya milk versus cow's milk were measured. Additionally, the main metabolic pathways used by the bacterium when growing in soya milk were characterized using a proteomic approach. *Streptococcus thermophilus* LMD-9 growth decreased soya milk pH, from 7.5 to 4.9, in 5 h. During fermentation, acidification thus occurred in tandem with lactate production and increasing population size (final population: 1.0×10^9 CFU/ml). As growth proceeded, sucrose was consumed, and fructose was produced. The proteomic analysis (LC-MS/MS) of the strain's cytosolic and cell envelope-associated proteins revealed that proteins related to amino acid transport and nitrogen metabolism were the most common among the 328 proteins identified (63/328 = 19.2% of total proteins). The cell-wall protease PrtS was present, and an LMD-9 deletion mutant was constructed by interrupting the *prtS* gene (STER_RS04165 locus). Acidification levels, growth levels, and final population size were lower in the soya milk cultures when the Δ*prtS* strain versus the wild-type (wt) strain was used. The SDS-PAGE profile of the soluble proteins in the supernatant indicated that soya milk proteins were less hydrolyzed by the Δ*prtS* strain than by the wt strain. It was discovered that *S. thermophilus* can grow in soya milk by consuming sucrose, can hydrolyze soya proteins, and can produce acidification levels comparable to those in cow's milk. This study comprehensively examined the proteomics of *S. thermophilus* grown in soya milk and demonstrated that the cell-wall protease PrtS is involved in the LAB's growth in soya milk and in the proteolysis of soya proteins, which are two novel findings. These results clarify how *S. thermophilus* adapts to soya milk and can help inform efforts to develop new fermented plant-based foods with better-characterized biochemical and microbiological traits.

1. Introduction

By 2030, the world population will reach 8.4 billion, and providing enough food for everyone will be challenging. However, one step towards this goal is incorporating more vegetables into our diets. The development of plant-based foods is driven by consumer concerns about health, ethics, lifestyle choices, dietary issues, and sustainability. Societal demand for new fermented plant-based products has increased; in Europe in particular, efforts are directed towards creating and diversifying alternatives to meat and dairy products (e.g., as part of the growing flexitarianism and veganism movements). Legumes could contribute to this process because of their agroecological and nutritional properties. Because of their symbiotic relationships with nitrogen-fixing bacteria (i.e., rhizobia), legumes can produce high-quality biomass without the need for large amounts of nitrogen fertilizer. Soya is a legume that has long been consumed in Asia and Africa and that has become a major plant-based alternative to meat and dairy in the Western world (Mäkinen et al., 2016), notably because of its nutritional properties. It is rich in high-quality proteins that are easily digested and that represent up to 40% of total dry matter (Mäkinen et al., 2016). It also has high levels of unsaturated fatty acids (Sarkar, 2006), dietary fibers, bioactive compounds (e.g., peptides, vitamins), and certain

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minerals (Ca, Fe, Mg, Zn; Sandberg, 2002); at the same time, its levels of saturated fatty acids are low (Sarkar, 2006). In addition, because they contain no lactose, soya-based products can serve as alternatives to dairy-based products for people who suffer from lactose intolerance (Hajirostamloo, 2009). However, like other legumes, soya has some disadvantages. It is rich in poorly digested or undigestible oligosaccharides (mainly stachyose and raffinose), whose fermentation can lead to digestive discomfort (e.g., flatulence, diarrhea). People from Western populations often perceive soya as having undesirable off-flavors because of its beany/green/grassy notes (Buono et al., 1990; Favaro Trindade et al., 2001; Lee et al., 1990). Soya also contains some anti-nutritional factors (e.g., anti-trypsic factors, phytic acid, polyphenols) as well as isoflavones, which are phytoestrogens whose effect on human health is still a subject of debate (Patisaul and Jefferson, 2010).

Among the numerous plant-based fermented products that exist worldwide (> 5000; Tamang and Kailasapathy, 2010), fermented soya products predominate. Soya products fermented by lactic acid bacteria (LAB) are appealing because of their potential health and nutritional benefits (Mäkinen et al., 2016; Tangyu et al., 2019; Vij et al., 2011). The technical, nutritional, and functional properties of plant matrices following fermentation are determined by the growth and metabolic activities of the microorganisms present. When growing in soya, LABs consume carbohydrates and subsequently produce acid, which leads to gel formation and, in turn, contributes to overall product aroma. Another result is that the fermented product is protected from the development of undesirable or pathogenic microorganisms. The bacteria's proteolytic enzymes hydrolyze proteins in the soya matrix, leading to changes in texture and to the production of free amino acids (Ghosh et al., 2013). Proteolysis also improves protein digestibility and bioaccessibility, creating a potential source of bioactive peptides (with immunomodulatory, antihypertensive properties mediated via ACE inhibition). LAB can degrade the indigestible oligo/α-galactosides found in soya milk (Donkor et al., 2007; Garro et al., 1998; Leblanc et al., 2004; Mital and Steinkraus, 1975; Wang et al., 2003), and some LAB can transform isoflavone glucosides (e.g., soya phytoestrogens) into their respective bioactive isoflavone aglycones (Chien et al., 2006; Hati et al., 2015; Kano et al., 2006; Pham and Shah, 2009; Wei et al., 2007; Zhao and Shah, 2014), which are better absorbed by the gut. LAB fermentation of soya can also reduce the abundance of some antinutritional factors such as phytic acids, which chelate divalent metals and thus limit their bioavailability (Lai et al., 2013; Rekha and Vijayalakshmi, 2011; Rui et al., 2016). The consumption of fermented soya milk has also been found to have a beneficial influence on fecal microbiota in animals (Ara et al., 2001; Butteiger et al., 2016; Liu et al., 2006) and humans (Cheng et al., 2005). Finally, LAB fermentation can improve the flavor of soya milk (Pinthong et al., 1980), notably by reducing the beany note (Favaro Trindade et al., 2001; Mital and Steinkraus, 1976; Wang et al., 1974).

The lactic acid bacterium *Streptococcus thermophilus* (*S. thermophilus*) is an essential starter species in the dairy industry and is notably important in the production of yogurt, mozzarella, and hard cheeses. Its behavior and physiology in cow's milk are well characterized, as are its techno-functional properties (acidification, effect on texture and flavor) in dairy products. Soya milk (i.e., the liquid obtained after soaking and grinding up soybeans) is inexpensive and has been used for years as a culture medium for LAB. A recent study showed that, among the 276 LAB strains tested, *S. thermophilus* most effectively acidified soya milk (Harlé et al., 2020).

Here, we characterized the growth and production of certain sugars and organic acids by *S. thermophilus* cultures grown in soya milk; cultures grown in cow's milk were used as a standard of reference. The objective was to better understand the behavior and physiology of *S. thermophilus* in soya milk. This study also identified the strain's important/limiting metabolites in soya milk and established the first overall proteomic profile for *S. thermophilus* grown in soya milk.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two bacterial strains were used: the *S. thermophilus* wild-type (wt) strain LMD-9 and its negative mutant for the cell-wall protease PrtS (Δ*prtS*, EryR; see below). The strains were grown in soya milk (Sojasun containing calcium and vitamin D; Triballat-Noyal, Noyal-sur-Vilaine, France) and in reconstituted cow's milk (nonfat dried milk, M7409; Sigma Aldrich).

Frozen stock culture of *S. thermophilus* LMD-9 was grown in the two media as follows. The first pre-culture was created over the course of a day: an isolated colony of an overnight culture grown on M17Lac was then grown at 37 °C in M17 broth (Difco) supplemented with lactose (10 g/l). The second pre-culture was created by inoculating 5 ml of M17 sucrose (10 g/l) with the first pre-culture (1%) and carrying out incubation at 37 °C overnight. The final cultures were generated by inoculating soya milk or cow's milk with the second preculture (OD_{600nm} 0.05) and carrying out incubation at 37 °C. Culturing was ended when the pH reached 5.8 for the soya milk (2.5 h) and 5.3 for the cow's milk (3 h); the cultures were then frozen in liquid nitrogen and stored at −80 °C. Chain disruption in the stock cultures was performed using a mechanical blender (Turax X620, Labo-Moderne, France) that was run for 40 s, and the resulting dilutions were then plated on M17Lac (1%) agar. Enumeration took place after 16 h of incubation at 42 °C under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany). Despite the 10 months of storage at −80 °C, the matrices protected *S. thermophilus* against freezing, and the cultures were 100% viable in both media (reaching 2×10^8 CFU/ml in soya milk and 8×10^8 CFU/ml in cow's milk).

Experimental cultures were created, in triplicate, by inoculating soya milk or cow's milk with the appropriate frozen stock culture (1×10^6 CFU/ml), and incubation was carried out at 37 °C. Acidification was tracked by measuring pH, and growth was quantified via enumeration following culturing on M17 Lac agar, as described above. The apparent growth rate (μ_{max}) was defined as the maximum slope of the semi-logarithmic growth curves established from population size (the CFU/ml counts).

2.2. Construction of the ΔprtS mutant

The negative mutant of the *S. thermophilus* LMD-9 strain, Δ*prtS*, was constructed via gene interruption using an erythromycin (Ery) resistance cassette and two additional PCR fragments. These three fragments were generated using PCR (TaqPhusion polymerase, 1 min elongation, 56 °C) and the primers ErmF (CCAAGGAGCTAAAGAGGT CCC) and ErmR (GGAGATAAGACGGTTCGTGTTCG) for the Ery resistance cassette; RG254 (TGGTAAGCACGTAGACC) and RG259 (CTA CTGACAGCTTCCAAGGAGCTAAAGAGGTCCAGGCTTGTCAATTCAT CTG) for the upstream fragment; and RG257 (CGTATGCTTACCAACA GAG) and RG260 (GCAAGTCAGCACGAACACGAACCGTCTTATCTCCG AAAGCCAACTTAGATGG) for the downstream fragment. The 3′ end of the upstream fragment contained a sequence complementary to the 5′ end of the Ery resistance cassette, whereas the 5′ end of the downstream fragment contained a sequence complementary to the 3′ end of the cassette. The three fragments (upstream, resistance cassette, and downstream) were joined via PCR (3 min elongation) using the primers RG254 and RG257 (TaqPhusion polymerase, activation at 98 °C for 3 min, 35 cycles of 30 s at 97 °C, 30 s at 55 °C, and 4 min at 72 °C). After purification with a Promega column, 10 µl of the resulting combination of fragments was used to transform natural competent cells of the wt *S. thermophilus* LMD-9 strain, as described in Gardan et al. (2009). Transformants were selected on M17Lac plates containing erythromycin (5 µg/ml); they were verified using PCR, and the flanking regions were sequenced to ensure that no unwanted mutations had been introduced.

2.3. Quantification of sugar and lactate concentrations

Bacteria were removed from the soya milk and cow's milk cultures at 0, 2, 2.5, and 3 h after culturing was initiated. Samples were centrifuged at 5000 ×*g* at 4 °C for 10 min to obtain the supernatants. The supernatants were then clarified with Carrez reagent: 100 µl of culture supernatant was mixed with 50 µl of Carrez I reagent (3.6% tetrapotassium hexacyanoferrate, w/v), 50 µl of Carrez II reagent (7.2% zinc sulfate, w/v), 100 µl of NaOH (0.1 N), and 1 ml of Milli-Q® water q.s. The mixture was centrifuged for 10 min at 35,000 ×*g* and filtered (0.45 µm, 4 mm PVDF membrane). The supernatants were then injected into an Aminex HPX-87H column (Biorad), which was heated to 40 °C. Sugars and organic acids were separated out during 60-min runs (rate of 0.6 ml/min); H_2SO_4 buffer (5 mM) was employed. The compounds were detected with a UV 2996 detector and a 2410 differential refractometer. Quantities were determined via comparisons with standard curves that were established using lactate, lactose, glucose, galactose, fructose, sucrose, stachyose, and raffinose.

2.4. Scanning electron microscopy

Microscopy analyses were performed at the Microscopy and Imaging Platform MIMA2 (INRA, Jouy-en-Josas, France) (INRA, http://www6.jouy.inra.fr/mima2/). Bacteria were recovered from 1-ml samples of cow's milk and soya milk cultures during two growth phases -the exponential phase (at 2 h of culture) and the stationary phase (at 3.5 h of culture)- by centrifugation (5000 ×*g* at 4 °C for 10 min). The bacteria were then washed twice with 3.5 ml of Tris 0.1 M (pH 7.5). One 40-µL drop of washed bacteria was deposited on a sterile cover glass (12 mm in diameter; Marienfeld, VWR, France). Each biofilm coupon was placed into one well of a 24-well polystyrene plate. Samples were fixed via careful immersion in a solution of 2.5% glutaraldehyde containing 0.1 M sodium cacodylate buffer (pH of 7.4) kept at room temperature. The samples remained immersed overnight at 4 °C. They were then rinsed three times for 10 min in sodium cacodylate solution (pH 7.4) and underwent progressive dehydration by being soaked in a graded series of ethanol solutions (50 to 100%) before being subjected to critical point drying using CO_2. The coupons were subsequently mounted on aluminium stubs (10 mm in diameter) with carbon adhesive disks (Agar Scientific; Oxford Instruments SAS, Gometz-la-Ville, France) and sputter coated with gold/palladium (Polaron SC7640; Elexience, Verrières-le-Buisson, France) for 200 s at 10 mA. The samples were viewed as secondary electron images (2 kV) using a field emission gun scanning electron microscope (SEM) (Hitachi S-4500; Elexience, Verrières-le-Buisson, France).

2.5. SDS-PAGE electrophoresis

Supernatant was obtained from the soya milk and cow's milk cultures by centrifugation (5000 ×*g* for 10 min), and levels of soluble proteins were quantified using Coomassie protein assay reagent (Pierce Biotechnology Inc., Rockford, Illinois, USA); bovine serum albumin served as the standard. A 10-µg sample of proteins was then mixed with 4 µl of a denaturation solution (4× Laemmli sample buffer; Biorad) and 20 µl of Milli-Q H_2O q.s.; the mixture was heated to 96 °C and kept there for 2 min before being placed in NuPage™ 4–12% Bis-Tris Gel (Invitrogen) containing 10 µl of SeeBlue™ Plus2 marker (Invitrogen). SDS-PAGE electrophoresis was then carried out. Migration occurred at 220 V–110 mA for 45 min. The gels were colored for 1 h with Coomassie Blue. Then, the gels were washed four times for 15 min and scanned to digitize the results.

2.6. Proteomic analysis

2.6.1. Protein extraction

Samples (100 ml) of the soya milk and cow's milk cultures collected during the exponential phase of growth were mixed with 100 ml of sodium citrate buffer (1 M) and centrifuged (32,000 ×*g* at 4 °C for 10 min). The resulting bacterial pellets were washed twice with Tris-EDTA buffer; they were then frozen and stored at −80 °C. Bacteria were broken up using a cell disruptor (one passage at 2.5 Kbar/sample; AZIC Z cell disruptor, Celld, Warwickshire, UK). Unbroken bacteria and large cellular debris were eliminated using centrifugation (20,000 ×*g*); the supernatant was recovered and ultracentrifuged (200,000 ×*g*) to separate the cell-envelope proteins from the cytosolic proteins. Proteins in the cell-envelope fractions and the cytosolic fractions were quantified as described above and stored at −80 °C.

2.6.2. Sample preparation, mass spectrometry analysis

Samples (10 µg) of the cell-envelope proteins and the cytosolic proteins were separated using one-dimensional electrophoresis (4–12% acrylamide gel, SDS-PAGE) with short migration times. Biological triplicates were loaded onto the gel, and the protein bands were cut and digested in gel. The resulting peptides were extracted as previously described (Gardan et al., 2009). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was carried out using an UltiMate™ 3000 RSLCnano system (Thermo Fisher Scientific) with a 50-cm column (PepMap 100, 3 µm; column: 75 µm × 500 mm; one run = 210 min) coupled to a LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Fisher Scientific) in CID mode, as described in Gardan et al. (2009). Protein identification was performed with X! Tandem software by comparing the experimental results against known proteins of *S. thermophilus* LMD-9 (GenBank v. 2013, 1710 entries), soya (Uniprot v. 16-11-14; 150,681 entries), and potential contaminants. Results were filtered using the built-in X! Tandem Pipeline (http://pappso.inra.fr/bioinfo/); the requirements were a peptide *E*-value of 0.01, a protein *E*-value of −4, and the presence of at least 3 specific peptides per protein. The proteins identified from the cell-envelope and cytosolic fractions are presented together (Tables S1 and S2).

The analysis of the soya milk samples was less extensive than the analysis of the cow's milk samples because the former contained a larger percentage of soya proteins (57% of soya milk proteins identified/total vs. 4.2% of cow's milk proteins identified/total). Because differences in the relative levels of protein identification in the two sample types could stem from detection differences, as opposed to true protein presence/absence, the two sets of data were not compared.

3. Results and discussion

This study examined growth, sugar production, and lactic acid production by an *S. thermophilus* strain (LMD-9) cultured in soya milk and cow's milk. It also characterized the main metabolic pathways that the bacterium employs in soya milk using a proteomic approach, which highlighted the important role of nitrogen metabolism.

1. *Streptococcus thermophilus* LMD-9 is metabolically active in soya milk.

First, an assessment was performed of the overall morphology of *S. thermophilus* LMD-9 cultures grown in the soya milk and cow's milk via SEM observations conducted during two growth phases: the exponential phase and the stationary phase. During both phases in both media, *S. thermophilus* exhibited ovococcus-shaped bacteria and constructed chains of > 10 cells (Fig. 1), traits typical of this bacterium. Consequently, *S. thermophilus* LMD-9 displayed no morphological differences when grown in soya milk. Furthermore, dividing cells had visible septa, which indicates that the bacteria were metabolically active in soya milk. In both media, bacteria were sticky in the matrix.

2. *Streptococcus thermophilus* LMD-9 rapidly drives down soya milk pH.

The growth of *S. thermophilus* LMD-9 resulted in the acidification of

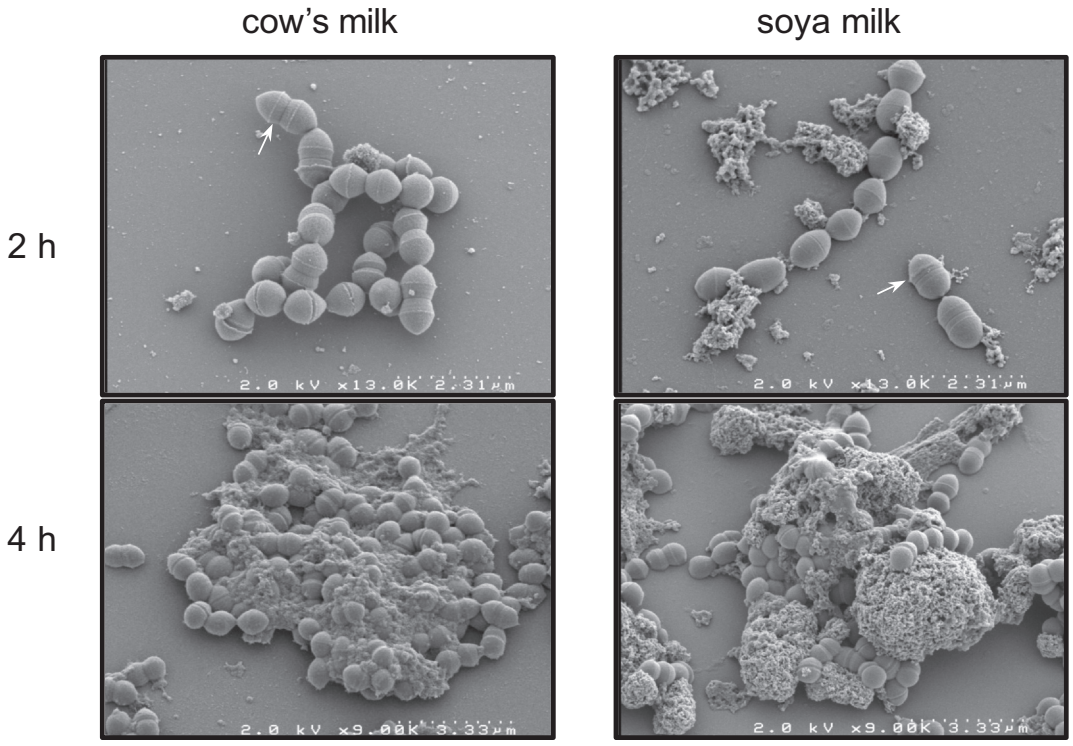

Fig. 1. Scanning electron microscope images of wild-type *S. thermophilus* LMD-9 cultures in cow's milk and soya milk illustrating the morphological similarities of the bacterium in the two growth media during its two growth phases: the exponential phase (2 h) and the stationary phase (4 h). The arrows indicate the bacterial septa.

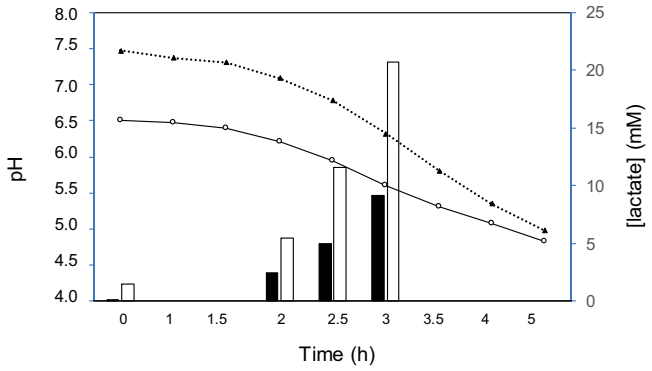

Fig. 2. Acidification kinetics and lactate production of wild-type *S. thermophilus* LMD-9 cultures grown in cow's milk (○) and soya milk (▲).

the cow's milk, with pH dropping from 6.5 to 4.7 in 5 h (Fig. 2); the acidification rate peaked between 2 and 4 h. Curding occurred 4 h after fermentation, at a pH of around 5.1 in the cow's milk cultures and 5.3–5.4 in the soya milk cultures.

Acidification kinetics in the soya milk cultures paralleled those in the cow's milk cultures in terms of the number and duration of the different phases: acidification was absent or very low during the first hour, accelerated and then peaked between 2 and 3.5 h, and finally decelerated. The pH decreased from 7.5 to 4.9 in 5 h (Fig. 2). The major difference between the two media was that the maximum acidification rate was 1.6 higher in the soya milk cultures (ΔpH/h = 0.64) than in the cow's milk cultures (ΔpH/h = 0.41); this result explains why final pH was similar for both media even though initial pH was higher in the soya milk cultures (pH = 7.5) than in the cow's milk cultures (pH = 6.5) (Fig. 2).

When *S. thermophilus* LMD-9 was grown in soya milk, it generated a higher level of acidification (ΔpH of 2.6 in 5 h) than other *S. thermophilus* strains studied up until now (ΔpH of 1.6–2.3; Champagne et al., 2009; Chumchere and Robinson, 1999; Garro et al., 1998; Mital and Steinkraus, 1975, 1976; Wang et al., 2003). These differences could result from the strains themselves and/or from differences in soya milk composition or preparation. A previous study found that the ST5 strain acidified laboratory-prepared soya milk in 8 h (ΔpH of 2) and a commercial soya milk in 12 h (Champagne et al., 2009).

As expected for a LAB, *S. thermophilus* LMD-9 produced lactate as it grew, acidifying both the cow's milk and the soya milk (Fig. 2). While the lactate concentration increased steadily in both media during initial culturing, it was lower in the soya milk than in the cow's milk. Levels of lactate production during the first 3 h of culture were 0.82 g/l (± 0.076) and 1.74 g/l (± 0.013) in the soya milk and cow's milk, respectively.

3. *Streptococcus thermophilus* LMD-9 grows in soya milk via sucrose consumption.

3.1. Growth kinetics

Streptococcus thermophilus LMD-9 displayed two growth phases in the soya milk and cow's milk: an exponential phase, where growth accelerated after 1 h, and a stationary phase, which began after 4 h of culture (Fig. 3). In both media, growth stopped when the pH reached around 5.2, which is expected for this bacterium. The growth rate was higher in the cow's milk than in the soya milk (2.5 h^{-1} vs 1.8 h^{-1}); after 5 h of culture, population sizes were higher in the cow's milk than in the soya milk (2.3×10^9 CFU/ml [$\pm 0.4 \times 10^9$] vs. 1.0×10^9 CFU/ml [$\pm 0.15 \times 10^9$]), which correlated well with the levels of lactate production in the two media (Fig. 2).

Mital and Steinkraus (1975) observed a similar difference in *S. thermophilus* growth: populations were 3–4 larger in cow's milk than in soya milk. Such differences could be related to the contrasted buffering capacities of the two media (Champagne et al., 2009; Lutchman et al., 2006). Therefore, β-glycerophosphate (19 g/l) was added to the soya milk as a buffer in an effort to reduce acidification, but the strain's

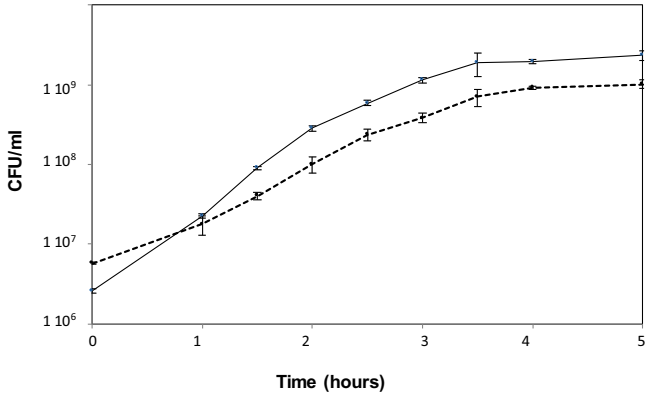

Fig. 3. Growth curve of the wild-type *S. thermophilus* LMD-9 cultures grown in cow's milk (○) and soya milk (▲). The curve was constructed from three independent replicates.

growth did not improve (data not shown). Then, lactose was added as it is the preferred sugar of *S. thermophilus* LMD-9 (Thomas et al., 2011). This supplementation (10 g/l) also failed to increase the strain's growth (data not shown). Since large amounts of usable sugars were still present at the end of the culturing period, both in the cow's milk and the soya milk, it was concluded that carbohydrate availability was not limiting the growth of *S. thermophilus* LMD-9 in the soya milk.

Streptococcus thermophilus strains vary dramatically in their ability to grow and/or reach large population sizes in soya milk. For example, in our study, the population size increased 170 fold during the stationary phase (from 5.8×10^6 to 1.0×10^9 CFU/ml); in other studies, this increase ranged between 36 and 10,000 fold (Champagne et al., 2009; Chumchere and Robinson, 1999; Garro et al., 1998; Wang et al., 2002). Like the results for acidification, the results for population size in soya milk could be related to strain traits (i.e., the capacity to use soya sugars and proteins), the properties of the soya milk matrices, the soya milk preparation method (e.g., the sterilization temperature), and/or the soya milk's composition (e.g., sugars and yeast extract are sometimes added to soya milk).

3.2. Sugar profiles

The concentrations of certain types of sugars were quantified in the cow's milk and soya milk cultures of *S. thermophilus* LMD-9 during the first 3 h of growth. Lactose and sucrose are the main sugars in cow's milk (170 mM = 58.2 g/l) and soya milk (72 mM = 24.6 g/l), respectively.

In the cow's milk cultures, the lactose concentration declined steadily (Δ of 8.64 mM [= 2.96 g/l]), while the galactose concentration increased (Δ of 8.6 mM [= 1.55 g/l]) (Fig. 4A); no glucose was detected in the medium. Sørensen et al. (2016) observed similar dynamics of lactose consumption (44 mM) and galactose production (36 mM) over a 30-h culture period. This result fits with what is known about lactose transport in *S. thermophilus*: lactose is hydrolyzed to produce galactose, which is most commonly released into the medium, and glucose, which is metabolized via glycolysis to produce ATP. The kinetics of lactose consumption and galactose production are thus well correlated with growth kinetics (Fig. 3).

In the soya milk cultures, the sucrose concentration decreased (Δ of 4 mM [= 1.36 g/l]), while the fructose concentration increased (Δ of 2.1 mM [= 0.38 g/l]) (Fig. 4B). Similar sucrose consumption dynamics by *S. thermophilus* LMD-9 in soya milk have previously been observed (0.4 g/L; Chumchere and Robinson, 1999; Wang et al., 2003). The growth of *S. thermophilus* LMD-9 in soya milk thus appears to be fueled by the consumption of sucrose, the main sugar in the soya milk used here and a good carbohydrate source for this particular strain (Thomas et al., 2011). The ability to exploit sucrose is shared by most *S. thermophilus* strains (Harlé et al., 2020; Mital and Steinkraus, 1975; van den Bogaard et al., 2004). In addition, genes related to sucrose transport and the production of fructose from sucrose are common in *S. thermophilus* species (Alexandraki et al., 2019; Bolotin et al., 2004; Goh et al., 2011; Hols et al., 2005).

4. Predominance of proteins related to nitrogen metabolism in soya milk

To obtain a more holistic view of the metabolism and physiology of *S. thermophilus* LMD-9 during growth in soya milk, a proteomics analysis of the strain's cell-envelope proteins and cytosolic proteins was carried out using LC-MS/MS. Indeed, when proteins linked to certain metabolic pathways are present under certain growth conditions, it can be inferred that particular pathways and nutrient sources are being used. A similar analysis was performed on the samples of *S. thermophilus* LMD-9 grown in cow's milk to obtain an appropriate reference for this study, although it should be noted that proteomic data already exist for another strain of *S. thermophilus* grown in cow's milk (Derzelle et al., 2005; Herve-Jimenez et al., 2008).

4.1. Proteomic profile in cow's milk

In the proteomic profile of *S. thermophilus* LMD-9 grown in cow's milk, there were 515 proteins that most commonly fell into the following clusters of orthologous groups (COGs): translation, ribosomal structure, and biogenesis ([J], 17.9%); amino acid transport and metabolism ([E], 17.3%); and cell wall/membrane/envelope biogenesis ([M], 8.7%) (Table S1, Fig. S1). The COGs [J] and [E] were thus predominant (around 35%), a result previously observed for another strain (Herve-Jimenez et al., 2008).

Streptococcus thermophilus needs amino acids (AAs) for protein synthesis and ensures its supply in two main ways: by creating AAs via intracellular synthesis and/or by transporting proteins and peptides in from the extracellular medium and hydrolyzing them with amino- and oligopeptidases. This study identified the presence of the cell-wall protease PrtS (one of the first enzymes to act in the proteolytic cascade); 8 of the 12 intracellular aminopeptidases (i.e., general aminopeptidases, proline-specific peptidases, dipeptidase, oligopeptidases) present in the LMD-9 genome (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA13773); and most of the AA, di/tripeptide, and oligopeptide transport systems (14 out of 20). These results suggest that all the different AAs required by *S. thermophilus* are likely provided by cow's milk thanks to the activities of these diverse proteins, which target all types of peptides (of varying lengths and AA composition). In addition, proteins from all the complete AA biosynthetic pathways (19) of LMD-9 were identified.

The profile also revealed the presence of 4 of the 7 transporters involved in carbohydrate metabolism that are predicted to occur in the genome (3 phosphotransferase system [PTS] transporters, 2 ATP-binding cassette [ABC]-type transporters, and 2 permeases), including lactose permease (LacS). All 9 of the glycolytic enzymes were identified as previously observed. This result is unsurprising given that glycolysis is the main process by which *S. thermophilus* forms ATP.

4.2. Proteomic profile in soya milk

In the proteomic profile of *S. thermophilus* LMD-9 grown in soya milk, there were 328 bacterial proteins (Table S2), which is fewer than the number in the cow's milk cultures. This difference was caused by the higher level of soya protein contamination in soya milk cultures. When classified into COGs, half the proteins were identified as being involved in basic cell functions: amino acid transport and metabolism (19.2%); translation, ribosomal structure, and biogenesis (15.2%); carbohydrate transport and metabolism (8.8%); and cell wall/membrane/envelope biogenesis (8.5%) (Fig. 5).

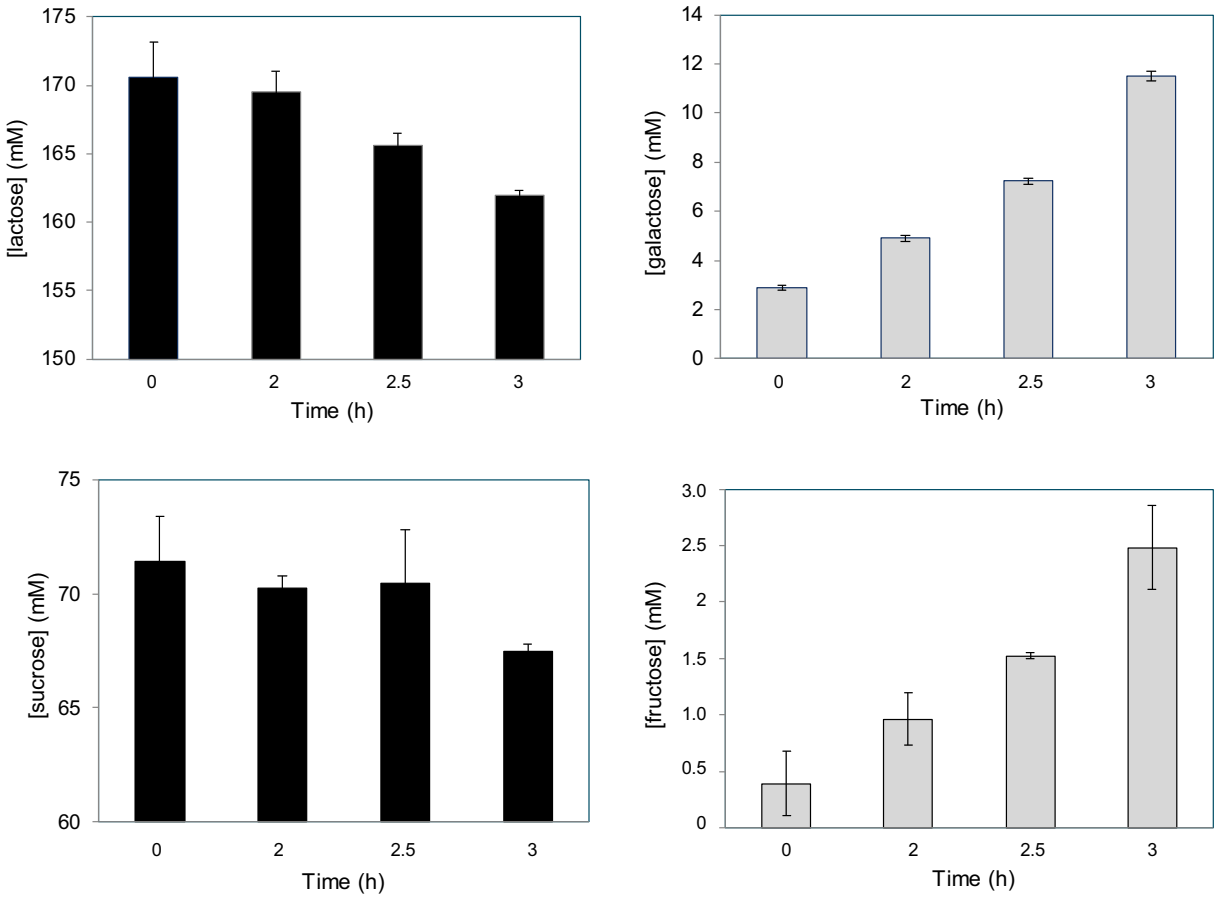

Fig. 4. Sugar concentrations in the wild-type *S. thermophilus* LMD-9 cultures: A) lactose and galactose concentrations in cow's milk, B) sucrose and fructose concentrations in soya milk.

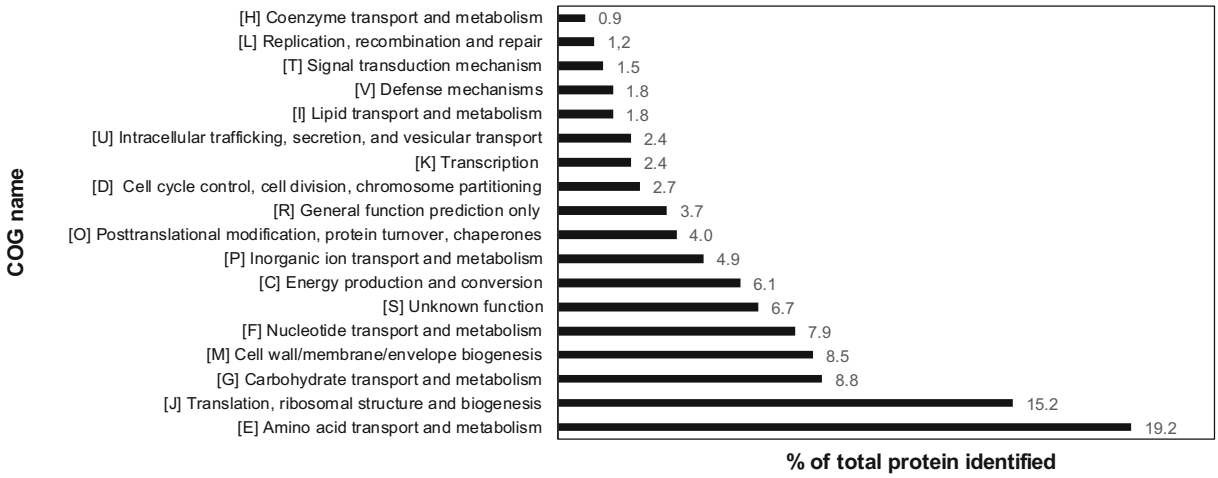

Fig. 5. Percentage of proteins in different COG categories found in *S. thermophilus* LMD-9 cultures after 3 h of growth in soya milk.

Nitrogen metabolism seems to play an important role in *S. thermophilus* LMD-9 growth in soya milk based on the number of proteins identified that were involved in nitrogen metabolism (63/328), which probably reflects the bacterium's significant need for nitrogen. The profile revealed the presence of the extracellular cell-wall protease PrtS, 11 amino acid transporters (for polar AAs, branched-chain AAs, Met, Ser/Thr, Ala, and Gln), and 1 oligopeptide transporter (Ami) out of the 20 transporters present in the genome. Ami transporters are capable of transporting peptides containing up to 23 AAs (Juille et al., 2005). Five of the 12 intracellular peptidases found in the genome were also present in the profile: the 2 oligopeptidases PepB/F and PepO (that hydrolyze peptides between 5 and 30 AAs in length; Chavagnat et al., 2000); the general aminopeptidase PepN that liberates all types of AAs; and 2 dipeptidases—the general dipeptidase PepV and the proline-specific prolidase PepQ. These results suggest that peptides of up to 23 AAs in length can potentially be transported and hydrolyzed intracellularly by *S. thermophilus* grown in soya milk. This study also identified the presence of biosynthesis pathways for all the AAs except histidine; evidence was particularly pronounced for the pathways for branched-chain AAs (Leu, Ile, Val), sulfur AAs (Met, Cys, Ser), and glutamic acid.

From a qualitative point of view, these results generally suggest that *S. thermophilus* LMD-9 grown in soya milk can produce peptides from soya proteins via the action of PrtS, peptide transport, the hydrolysis of certain peptides, and the synthesis of most of the different amino acids.

Carbohydrate metabolism was of particular interest in this study, and the proteomic profile revealed the presence of proteins potentially involved in the utilization of glucose polymers (AmyL, GlgP, MalQ), sucrose, lactose (LacS-Z), and possibly glucose (GlcK) and fructose (ManMNL PTS transporters). More specifically, the following proteins were observed: ScrA, PtsI, and PtsH- which have been implicated in sucrose transport in other bacteria- and ScrB hydrolase (β-fructofuranosidase = sucrose-6 phosphate hydrolase). Once imported into the cell, sucrose is likely hydrolyzed into glucose-6-phosphate and fructose by ScrB; the glucose-6-phosphate then enters the glycolysis pathway, and part of the fructose is released into the extracellular medium. Previous studies have shown that the growth of *S. thermophilus* in soya milk can lead to a similar accumulation of fructose in cultures (Fig. 4B; Champagne et al., 2009; Garro et al., 1998; Wang et al., 2003). All 9 glycolytic enzymes were present, as was lactate dehydrogenase (Ldh), which converts the end product of glycolysis, pyruvate, into lactate. The latter is toxic to the bacterium and is thus released into the extracellular medium, as was observed here (see above, Section 2.2).

5. The cell-wall protease PrtS contributes to *S. thermophilus* LMD-9 growth via its role in soya protein hydrolysis

As discussed above, the proteomic profile showed that nitrogen metabolism seems to play an important role in *S. thermophilus* LMD-9 growth in soya milk. We therefore explored this issue further using two approaches: 1) soya milk was supplemented with free amino acids and peptides and 2) an *S. thermophilus* LMD-9 mutant was used whose cell-wall protease PrtS had been deleted. When *S. thermophilus* is cultured in cow's milk, PrtS helps the bacterium grow and is responsible for casein hydrolysis (Courtin et al., 2002; Dandoy et al., 2011).

5.1. Effects of amino acid and peptide supplementation on S. thermophilus LMD-9 growth in soya milk

To test the effects of nitrogen supplementation on *S. thermophilus* LMD-9 growth in soya milk, we used a casein hydrolysate (mostly composed of free amino acids/dipeptides; Bacto™ Casamino Acids) and either pure or mixed amino acids. In particular the bacterium was supplemented with lysine because, like other *S. thermophilus* strains, LMD-9 lacks the complete biosynthesis pathway for this AA (Alexandraki et al., 2019), suggesting it might be a lysine auxotroph.

Supplementation with casein hydrolysate did not modify the growth or acidification patterns of *S. thermophilus* LMD-9 in soya milk over the first 6 h of growth (with supplementation: ΔpH of 2.33 [± 0.17] and Δpopulation size of 1.64×10^9 CFU/ml [± 0.92×10^9]; without supplementation: ΔpH of 2.34 [± 0.15] and Δpopulation size of 1.6×10^9 CFU/ml [± 1.26×10^9]). The addition to the soya milk of lysine, various branched-chain AAs (Ile, Leu, Val), and various sulfur AAs (Met, Cys, Ser) at concentrations of 1 or 5 mM did not significantly increase the growth of *S. thermophilus* LMD-9 (data not shown). These results suggest that neither the AAs/dipeptides in the casein hydrolysate nor the previously mentioned free AAs limited the bacterium's growth in soya milk.

5.2. Effect of the cell-wall protease PrtS on S. thermophilus LMD-9 growth in soya milk

The proteomic profile revealed the presence of the cell-wall protease PrtS in *S. thermophilus* LMD-9 grown in soya milk, the next step was to explore the role of this protease in the acidification of soya milk. A deletion mutant of the LMD-9 strain (Δ*prtS* strain) was constructed by interrupting the *prtS* gene (locus: STER_RS04165) with an antibiotic gene cassette (see Materials and methods section). Acidification was less pronounced in the Δ*prtS* strain cultures (ΔpH of 1.77 [± 0.036]) than in the wild-type (wt) strain cultures (ΔpH of 2.22 [± 0.01]), underscoring the involvement of PrtS in the process. A similar pattern was seen for population size, which was 3 fold lower for the Δ*prtS* strain (2.7×10^8 CFU/ml [± 1.24×10^8]) than for the wt strain (7.6×10^8 CFU/ml [± $2.5\ 10^8$]) after 6 h of culture. We thus concluded that the cell-wall protease PrtS is involved in *S. thermophilus* LMD-9 growth in soya milk. This finding fits with the results of a previous study, which suggested a link between the bacterium's proteolysis capacity and growth in soya milk (Donkor et al., 2007).

There was no effect of nitrogen supplementation (bactocasitone peptone, 1 g/L) on the growth and acidification dynamics of the wt strain grown in soya milk. In contrast, there was an effect on the Δ*prtS* strain: over the first 4 h of culture, pH and population size were higher with supplementation (pH = 2.15 [± 0.21], population size: 1.22×10^8 CFU/ml [± 0.44]) than without supplementation (pH = 1.7 [± 0.17], population size: 2.7×10^8 CFU/ml [± 0.7]). This result suggests that PrtS was active when the bacterium was grown in soya milk; it was then studied if PrtS was able to degrade soya milk proteins. Using SDS-PAGE, the soluble proteins in the cultures of the wt and Δ*prtS* strains grown in soya milk were separated out (Fig. 6). Based on the bands observed, the most common proteins present are probably β-conglycinin and glycinin, which are the main storage proteins in soya seed (Hsieh et al., 2012; Krishnan et al., 2009).

After 6 h of culture, the protein profile of the wt strain (lanes 2 & 3) was markedly different: bands associated with heavier proteins (> 62 kDa) had completely disappeared, while bands associated with lighter proteins (49–62 kDa and < 38 kDa) had appeared. This result indicates that some of the proteins had been proteolyzed during the bacterium's growth. In contrast, the protein profile of the Δ*prtS* strain suggested that less hydrolysis had taken place: the two bands associated with heavier proteins were more pronounced. However, the bands associated with the lighter proteins were similar to those of the wt strain. These findings show that the wt strain was more proteolytic than the Δ*prtS* strain and thus probably generated more peptides, which could

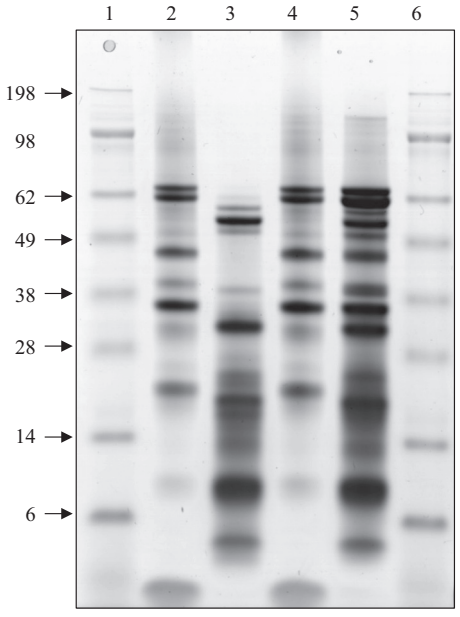

Fig. 6. SDS-PAGE results for the proteins found in cultures of the *S. thermophilus* LMD-9 wt strain and Δ*prtS* strain grown in soya milk. The numbers on the left are the reference molecular masses. Lanes 1 and 6: molecular mass standard (SeeBlue™ Plus2 marker); Lane 2: *wt* strain at the start of culturing (T0); Lane 3: wt strain after 6 h of culture (T6); Lane 4: Δ*prtS* strain at the start of culturing (T0); Lane 5: Δ*prtS* strain after 6 h of culture (T6).

explain its better growth. Just as in the cow's milk cultures, the hydrolytic activity of PrtS in the soya milk cultures could have promoted the growth of wt *S. thermophilus* LMD-9 via the production of peptides that could serve as an exogenous supply of nitrogen.

6. Conclusion

We showed that *S. thermophilus* LMD-9 could grow in and rapidly acidify soya milk. Furthermore, the bacterium displayed similar growth and acidification dynamics in soya milk and cow's milk. It consumes sucrose to grow, which leads to lactate production and, in turn, acidification, a process that can preserve soya milk from microbial contamination. This work also established the first proteomic map for *S. thermophilus* grown in soya milk, which highlighted the importance of nitrogen metabolism. Additionally, it was discovered that the LAB cell-wall protease PrtS is involved in the bacterium's growth in soya milk and its proteolysis of soya proteins. The ability of *S. thermophilus* to hydrolyze soya proteins could help improve the digestibility of soya-based products and provide health benefits, such as reducing the allergenicity of soya proteins or modulating the production of potentially bioactive peptides.

The identification of the peptides and, more generally, the metabolites present after fermentation should enhance understanding of the organoleptic and probiotic properties of soya-based products fermented with *S. thermophilus* or with other LAB.

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CRediT authorship contribution statement

Boulay M.: conceptualization, formal analysis, investigation, methodology, validation, visualization. Al Haddad M.: conceptualization, formal analysis, investigation, validation. Rul F.: conceptualization, funding acquisition, investigation, project administration, supervision, visualization, writing – original draft

Declaration of competing interest

None.

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