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Streptococcus thermophilus urease activity: physiological role and technological relevance in dairy and non-dairy applications

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1. STATE OF THE ART

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are historically defined as a group of microaerophilic, Gram-positive organisms that ferment hexose sugars to produce primarily lactic acid. This functional classification includes a variety of industrially important genera, comprising *Lactococcus, Enterococcus, Oenococcus, Pediococcus, Streptococcus, Leuconostoc,* and *Lactobacillus* species (Makarova *et al.*, 2006). Biochemically, LAB employ two pathways to metabolize hexose: homofermenters produce primarily lactic acid, while heterofermenters yield also a variety of fermentation by-products, including lactic acid, acetic acid, ethanol, carbon dioxide and formic acid (Klaenhammer *et al.*, 2005). The metabolism of LAB has been exploited throughout history for the preservation of foods and beverages in nearly all societies dating back to the origins of agriculture (Kiple *et al.*, 2000), leading to their widespread human consumption and generally recognized as safe (GRAS) status. Today, LAB are widespread in the world food supply, performing the main bioconversions in fermented dairy products, meats, and vegetables and they are also involved in the production of wine, coffee, silage, cocoa, sourdough, and numerous indigenous food fermentations (Wood, 2012), in which they are exploited not only for their acidification capacity, but also to contribute to other product characteristics like flavor, texture and nutrition.

LAB species are indigenous to food-related habitats, including plant, e.g. fruits, vegetables, and cereal grains, and milk environments. In addition, they are naturally associated with the mucosal surfaces of animals, e.g., small intestine, colon, and vagina (Makarova *et al.*, 2006); indeed, LAB are considered to be important components of the normal intestinal microbiota, contributing to a variety of functions including intestinal integrity, immunomodulation, and pathogen resistance (Klaenhammer *et al.*, 2005). Selected groups of *Lactobacillus* are used widely as probiotics primarily in dairy products and dietary supplements (Reid, 1999).

LAB genomes are characterized by low GC content and small genomes ranging in size between 1.8 and 3.3 Mb (Klaenhammer *et al.*, 2005). The number of predicted protein-coding genes in the LAB differs from 1,700 to 2,800: considering the close phylogenetic relationship between this group, such a difference suggests that during their evolution LAB underwent substantial gene loss and/or gain. In addition, all LAB genomes harbor pseudogenes, indicating for those species whose number is high, an active, ongoing process of genome degeneration (Makarova *et al.*, 2006). Many LAB harbor plasmids, some of which are essential for their growth in specific environments since they carry genes encoding for metabolic pathways, membrane transport and bacteriocin production (McKay and Baldwin, 1990).

Thermophilic lactic acid bacteria (T-LAB) are traditionally used for the manufacture of yogurt and hard 'cooked' cheeses (e.g. grana, parmigiano, emmental, gruyere etc.), whose production processes require an incubation of the milk or curd at a relatively high temperature (45 °C or above). Not many LAB strains are able to grow or survive at such high temperatures, and those that are mainly used as dairy starters belong to three species: *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or *lactis* (Delcour *et al.*, 2000).

1.2 *Streptococcus thermophilus*

S. thermophilus is a thermophilic lactic acid bacterium, belonging to Bacteria superkingdom, Firmicutes phylum, Bacilli class, Lactobacillales order, Streptococcaceae family, *Streptococcus* genus, *Streptococcus thermophilus* species. The taxonomic status of *S. thermophilus* has been controversial: for some years it was classified as a *S. salivarius* subspecies (*Streptococcus salivarius* subsp. *thermophilus*) (Farrow and Collins, 1984); then Schleifer *et al.* (1991) provided evidences, based on DNA–DNA reassociation experiments under stringent conditions, that justifies the conferment of the full species status to *S. thermophilus* (Delorme, 2008).

S. thermophilus is frequently isolated from dairy environments, but strains have been isolated also from plant samples (Michaylova *et al.*, 2002). The isolates are identified as anaerobic, aerotolerant, catalase-negative and Gram-positive, growing as linear chains of ovoid cells and unable to grow at 10 °C, at pH 9.6 or in 6.5% NaCl broth (Delorme, 2008). Identification of *S. thermophilus* species is based on hydrolysis of arginine and esculin, acid fermentation in amygdalin, cellobiose, inulin, maltose, mannitol, raffinose and N-acetylglucosamine broths and ability to grow at 45 °C (Facklam, 2002).

S. thermophilus is considered the second most important species of industrial lactic acid bacteria after *Lactococcus lactis*, with a market value of around 40 billion US$ (Chausson and Maurisson, 2002). It is extensively used for the manufacture of dairy products: besides the traditional use in combination with *L. delbrueckii* subsp. *bulgaricus* in yoghurt, *S. thermophilus* is used to produce several varieties of cheese, such as Swiss cheese, Brick cheese, Parmesan, Provolone, Mozzarella, and Asiago (Parente *et al.*, 2017) and also for the production of Cheddar in combination with mesophilic starters (Iyer *et al.*, 2010). The main role of *S. thermophilus* in milk fermentations is to provide rapid acidification producing lactic acid from the disaccharide lactose, but it also contributes to the formation of flavor and texture; besides lactic acid, other fermentation products are low levels of formate, acetoin, diacetyl, acetaldehyde, and acetate. Five different sugars are fermented by *S. thermophilus*: lactose, sucrose, glucose, galactose, and fructose. The latter two sugars are fermented only by a limited number of strains (Hols *et al.*, 2005).

So far, the complete genome sequence of 23 *S. thermophilus* strains were made publicly available: the genomes are on average 1.8 megabases in length and contain about 1,663 proteins and 1,978 genes with a GC content of 39% (https://www.ncbi.nlm.nih.gov/genome/genomes/420? – updated to September 2018).

S. thermophilus is closely related to the nonpathogenic *L. lactis*, but it is even more closely related to other streptococcal species that are considered harmful human pathogens (Hols *et al.*, 2005) (Figure 1), such as *Streptococcus pneumoniae*, the agent of bacterial sepsis, pneumonia, otitis media and meningitis; *S. pyogenes*, which causes pharyngitis, impetigo and rheumatic fever; *S. agalactiae* which causes bacterial sepsis, pneumonia or meningitis in neonates and, at last, *S. thermophilus* is also related to *S. mutans*, the etiologic agent of tooth decay (Mitchell, 2003).

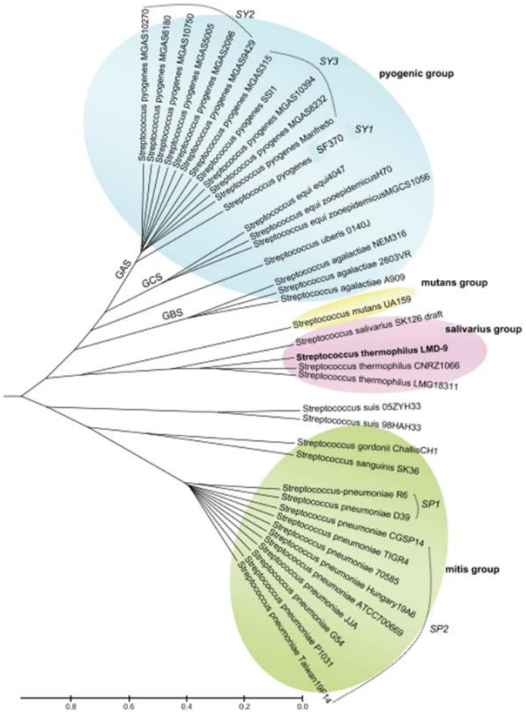

Figure 1. Functional genome distribution (FGD) tree of 39 *Streptococcus* genomes (Goh *et al.*, 2011).

Ten percent of the *S. thermophilus* genes are pseudogenes, which means genes that are not functional in consequence of frameshift, nonsense mutation, deletion or truncation. Interestingly, the most abundant pseudogenes belong to the "transport proteins" and the "energy metabolism" functional groups and, conversely, a specific symporter for lactose (the main milk carbohydrate) is present in the *S. thermophilus* genome but absent in other streptococci (Bolotin *et al.*, 2004), in agreement with the low variety of

carbon sources in milk (Geertsma *et al.*, 2005). Moreover, the comparison of the *S. thermophilus* genomes with published genomes of streptococcal pathogens highlighted that many streptococcal virulence-related genes (VRGs) are absent in the *S. thermophilus* genome or are present only as pseudogenes (Bolotin *et al.*, 2004). Between them, we can find genes encoding for cell-surface proteins known to interact with human cells and the immune system (lipoproteins, adhesins, choline-binding proteins, cell wall anchored proteins, IgA proteases) and proteins involved in anchoring these proteins on the cell surface (sortases). It is interesting to notice that some of the VRGs involved in the biosynthesis of the polysaccharide capsule that surrounds pathogenic streptococci are maintained in *S. thermophilus*: they contribute to the production of external polysaccharides that confer the desirable texture of yogurt (Tettelin, 2004). In conclusion, *S. thermophilus* evolved from those closely phylogenetically related pathogenic streptococci through loss-of-function events counterbalanced by the acquisition of relevant traits, such as lactose utilization (Bolotin *et al.*, 2004), that have allowed the assembly of new genomic organization suitable for the colonization of the dairy niche (Hols *et al.*, 2005). All these findings support that the massive consumption of *S. thermophilus* does not represent a danger, since it is estimated that over 10^{21} live cells are ingested annually by the human population (Hols *et al.*, 2005), and support the "Generally Recognized as Safe'' (GRAS) status (or "Qualified Presumption of Safety" – QPS, according to EFSA guidelines).

1.3 *S. thermophilus* in dairy applications

S. thermophilus as starter culture is traditionally used in the production of yogurt and many cheeses, for example hard-cooked cheeses, like Emmental, Gruyère, Parmigiano and Grana-types, as well as Mozzarella and Cheddar. *S. thermophilus* is able to grow or survive at the high temperatures (45 °C) required in the production processes of these products. For cheese-making, *S. thermophilus* is used alone or in combination with several lactobacilli and mesophilic starters, but for yogurt it is always used with *L. delbrueckii* subsp. *bulgaricus* (Auclair and Accolas, 1983). In particular, the interactions that occur between the two lactic acid bacteria species in milk, during the yogurt production, have been described as a proto-cooperative process, which has positive effects on the growth rate and size of each population, in contrast to the independent growth of the two species in milk, in which we assist to a slower growth rate and a smaller population size (Sieuwerts *et al.*, 2010). The molecular interactions that play key roles in the mutualistic behavior of the yogurt consortium have been described in relation to:

- the availability of nitrogen in milk: $PrtB^+$ *L. delbrueckii* subsp. *bulgaricus* supplies peptides to *S. thermophilus*, thus enhancing the acidification potential of the latter species (Courtin *et al.*, 2002);
- the exchange of formic acid, pyruvic acid and folic acid: *S. thermophilus* supplies *L. delbrueckii* subsp. *bulgaricus* with crucial components for purine nucleotide biosynthesis, including the precursor formic acid and the cofactor folic acid (Sieuwerts *et al.*, 2010);
- the production and utilization of carbon dioxide: carbonate dehydratase, encoded by *cah* gene in *S. thermophilus*, is up to 15-fold upregulated in mixed culture, especially in the earlier growth phases. The releasing of CO_2 from carbonate, may play a fundamental role in providing the CO_2 required for the biosynthesis of aspartate, glutamate, arginine, and nucleotides in both species (Sieuwerts *et al.*, 2010);
- the metabolism of amino acid, long-chain fatty acids (Sieuwerts *et al.*, 2010) and iron (Herve-Jimenez *et al.*, 2009).

Besides the rapid conversion of lactose into lactic acid, the main role of *S. thermophilus* in milk fermentation is the production of metabolites important for their technological properties.

Extracellular polysaccharides are produced by a variety of bacteria and are present as capsular polysaccharides (CPS and LPS), bound to the cell surface, or are released into the growth medium (EPS). They can consist of a single type of sugar, the so called homopolysaccharides, or they consist of different sugars units repeating regularly, the heteropolysaccharides (Hols *et al.*, 2005). Most strains of *S. thermophilus* synthesize heteropolymer EPS (Cerning, 1995), but some *S. thermophilus* strains can also be encapsulated (Hassan *et al.*, 1996). The production of EPS by *S. thermophilus* during milk fermentation provides a desirable viscous, smooth texture to the fermented product (Laws and Marshall, 2001), which contributes to mouthfeel and taste perception typically associated with certain fermented dairy products. In addition, production of EPS could contribute to the maintenance of texture properties and avoid syneresis in products with reduced fat levels such as yogurt, sour cream or cheeses (Hols *et al.*, 2005). Most *S. thermophilus* EPS are highly variable but mainly composed of galactose, glucose, and rhamnose; however, polymers containing acetyl-galactosamine, fucose and acetylated galactose moieties have also been reported (Laws *et al.*, 2001). More than 60 different *S. thermophilus eps* gene clusters may exist (Rallu *et al.*, 2002): the mobile genetic elements play a fundamental role in this deep heterogeneity.

Antagonistic compounds produced by lactic acid bacteria are interesting from a research point of view because of their potential use in fermented food and feed. Among the different types of antagonistic compounds produced by this group of bacteria, bacteriocins have attracted increasing interest (Aktypis

et al., 1998). Bacteriocins are proteinaceous or peptidic toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain species. Regarding the bacteriocins produced by lactic acid bacteria, both those active against lactic acid bacteria and non-lactic acid bacteria are of interest: the production of the formers is a potential important property for starter cultures for food and feed, since they could help in suppressing the growth of non-starter lactic acid bacteria; the latter, potentially active against genera like clostridia, listeria, entero-pathogens and undesired microorganism in general, would be the most important regarding food preservation and the development of probiotics, also in the perspective of the general tendency to decrease the use of chemical additives in food product. Bacteriocins of *S. thermophilus* strains, known as thermophilins, are thermostable, active over a wide range of pH values and safe because of the GRAS status of *S. thermophilus*. Although several bacteriocin producing strains of *S. thermophilus* have been reported, only few bacteriocins have been characterized (Iyer *et al.*, 2010):

- thermophilin 347, produced by a yogurt strain, active against *Lysteria monocytogenes* and several LAB species (Villani *et al.*, 1995);
- thermophilin A (Ward and Somkuti, 1995);
- thermophilin T, produced by a *S. thermophilus* strain isolated from feta cheese, active against several lactic acid bacteria strains of different species and food spoilage bacteria, such as *Clostridium sporogenes* and *Cl. tyrobutyricum* (Aktypis al., 1998);
- thermophilin 13, with antilisterial activity (Marciset *et al.*, 1997);
- a bacteriocin from *S. thermophilus* 81, which does not resemble any other *S. thermophilus* bacteriocin because of its broad inhibitory spectrum: it is, indeed, efficient against several *Bacillus* species, *L. monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Yersinia pseudotuberculosis* and *Y. enterocolitica* and because of its heat sensitivity. Moreover, the low sensitivity of *L. delbrueckii* subsp. *bulgaricus* to the isolated bacteriocin suggests that *S. thermophilus* 81, the producer, may be used in yogurt starters (Ivanova *et al.*, 1998);
- a bacteriocin from *S. thermophilus* 580, not thermoresistant with an inhibitory spectrum limited to other thermophilic streptococci, *Brochothrix* and sporulated gram-positive rods (Mathot *et al.*, 2003).

The criteria to recognize a strain as probiotic, which can be used for manufacturing of functional foods, are the survival in gastrointestinal (GI) conditions, non-pathogenicity, proved beneficial health effects and resistance to industrial process (Miquel *et al.*, 2015). Even if most of *S. thermophilus* strains appeared to be sensitive to acid pH and bile salts (e.g. Del Campo *et al.*, 2005), human studies have demonstrated

their ability to survive through the GI tract and transiently colonize it during ingestion (e.g. Elli *et al.*, 2006). Moreover, some strains of *S. thermophilus* have showed the ability to adhere to the intestinal epithelial cells (Junjua *et al.*, 2016), which is an important criterion for probiotic strain selection, as it may increase their ability to colonize the digestive tract and to protect the gut (Uriot *et al.*, 2017). Numerous in vivo studies in human or animal models have also shown beneficial health effects of *S. thermophilus*, such as alleviation of lactose intolerance, since *S. thermophilus* cells are able to produce an active β-galactosidase in the GI tract (Drouault *et al.*, 2002), prevention of gastritis, with the gastroprotective effect attributed to the EPS produced by the *S. thermophilus* strain, which could stimulate the immune system and exert an inhibitory effect on ulcer in the host (Rodríguez *et al.*, 2009) and prevention of infectious diarrhea (e.g. Canani *et al.*, 2007). Regarding the mode of action of *S. thermophilus*, it seems to act mainly through the production of antimicrobial compounds (such as thermophilins), but also through its antioxidant and anti-inflammatory properties or its ability to enhance epithelial barrier function (Uriot *et al.*, 2017).

As previously mentioned, the main role of *S. thermophilus* in milk acidification is to provide a rapid acidification. The rate of acidification is an important technological feature because delay in the acidification time may have severe effects on the quality of the product and economic consequences (Mora *et al.*, 2004). The rate of acidification is a strain-dependent metabolic trait that may be influenced by several factors; the most important are the lactose-galactose metabolism, the proteolytic system and urease activity.

Lactose, the main milk carbohydrate, is transported into the cell of *S. thermophilus* by a permease, LacS, belonging to the glycoside-pentoside-hexuronide–cation symporter family (Poolman *et al.*, 1996); LacS operates as a lactose-galactose antiporter or as a galactoside-proton symport system (Foucaud and Poolman, 1992). Lactose is hydrolyzed inside the cell into glucose and galactose by the β-galactosidase: glucose is metabolized to lactic acid via the glycolytic, Embden-Meyerhof-Parnas pathway, whereas in most strains the galactose moiety cannot be metabolized (Mora *et al.*, 2002) and is excreted into the medium in equimolar amounts with the lactose uptake (Vaillancourt *et al.*, 2002). The inability of *S. thermophilus* to metabolize galactose is not caused by the absence of the genetic information required for the synthesis of the suitable metabolic pathways, since the galactose operon coding for the Leloir pathway enzymes has been described (Vaughan *et al.*, 2001).

The concentrations in milk of the essential amino acids glutamic acid and methionine is 45 and less than 1 mg per liter, respectively (Juillard *et al.*, 1995), meanwhile the requirements of *S. thermophilus* are 200 and 60 mg per liter, respectively (Letort and Juillard, 2001). Consequently, during the growth in milk, *S.*

thermophilus should find complementary sources of amino acids. The proteolytic system of *S. thermophilus* comprises more than 20 proteolytic enzymes that enables *S. thermophilus* to use milk proteins; it is composed of:

- an extracellular cell-anchored protease capable of casein hydrolysis (PrtS);
- a set of amino acid and peptide transport systems required for their import inside the cells;
- a set of intracellular peptidases involved in the hydrolysis of casein-derived peptides essential for various housekeeping processes (Hols *et al.*, 2005).

In milk, Prt^+ *S. thermophilus* strains display two distinct exponential growth phases, separated by a non-exponential one: during the first stage of growth it is observed a decrease in the concentration of several free amino acids of the milk, suggesting that PrtS is not expressed during these initial stages of growth, because *S. thermophilus* relies on the free amino acids and peptides of the milk. When the concentration of these compounds decreases, *S. thermophilus* enters the non-exponential growth phase, during which it synthetizes the PrtS, that is necessary to hydrolyze the caseins of the milk, to supply more amino acids and peptide that sustain the second exponential growth phase (Letort *et al.*, 2002). PrtS is present in only a limited number of strains of *S. thermophilus*: it is essential for the optimal growth of *S. thermophilus* when it is present alone in milk; however, when co-cultivated with a proteinase-positive *L. delbrueckii* subsp. *bulgaricus* strain, *S. thermophilus* can grow using the peptides released by the latter species (Courtin *et al.*, 2002). This cooperation could explain the absence of PrtS in lot of *S. thermophilus* strains (Hols *et al.*, 2005).

1.4 Urease activity of *S. thermophilus*

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel metalloprotein that catalyzes the hydrolysis of urea to yield ammonia and carbamate, which spontaneously decomposes to yield a second molecule of ammonia and carbonic acid. The released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms respectively, and the net effect of these reactions is an increase in pH of the environment that surrounds the urease-positive microorganisms. The reaction is schematically presented in Figure 2.

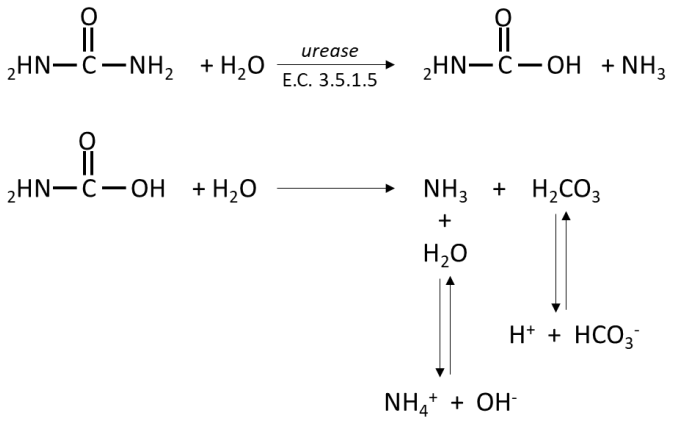

Figure 2. Schematic representation of the urea hydrolysis reaction.

S. thermophilus is the only lactic acid bacterium displaying urease activity; it is a phenotypic trait widely distributed between the species (Spinnler and Corrieu, 1989) and only a few slow or negative urease strains have been described (Mora *et al.*, 2002); within the species of the genus *Streptococcus*, the urease operon is restricted to *S. alactolyticus*, *S. hyontestinalis*, *S. salivarius*, *S. thermophilus* and *S. vestibularis*. Urea is present in milk in concentration ranging from 0.2 to 0.4 g per liter, so, during the milk acidification process, the *S. thermophilus* urease cleaves urea releasing ammonia. These reactions lead to a slower decrease in pH, buffering the lactic acid production during the fermentation process (Mora *et al.*, 2004) (Figure 3).

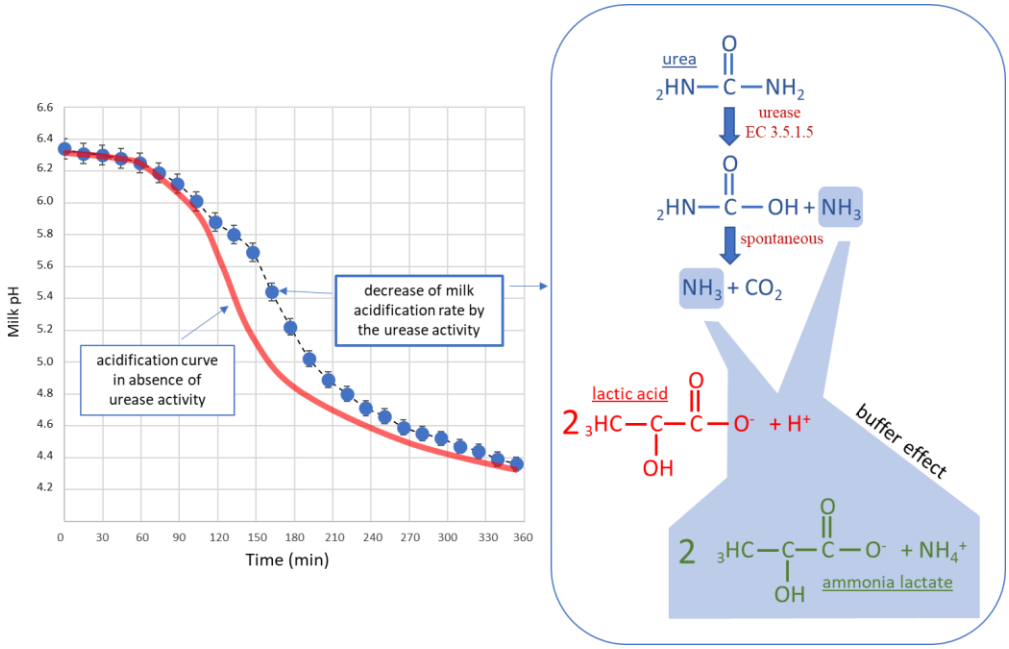

Figure 3. The modelling of *S. thermophilus* milk acidification in presence and absence of urea. The buffering effect on lactic acid due to urease activity is shown in the right panel of the figure.

The influence of urease activity on acidification rate is relevant to various milk fermentation processes: in cheese manufacturing for example, the technological operations (cutting of the curds, stirring, etc.) should take place at given values of pH, but in practice these operations are generally carried out at predetermined times; the variations in acidifying activity caused by urea hydrolysis lead to high variability in the cheeses and defect in their texture, percentage of moisture, ripening (Sepulchre *et al.*, 2005). For example, Martin *et al.* (1997) observed that during the manufacture of Reblochon cheese, the variations in urea content determined changes in the acidification kinetics and in the texture of the final product. Moreover, delays in the acidification process determine the increase of the heating costs of the production, since it is carried out at around 40 °C, the optimal growth temperatures of the microbial starters used. The longer fermentation time and the higher pH could also promote the risk of contaminations by undesirable microorganisms, especially when the dairy process is carried out using raw milk (Mora *et al.*, 2004). Furthermore, it is desirable that the residual whey of the cheese-making process does not contain an excessive amount of ammonia, since it is often used for animal feed. Besides, as previously mentioned, the urea content in milk is variable from one batch to another, especially depending on the feeding of the livestock (DePeters and Ferguson, 1992), determining big difficulties in controlling this phenomenon.

1.4.1 Urease gene cluster of *S. thermophilus*

The organization of the urease operon in *S. thermophilus* shows the same genetic organization observed in the closest phylogenetic neighbor *S. salivarius* (Mora *et al.*, 2004). The urease genes of *S. salivarius* are organized as an operon, beginning with *ureI*, followed by *ureABC* (structural genes) and *ureEFGD* (accessory genes) (Chen and Burne, 2003). In *S. thermophilus*, a nucleotide sequence of the 6700 bp revealed the presence of eight complete ORFs that showed high homologies (97–100% of identity) with the previously characterized urease genes of S. *salivarius*, and also the same organization observed in that species. The eight ORFs have been designated as *ureI* (putative membrane urea transporter gene), *ureA, ureB, ureC* (structural genes), *ureE, ureF, ureG and ureD* (accessory genes) (Figure 4). A putative promoter was identified immediately 5' to *ureI* based on proximity and high sequence conservation with the promoter region described for the *S. salivarius* urease operon (Mora *et al.*, 2004). Later, three further genes, *ureMQO* dedicated to nickel ions transport, were identify and characterized in *S. salivarius* and then in all *S. thermophilus* strains whose genome was sequenced. The urease operon, with its 11 genes, accounts for the 0.9% of the estimated core genome size of *S. thermophilus* (Rasmussen *et al.*, 2008).

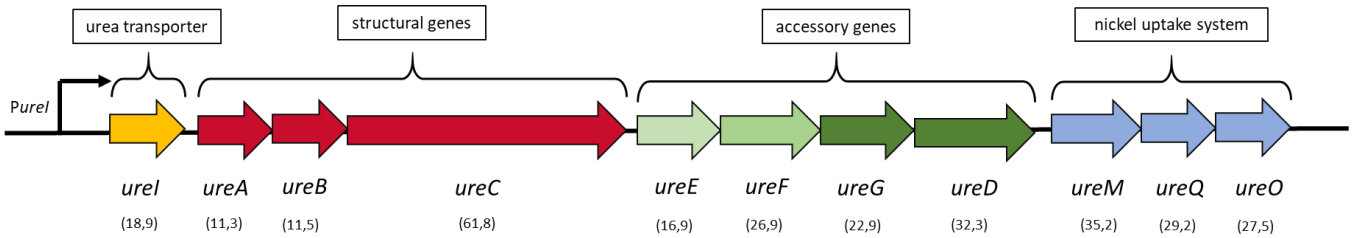

Figure 4. Schematic representation of *S. thermophilus* urease operon organization; the molecular mass in kilodaltons of each gene product is indicated in parenthesis.

1.4.2 Urease biogenesis in *S. thermophilus*

The studies regarding the urease biogenesis show that when *S. thermophilus* is cultivated in M17 broth, urease activity is detectable starting from the late exponential growth phase, when the pH fell below 6, as previously observed in *S. salivarius*, but, in contrast to this close related species, a reduction in active urease biogenesis, but not complete repression, is observed when growing *S. thermophilus* at neutral pH; in this context, the availability of nickel is considered a key factor in the biogenesis of the active urease (Mora *et al.*, 2005). The expression of *ure* genes is not induced by urea, but urea-dependent changes in urease activity can be observed, suggesting that the activity level of the enzyme could be moderately regulated by the presence of the enzyme substrate itself. In contrast to *S. salivarius*, the expression of *ure* genes in *S. thermophilus* is not induced by an excess of carbon source; two possible explanations have been proposed by Mora and colleagues (2005): i) sequence differences in the urease operon promoter region of the two species have been observed; ii) the ecological explanation of the absence of regulation of expression of *ure* genes by carbohydrate availability is that *S. salivarius* colonize the oral cavity, whose carbohydrate availability is strictly depending on diet intake, meanwhile *S. thermophilus* is adapted to the milk environment, where the lactose availability is practically unlimited. Therefore, unlike *S. salivarius*, *S. thermophilus* does not need to regulate the expression of urease, in function of environmental lactose concentration. The increase of urease biogenesis at pH 6 is interpreted as a stress response of *S. thermophilus* metabolism to the acidic environment, but the production of urease at pH 7, although at low level, could be linked to a physiological mechanism of *S. thermophilus* to supply ammonia and carbon dioxide to its metabolism (Mora *et al.*, 2005).

1.4.3 The physiological role of urease in *S. thermophilus* metabolism

Urease activity has been linked to several biosynthetic pathways. The ammonia released from urea has been demonstrated to be used in the glutamine synthesis by Monnet and colleagues (2005): the glutamine

synthesis is essential for the growth of *S. thermophilus*, since the natural sources of glutamine in the milk do not fulfill its requirements, so some glutamine should be produced via the glutamine synthetase. They observed that glutamine addition stimulates the growth of *S. thermophilus* only when the catabolism of urea is inhibited, suggesting that ammonia production from urea is essential for the glutamine synthetase to have a sufficient in vivo activity (Monnet *et al.*, 2005).

Later, urease activity was also linked to aspartate: it was observed that the supplementation of milk with aspartate significantly affected the level of urease activity and that expression of the urease operon was sensitive to the aspartate concentration in milk and to the cell availability of glutamate, glutamine, and ammonium ions, suggesting that the modulation of urease activity should represent a cellular response to a nitrogen-limited condition (Arioli *et al.*, 2007).

Also the carbon dioxide generated by urea hydrolysis finds its role in *S. thermophilus* metabolism. It was observed that when *S. thermophilus* is cultivated in M17 medium with labelled ^{13}C-urea, the labelled CO_2 generated by urea hydrolysis was actively metabolized and detected in a significant amount in cells collected in stationary phase of growth, in particular in their nucleic acids (Arioli *et al.*, 2009).

The role of urease as a stress response to counteract acidic environmental pH, as it is described for several bacterial species (Cotter and Hill, 2003), is of difficult application for *S. thermophilus*, since it is produced also at pH 7 (Mora *et al.*, 2005). Moreover, urease is not a common trait in the close pathogenic streptococci, so its acquisition and maintenance should be linked to how it contributes to the environmental fitness of the species (Arioli *et al.*, 2010). Measuring the intracellular ATP concentration in the presence of D-luciferin via light emission by a bioluminescent *S. thermophilus* strain, it was observed that the light emission was significantly higher when the cells were supplemented with lactose and urea or lactose and ammonia, compared to lactose alone, indicating that urea hydrolysis (or ammonia supplementation) accelerate the glycolytic flux and homolactic fermentation thanks to the intracellular alkalization consequence of the release (entrance) of ammonia into the cytoplasm. These phenomena result in the increasing of the lactose consumption and the lactic acid production, since the glycolytic enzymes have their maximum of activity at alkaline pH. Interestingly, no significant changes in the transcript level of any of the analyzed genes in presence of urea or ammonia have been recorded, suggesting that, in *S. thermophilus*, the modulation of the intracellular pH toward alkaline values, in consequence of urea hydrolysis or ammonia supplementation, and not a transcriptional regulation, represents the main regulatory mechanism of cellular bioenergetics (Arioli *et al.*, 2010).

Since urea hydrolysis increases both intracellular and extracellular pH, in consequence of the rapid diffusion of ammonia outside of the cell, in presence of urea and a urease-positive microorganism, a

urease-negative microorganism should share the environmental benefit derived from the release of ammonia and the related transient local pH increase. Arioli and colleagues (2017) studied the effect of urea hydrolysis by *S. thermophilus* on variations in the intracellular pH and lactic acid fermentation of the urease-negative species *L. delbrueckii* subsp. *bulgaricus* to obtain new information related to the protocooperation interactions of the yogurt consortium. They observed that when a urease-positive *S. thermophilus* strain is co-cultured with a urease-negative *L. delbrueckii* subsp. *bulgaricus* strain:

- the urea supplementation determined not only the increase of the intracellular pH of the *S. thermophilus* population, but also of the *L. delbrueckii* subsp. *bulgaricus* one, in consequence of the release and diffusion of ammonia inside the urease-negative cells;
- like *S. thermophilus*, the homolactic fermentation of *L. delbrueckii* subsp. *bulgaricus* appeared to be boosted by ammonia, as demonstrated by the increased amount of lactic acid produced and lactose consumed, measured in presence of ammonia;
- like *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* showed an optimum of glycolysis and homolactic fermentation at the alkaline pH.

The authors, therefore, propose that urease activity should be considered an altruistic cooperative trait, which is costly for urease-positive species but provides a local benefit to the urease-negative species sharing the same environment, which can take advantage of the release of ammonia (Arioli *et al.*, 2017).

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2. AIMS AND RATIONALE

The aim of this Ph.D. work was to investigate the physiological importance and the overall metabolic implication of urease activity of the dairy bacterium *Streptococcus thermophilus*. It is the only lactic acid bacterium of dairy interest displaying this enzymatic activity and in the light of the fact that *S. thermophilus* genome has evolved mainly through loss of function events but the urease operon, composed by 11 genes, accounts for the 0.9% of its estimated core genome size, it should be involved in some essential physiological functions. It has been previously investigated and demonstrated in laboratory condition this connection and here we tried to move to the natural environment in which *S. thermophilus* exerts its role as starter culture, milk.

Chapter 3 presents the development of an automatized protocol for the monitoring of milk acidification in 96-well plates; the method had been useful whenever there was the need of monitoring several and different acidification conditions and the traditional method of the pH monitoring fail in the management of many samples.

In Chapter 4 it is presented the development of a milk-based medium that allows the discrimination between urease-positive *S. thermophilus* strains and urease-negative ones based on the colonies morphology. Firstly, the effectiveness of the medium was set up using reference strains, then allowed us to easily select urease-negative mutants after UV mutagenesis of urease-positive strains of interest and it was used whenever there was the need of easily distinguishing the two phenotypes.

Then we move to deeply investigate the physiological and ecological role of urease activity and how it contributes to the fitness of the species. In this context, Chapter 5 presents the investigation of this aspects that had been conducted cultivating the species in milk, instead of in model systems, as previously reported in literature.

Chapter 6 is dedicated to the development of a cytofluorimetric protocol for the evaluation of urease activity, using the fluorescent probe 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE): after a development phase, the method was successfully applied, in parallel with standard procedures, to evaluate the quality of a multi strain probiotic product, in terms of enumeration of the *S. thermophilus* population, and of starter culture biomasses, in terms of abundance of urease activity within the populations.

Considering the detrimental effects of urease activity in the industrial milk acidification processes, in Chapter 7 we evaluate the possibility to modify some parameter of the standard production process of the *S. thermophilus* biomass with the aim of reducing the urease activity of the biomass itself.

Chapters 8 and 9 close this manuscript presenting the conclusion of the overall work and the materials produced during this period, respectively.

3. DEVELOPMENT OF AUTOMATIZED FLUORESCENT-BASED METHOD FOR THE CHARACTERIZATION OF *STREPTOCOCCUS THERMOPHILUS* MILK ACIDIFICATION PROFILES

3.1 Introduction

The selection of lactic acid bacteria for dairy applications is mainly based on their ability to rapidly acidify milk. Milk acidification by lactic acid bacteria can be affected by the several metabolic properties that are usually strain-dependent features (Mora *et al.*, 2002). Among them, the most relevant are the presence of cell envelope proteinases (Letort *et al.*, 2002), the auxotrophy towards amino acids (Arioli *et al.*, 2009), the lactose transport (Foucaud and Poolman, 1992), the efficiency of the glycolytic/homolactic fermentation pathway (Hutkins *et al.*, 1987) and the urease activity (Mora *et al.*, 2002; Monnet *et al.*, 2004). Moreover, also milk composition, specifically the urea content, and the milk heat treatment can significantly affect milk acidification. It follows that, in quality control or research and development phases, high number of variables need to be monitored: for example, the simultaneous comparison of several strains or several milk treatments or milk formulations using one or few strains. In this context, nowadays, when many conditions of milk acidification and their replicates have to be analyzed, it is usually used a multichannel pH analyzer (AMS, Guidonia, Rome, Italy), which is currently commercially available up to a 32 measurement channels, therefore allowing the analysis of 16 samples in duplicate at maximum (or a lower number of conditions with higher replicates). So, despite the relative simplicity of pH measurement, in case of need of measuring higher number of different conditions, the multichannel pH analyzer turns to be inefficient. Probes such as the 6-carboxyfluorescein have been used in several applications due to the pH-dependent fluorescence of the molecule, even if the main applications of 6-carboxyfluorescein and its derivative probes are related to the evaluation of intracellular pH (Breeuwer *et al.*, 1996; Sawatari and Yokota, 2007; Arioli *et al.*, 2017). To our knowledge there is only one previous publication (John *et al.*, 2003) in which 6-carboxyfluorescein is used to monitor milk acidification. In particular, the authors developed microplates with integrated fluorescent pH-sensors: each wells of 96-well microplates were coated with a polymer containing covalently bound 6-carboxyfluorescein, the pH-sensitive fluorophore, and sulforhodamin, as reference fluorophore. Here we present a revised and simplified version of the previous methods that allow a complete automation through a liquid handling system, since 6-carboxyfluorescein is added directly in milk. The new protocol was applied to monitor the milk acidification of the dairy bacterium *S. thermophilus* in 96-well plates, with focus on how urease activity affects the acidification profiles.

3.2 Materials and methods

3.2.1 Procedure

The 96-well plates are automatically charged by EPMotion 5070 (Eppendorf, Amburg, Germany); each well expect the filling with 200 µl of sterile milk, previously supplemented with 6-carboxyfluorescein, and then the inoculum. The milk formulation and the characteristics and size of the inoculum can vary, depending on the condition intended to test. The pH-dependent carboxyfluorescein fluorescence is then recorded every 10 min for 24 h using a fluorometer (Perkin-Elmer, Waltham, MA). The optimal concentration of 6-carboxyfluorescein to be used has been set up at 0,8 µM, since lower and higher amount did not allow a stable detection of the milk acidification over the time. The addition of urea to the milk at a final concentration of 10-20 mM, prior to the dispensation of the milk in the wells, is necessary to highlight the pH variation caused by *S. thermophilus* urease activity.

3.3 Results

3.3.1 The automatized fluorescent-based method allows the discrimination of different milk acidification profiles

The monitoring of milk acidification following the pH-dependent fluorescence of the carboxyfluorescein allows the discrimination of the acidification profiles of different *S. thermophilus* strains. Figure 1 shows three explanatory examples. Figure 1A represent the acidification curves of a urease-negative strain, indeed, no differences are observed in the acidification performed in absence (solid line) or presence (dashed line) of urea. Figures 1B and 1C show, instead, the acidification curves of two urease-positive strains, as the increase of fluorescence in presence of urea demonstrates, but the method is sensitive enough to discriminate in the intensity of the urease activity, which is less intense in the case of the strain of Figure 1B compared to the strain reported in Figure 1C.

3.4 Discussion

Nowadays, in the work routine of a laboratory or an industry, the need to have rapid, practical and ready-to-use procedures to analyze specific samples is crucial. The measurement of milk acidification rate represents a kind of analysis of tricky management, not for the difficulty of the procedure, since there is only the need of a pH meter to do it, but for the hard handling of high number of samples and conditions that have to be analyzed at the same time.

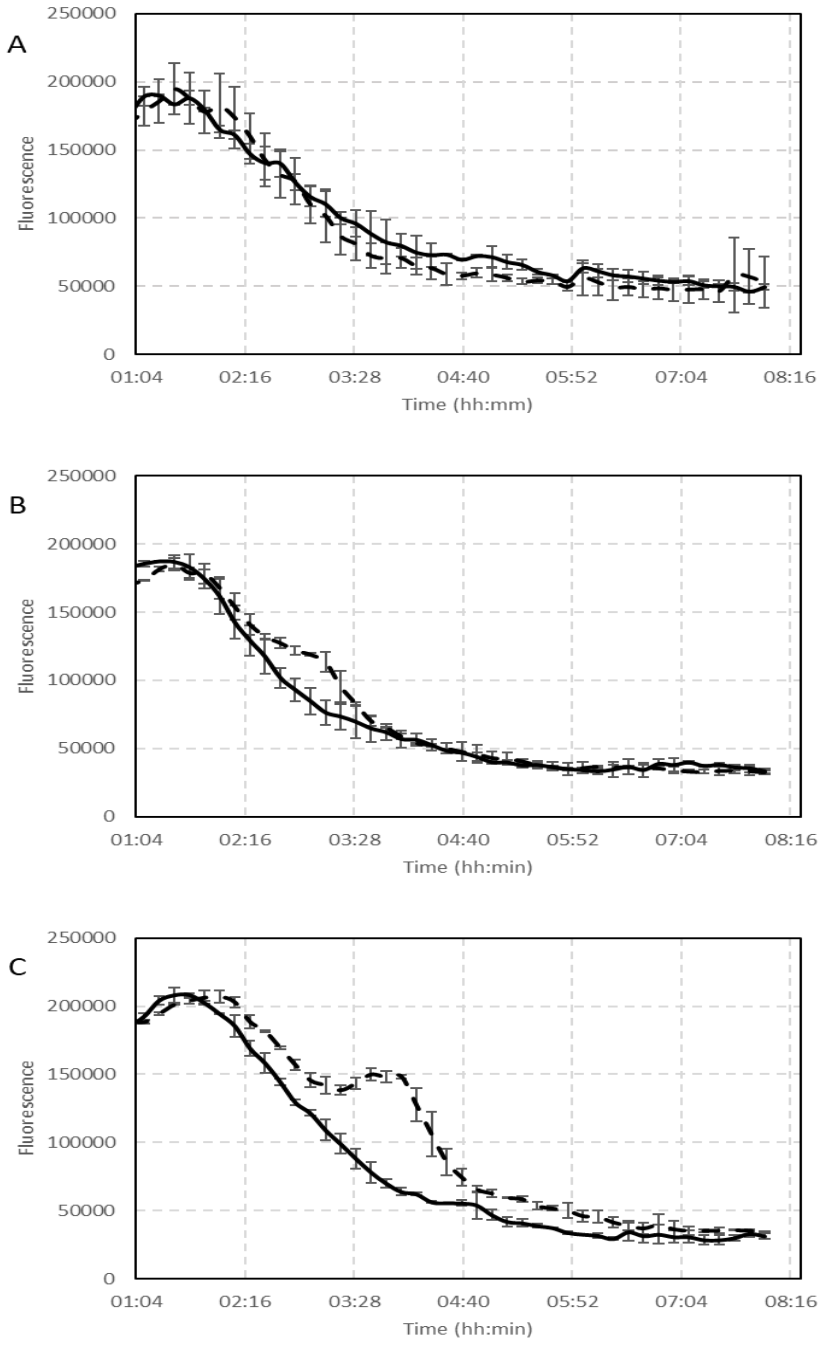

Figure 1. Milk acidification curves built by monitoring the fluorescence of carboxyfluorescein over the time. A) A urease-negative profile; B) and C) urease-positive profiles. The solid line represents the curves performed in absence of urea; the dashed line those performed in presence of urea.

To this purpose, we developed an automatized fluorescent-based method for the monitoring of milk acidification, that allows to work in small volumes (200 µl) in 96-well plate. The method is an indirect measurement of the pH decrease that occur in consequence of the release of lactic acid by the cells in milk, since we are actually monitoring the fluorescence of carboxyfluorescein, whose fluorescence is

pH-dependent. The sensibility of the method is enough not only to monitor the milk acidification but also to discriminate the effects on the acidification curve of urease activity. The possible uses of the method are practically unlimited: whenever there is the need to screen different milk compositions, strains, temperatures and other parameters that can affect the acidification profile.

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4. DEVELOPMENT OF A MILK-BASED MEDIUM FOR THE SELECTION OF *STREPTOCOCCUS THERMOPHILUS* UREASE-DEFECTIVE MUTANTS

4.1 Introduction

Streptococcus thermophilus is one of the most widely used lactic acid bacteria in dairy applications such as yogurt, other fermented milk, and cheeses, for an estimated annual market value of about $40 billion (Chausson and Maurisson, 2002); it is estimated that over 10^{21} live cells are ingested annually by the human population (Bolotin *et al.*, 2004). In this context, *S. thermophilus* has the "Generally Recognized as Safe" (GRAS) and the "Qualified Presumption of Safety" (QPS) status. The main role of *S. thermophilus* in dairy process is to provide a rapid acidification of the milk producing lactic acid from lactose. Lactic acid contributes to milk coagulation and curd draining, imparts a fresh acid flavor and helps to restrain the development of pathogens and spoilage microorganisms (Pernoud *et al.*, 2004). The rate of acidification is an important technological trait because delay in acidification time may have severe effects on the quality of the product or economic consequences in the industrial process (Mora *et al.*, 2004). The rate of acidification is a strain-dependent metabolic feature that may be influenced by several factors; between them, urease activity is one of the main actors. *S. thermophilus* is the only lactic acid bacterium displaying urease activity (Hols *et al.*, 2005): urease is a urea amidohydrolase (EC 3.5.1.5) that catalyzes the hydrolysis of urea to yield ammonia and carbamate, which spontaneously decomposes to yield a second molecule of ammonia and carbonic acid. The net effect of the release of two molecules of ammonia is an increase in pH. When *S. thermophilus* is growing in milk, the production of ammonia from urea, naturally present in milk, slows down the desired acidification, sometimes inducing a temporary increase of pH, thereby prolonging the fermentation time: delays in the acidification time could have detrimental effects on the texture and the percentage of moisture of the fermented products (Martin *et al.*, 1997). It could further lead to the development of contaminants, especially when the fermentation is carried out using unpasteurized raw milk; moreover, delays in the acidification process may increase the heating cost of the production plant, since the fermentation is carried out al 37-42 °C. Furthermore, since milk contains different amounts of urea (ranging from 3 to 6 mM) from one batch to another, a further negative consequence of urease activity is an unpredictable rate of acidification during the fermentation processes (Mora *et al.*, 2004). Considering these aspects and that urease activity is a phenotypic trait widely distributed among the *S. thermophilus* species (Tinson *et al.*, 1982; Spinnler and Corrieu 1989) and that urease-negative strains have been rarely described (Louaileche and Bracquart 2001; Mora *et al.*, 2002), it could be interesting to build a collection of urease-defective mutants of *S. thermophilus* strains, with attractive technological properties for their exploitation in fermentation

processes. For this purpose, the recombinant DNA technology would be an ideal method to eliminate urease as an unwanted property, thanks to its precision and versatility; however, the restricted food legislation and the doubtful consumer acceptance for genetically modified food ingredients discourage the use of this technique (Derkx *et al.*, 2014). In this context, today, all efforts to improve strains for industrial applications are based on natural strategies such as random mutagenesis. This approach is based on the introduction of random mutations into the genome of the strain of interest and the selection of mutants with the desired property (Derkx *et al.*, 2014). This last step is often the hardest in terms of success and use of time. The aim of this study was to develop a new medium for the efficient selection of urease-defective *S. thermophilus* mutants based on their colony morphology and on the physiology of *S. thermophilus*.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

All *S. thermophilus* strains used in this work and all urease-defective mutants selected and characterized are listed in Table 1. All strains were cultivated in M17 broth (DIFCO Laboratories, Detroit, MI) containing 20 g/l of lactose at 37 °C and maintained as cultures supplemented with glycerol (25% v/v final concentration) at – 80 °C.

Table 1. List of *S. thermophilus* strains used in this work

Strain	**Urease phenotype**	**Urease genotype ***	**Reference**
DSM20617^T	urease-positive	functional urease operon	(Mora *et al*., 2004)
A16*(ΔureC3)*	urease-negative	DSM20617^T derivative mutant with an *in frame* deletion of 639 bp in *UreC* gene	(Mora *et al*., 2004)
MIMO1	urease-positive $NiCl_2$-dependent	amino acids substitutions in *ureE* (Asp_{29} → Asn_{29}) and *ureQ* (Asp_{270}→Gly_{270})	this study (MH646550)
MIM13	urease-positive	functional urease operon	this study (MH681782)
MIM22	MIM13 derivative urease-negative	nonsense mutation in *ureA* (Glu_{71} → $Stop_{71}$)	this study (MH716244)
MIM772	urease-positive	functional urease operon	this study (MH681781)

MIM10	MIM772 derivative urease-negative	missense mutation in *ureC* (Pro_{172} →Leu_{172})	this study (MH700461)
MIM12	MIM772 derivative urease-weak	missense mutation in *ureG* (M_{140} →Leu_{140})	this study (MH700462)

* Mutation in urease gene of urease-defective mutants are described using as reference the urease gene sequences of the respective wild-type strain except for strain MIMO1 for which the reference was strain DSM 20617^T.

4.2.2 Measurement of temperature-dependent urease activity and homolactic fermentation

To measure the temperature-dependent urease activity, *S. thermophilus* cells growing in M17 at 37 °C were collected by centrifugation in the late exponential phase of growth ($O.D._{600nm}$ 1.0), washed twice and suspended in saline solution (9 g/l, NaCl). Cell concentration was quantified by flow cytometry (Accuri C6, BD Biosciences, Milan, Italy) as described in Arioli *et al.* (2017). The phenol red assay (Lanyi, 1987) was carried out by mixing 30 µl of solution A (urea, 2 g dissolved in 2 ml of ethanol and 4 ml of sterilized water) to 470 µl of solution B (KH_2PO_4, 1 g/l; K_2HPO_4, 1 g/l; NaCl, 5 g/l; phenol red, 20 µg/ml) and 10^8 *S. thermophilus* cell. Cell suspension, was dispensed (100 µl) in PCR tubes and incubated for 6 h at the following temperatures: 25.0, 30.0, 30.5, 31.7, 33.6, 36.2, 38.8, 41.2, 43.8, 46.4, 48.3, 49.5 and 50.0 °C using a thermal-cycler (Mastercycler Nexus Gradient, Eppendorf, Milan, Italy) with a gradient temperature ranging from 25 °C to 50 °C. After incubation, the development of a red-violet color due to the release of ammonia by urease was measured using a spectrophotometer ($O.D._{555nm}$). Urease activity was expressed as % of the maximum activity using as reference the maximum $O.D._{555\ nm}$ measured. To measure the temperature-dependent homolactic fermentation, *S. thermophilus* was inoculated in liquid milk-based medium avoiding the addition of urea. Cell suspension, was dispensed (100 µl) in PCR tubes and incubated as described above for the evaluation of urease activity. After incubation, the development of a green/yellow color, indicating the milk acidification, or a blue color, indicating the absence of acidification, was recorded.

4.2.3 Milk-based medium for the identification of urease-negative *S. thermophilus* strains

The milk-based medium was formulated with the aim of discriminating *S. thermophilus* strains based on their urease activity. The medium composition was designed with the aim of distinguishing urease-positive and urease-defective strains on the basis of the colony morphology and on the color of the medium surrounding the colonies, consequence of its pH. To prepare the medium, skimmed milk (DIFCO, Sacco srl, Italy) (90 g/l), sucrose (Sigma Aldrich, Milan, Italy) (10 g/l), yeast extract (DIFCO,

Sacco srl, Italy) (1 g/l) and the mix of pH sensitive dyes (50 ml/l) (bromocresol green, pKa <50, 1 g/l, bromocresol purple, pKa 6.3, 1 g/l, NaOH 4 mM) (Sigma Aldrich, Milan, Italy) were dissolved in half of the final volume and sterilized at 110 °C for 15 min. The agar was dissolved in the remaining volume (15 g/l) and sterilized at 110 °C for 30 min. After sterilization, the components were mixed in sterile condition, urea was added at a final concentration of 20 mM, and the medium poured in Petri dishes. To identify the urease-phenotype, overnight cultures of *S. thermophilus* grown in M17 (2% lactose) were diluted in sterile saline solution and plated on the milk-based medium. After incubation of 18 h at 37 °C the plates were further incubated at 25 °C for at least 5 h to allow the slowing-down of the homolactic fermentation and the appearing of the urease activity. If present, it determined the change of the color of the mix of pH indicators, around the colonies, from yellow to blue, due to the ammonia released from urea.

4.2.4 UV mutagenesis and screening of urease-defective strains of *S. thermophilus*

Streptococcus thermophilus MIM13 and MIM772 were cultured in M17 broth containing 20 g/l of lactose at 37 °C, until the culture reached an $O.D._{600\ nm}$ of 0.2. Cells were collected by centrifugation and washed twice in sterile saline solution (9 g/l NaCl). One ml of cell suspension was poured in a Petri dish and exposed to UV lights (distance 10 cm, exposure time 30 s). After UV treatment cells were diluted to obtain theoretically 1 CFU/µl, plated (200 µl) on milk-based medium and incubated at 37 °C for 24-36 h in anaerobic condition followed by at least 5 h of incubation at room temperature in aerobic condition. Based on colony morphology the potential urease-defective mutants were isolated, cultivated in M17 (2% lactose), tested for their urease activity using the phenol red assay, and screened for their ability to coagulate milk. To this aim, M17 cultures, obtained after 24 h of incubation at 37 °C, were used to inoculate (1% v/v) 10 ml of reconstituted skimmed milk (DIFCO, Sacco srl, Cadorago, Italy). After incubation at 37 °C for 12 h milk coagulation was visually quantified.

4.2.5 Determination of urease activity

Urease activity of *S. thermophilus* was evaluated by the phenol red assay described by Lanyi (1987), with some modifications. *S. thermophilus* were collected and suspended in solution A and B as previously described. Cell suspension, was dispensed (150 µl) in a 96-well microtiter plates and incubated at 37 °C for 6 h. The development of a red-violet color due to the release of ammonia by urease was monitored using a spectrophotometer EON (Biotek, Winoosky, VT, USA) that was programmed for readings (O.D. 555 nm) every 15 min, for 6 h, at 37 °C. At the end of the incubation, the urease activity expressed as maximum velocity ($mO.D._{555nm}$/min) was calculated using the software program Gene5 (Biotek,

Winoosky, VT, USA). The assay was performed in triplicate. For qualitative evaluation of urease activity cell suspensions in solution A and B were incubated 24 h at 37 °C. Urease-positive cell suspensions developed a purple color due to ammonia release and the consequent alkalization.

4.2.6 Milk acidification

The acidification rates of the mutants were evaluated in reconstituted skim milk compared to their wild type in absence or presence of 20 mM of urea. Cells from an overnight growth in M17 broth were used to inoculated (1% v/v) 10 ml of pre-heated skimmed milk and incubated at 37 °C until complete coagulation. These cultures were then used to inoculate (1% v/v) 200 ml of pre-heated skimmed milk without or with addition of urea filter sterilized. The pH was measured continuously and recorded every 30 min for 24 hours using a iCINAC system (AMS, Guidonia, Rome, Italy).

4.2.7 PCR protocols and DNA sequencing

Total bacterial DNA was extracted as previously described (Mora *et al.*, 2004) starting from 100 µl of M17 broth culture. The amplification of a DNA regions encompassing the complete urease cluster was performed as recommended by the suppliers (Takara Bio Europe., Saint-Germain-en-Laye, France) using the following primers: UreF 5'- GAGTGTCCAGGCTCCGATAA -3', UreR 5'- CTAAGATACGTAACACCAGA -3', NICKF1 5'- TCCTTAGATATCTCAGGTTTG -3', NICKR1 – 5' TTGTAACAGAATTCACTAAGC – 3'. The PCR conditions were: 35 cycles at 94 °C for 1 min, 56 °C for 35 s and 72 °C for 10 min and a single final extension at 72 °C for 10 min. All amplification reactions were performed in a CFX96 instrument (BioRad, Laboratoires, Milano, Italy). The PCR product was purified (QIAquick PCR Purification Kit QIAGEN, Germany) and sequenced using the above primers followed by primer walking (Microsynth AG, Balgach Switzerland). The sequences obtained was analyzed with BLAST services at the National Center for Biotechnology Information and subsequently manually aligned. The amplification of a DNA region encompassing *ureC* gene was developed to distinguish between *ureC* and the deleted version Δ*ureC*3 was carried out on DNA extracted from M17 cultures of strain DSM 20617^{T}, A16(Δ*ureC*3), and from colonies grown in milk-based medium. PCR was carried out using the following primer set (UreCd0f 5' – CTGTTCATGATCCTATTCAG – 3'; UreCd0r 5' – CAACACCAATAGCTAGGACA – 3') which allow the amplification of a 2439 bp and 1800 bp fragments respectively in the wild-type and in the urease-negative mutant A16(Δ*ureC*3). PCR reactions were performed in a 25 µl reaction mixture containing 1 colony (picked up by a sterile wooden stick), or 50 ng of DNA, 2.5 µl 10× reaction buffer Dream TaqTM, 200 µM of each dNTP, 0.5 mM $MgCl_2$, 0.5 µM each primer and 0.5 U Dream Taq^{TM}

DNA polymerase (ThermoFisher Scientific, Italy). Amplifications were carried out using a PCR-Mastercycler 96 (Eppendorf, Milan, Italy). The PCR mixtures were subjected to the following thermal cycling: initial hold at 95 °C for 3 min, and 39 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 50 s. Amplification products were electrophoresed in 1.5 % (w/v) agarose gel (with 0.2 μg/ml of ethidium bromide) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and photographed.

4.3 Results

4.3.1 Design of a medium showing a different colony morphology for urease-defective mutants

The milk-based medium was designed with the aim of highlighting differences in colony morphology and color of the medium using pH sensitive dyes. Milk contains lactose and *S. thermophilus* catabolizes the glucose moiety through the glycolytic pathway and the homolactic fermentation producing lactic acid. The galactose moiety of the lactose is excreted in the medium. Lactic acid production decreases the pH and in presence of the pH sensitive dyes bromocresol green and bromocresol purple, the medium turns from a blue/green color to yellow. In presence of urease activity, each urea molecule contained in milk is hydrolyzed generating two molecules of ammonia, which exert a buffering effect on the lactic acid produced by the homolactic fermentation. At high urea concentration, the ammonia generated by urea hydrolysis overcomes the lactic acid produced, resulting in medium alkalization, which turns the medium color from yellow to blue/dark blue (Figure 1). After 18 h of incubation at 37 °C the milk-based Petri plates containing the colonies were further incubated at room temperature for at least 5 h to allow the medium alkalization resulting from urea hydrolysis. At room temperature (25 °C) homolactic fermentation strongly slow down whereas the urease activity is still active, maintaining 74% of its maximum activity (Figure 2). The use of sucrose as additional carbon source in the milk-based medium was necessary to obtain larger colonies compared to those just having the lactose of the milk available. The use of an opaque milk-based medium instead of other laboratory media was dictated by the need to have a strong contrast between the color of bacterial colony and the color generated by the pH sensitive dyes in the medium. Further, using a milk-based medium, instead of a laboratory medium, reduces the risk of isolating mutants with secondary mutations that are not able to grow well in the industrial dairy application.

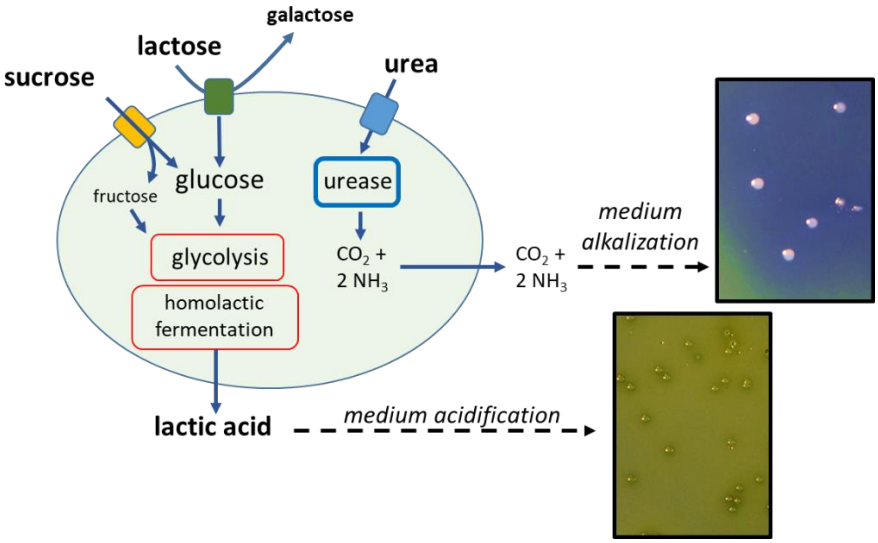

Figure 1. Schematic representation of the physiology of *S. thermophilus* cells cultivated in milk-based medium. As example, pictures of urease-positive and urease-negative *S. thermophilus* colonies grown on milk-based medium are reported.

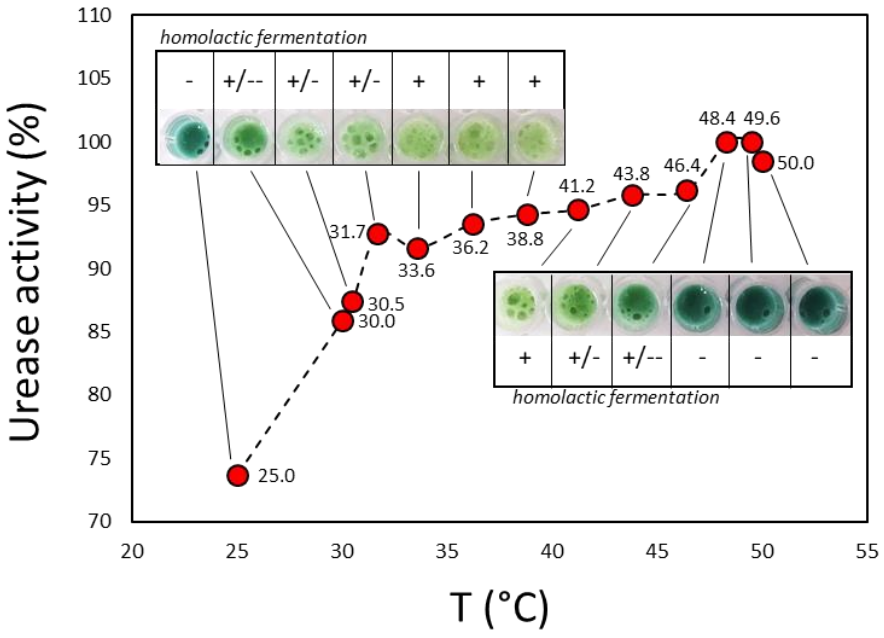

Figure 2. Temperature-dependent urease activity (red circles) and homolactic fermentation (graphical insets) of *S. thermophilus* MIM13. The temperature of each assay is also reported close to the red circles. For the homolactic fermentation the maximum acidification (+) recorded referred to a yellow/green color of the milk-based medium. The absence of acidification (-) referred to a blue-green color of the milk-based medium.

The efficacy of the developed medium to discriminate between urease-positive and urease-defective strains was tested using *S. thermophilus* DSM 20617^T which is urease-positive, and its derivative urease-negative mutant A16(Δ*ureC*3) (Mora *et al.*, 2004) (Table 1). On the milk-based medium DSM 20617^T showed white smooth colonies against a blue background. On the other hand, the urease-negative derivative, strain A16(Δ*ureC*3) showed yellow smooth colonies on a yellow background (Figure 3a, b). To confirm the actual screening procedure to distinguish urease-positive and urease-negative colonies on the same plate, a mixed culture of *S. thermophilus* DSM 20617^T and A16(Δ*ureC*3) was prepared and plated. As shown in Figure 3c the two colony morphotypes were easily identified. The urease phenotype of each colony morphotype was assessed using the colorimetric assay, whereas the genetic identity of the strains was confirmed by PCR (Figure 3d), designed to distinguish the wild-type and the A16(Δ*ureC*3), based on an in-frame deletion of 649 bp in *ureC* gene (Table 1).

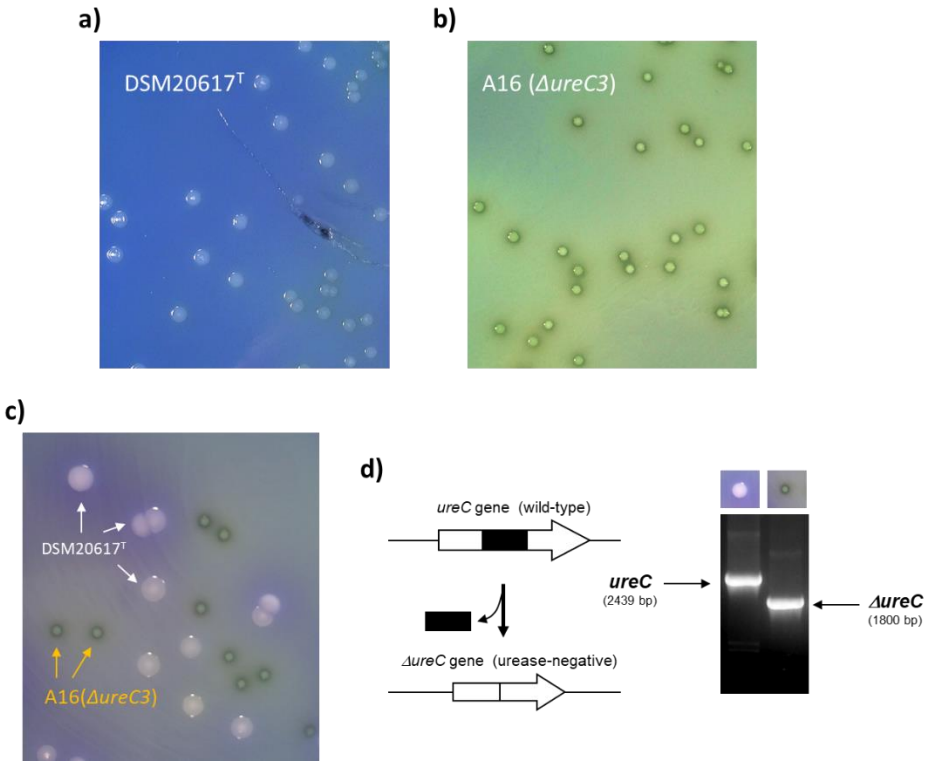

Figure 3. Colony morphology of urease-positive and urease-negative *S. thermophilus* in milk-based medium. a) Colony morphology of *S. thermophilus* DSM 20617^T urease-positive. b) Colony morphology of *S. thermophilus* A16(Δ*ureC*3), a DSM 20617^T urease-negative recombinant. c) Colony morphology of mixed culture of *S. thermophilus* DSM 20617^T and A16(Δ*ureC*3). d) Schematic representation of the genetic strategy adopted to generate the recombinant urease-negative A16(Δ*ureC*3) and example of the PCR assay used to confirm the genetic identity of the two colony morphotypes detected in the milk-based medium.

The discriminatory power of the milk-based medium was also tested on the urease-positive nickel-dependent *S. thermophilus* MIMO1 isolated from commercial yogurt (Mora *et al.*, 2002) and previously

characterized. Strain MIMO1 shows urease activity only if cultivated in presence of 1 µM $NiCl_2$ minimum, due to a defective nickel transport system (Table 1, Table 2). When strain MIMO1 was cultivated on the milk-based medium supplemented with $NiCl_2$, it showed the urease-positive morphology of the colonies, whereas it showed a urease-negative morphotype when nickel was not added to the milk-based medium. Due to the high level of urease activity of strain MIMO1 when cultivated in presence of $NiCl_2$, that determined a high ammonia release, its colonies appeared smaller than those of strain DSM 20617^T, probably as a consequence of the excessive environmental alkalization, as evidenced by the deep blue color of the medium (Figure 4).

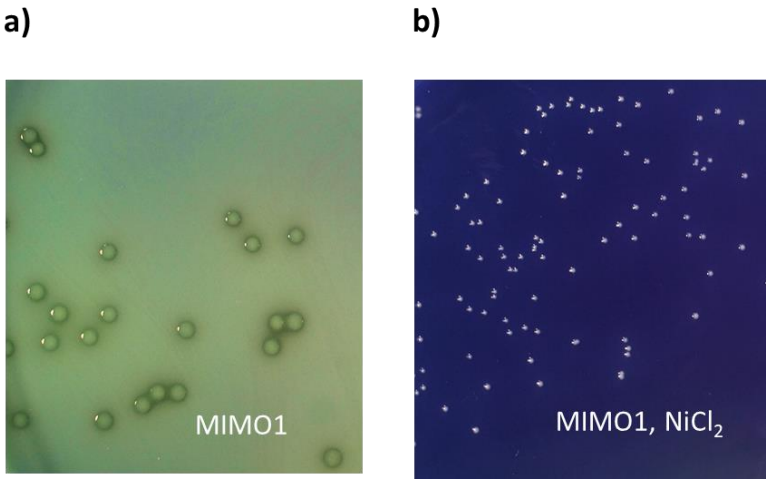

Figure 4. Colony morphology of urease-positive Ni-dependent *S. thermophilus* MIMO1 in milk-based medium without (a) and with addition of 5 µM $NiCl_2$ (b).

4.3.2 Selection and genetic characterization of *S. thermophilus* urease-defective mutants

Two industrial relevant *S. thermophilus* strains, MIM13 and MIM772, were subjected to UV mutagenesis to screen for urease-defective mutants using the above assay. After the mutagenesis process, the UV treated cultures were plated on the milk-based medium and approximately 30 potential urease-negative clones for each strain mutagenized were isolated based on colony morphology and color as above. The potential urease-negative colonies were further investigated through the evaluation of urease activity by the phenol red assay (see Materials and Methods). Only three mutants, showing absence or weak urease activity, maintained also the ability to acidify milk after 12 h of incubation at 37 °C. Two mutants, MIM22 and MIM10, respectively derived from MIM13 and MIM772, were found to completely lack urease activity, meanwhile mutant MIM12 was characterized as having a weaker urease activity than the respective wild-type MIM772 (Table 2). The genetic characterization of the genomic locus encompassing

the urease operon of wild-types and urease-defective derivatives highlighted mutations that justified the observed phenotype. Specifically, sequence analysis of urease operon of mutant MIM22 revealed a nonsense mutation in *ureA* gene (coding for urease gamma subunit) which generates a truncated UreA protein. Sequence analysis of the urease operon of mutant MIM10 showed only a single nucleotide substitution, which determined a missense mutation in the *ureC* gene (Pro172 → Leu172) in a non-conserved *ureC* domain generating a urease-negative phenotype. A single nucleotide substitution determining a missense mutation in the *ureG* gene (coding for urease accessory protein) was identified in the mutant MIM12 characterized by a weak but not absent urease activity.

Table 2. Urease activity of *S. thermophilus* strains and selected mutants

Strain	Urease activity ($mO.D._{555nm}$/min)
$DSM20617^{T}$	2.66 ± 0.09
A16*(ΔureC3)*	nd
MIMO1	nd / 3.4 ± 0.1*
MIM13	4.9 ± 0.3
MIM22	nd
MIM772	3.9 ± 0.4
MIM10	nd
MIM12	1.0 ± 0.1

* $NiCl_2$ was added in the M17 medium at a final concentration of 5 µM. nd, not detected.

4.3.3 Evaluation of the milk-acidification performance of wild-type and urease-defective mutants

The milk acidification assay is the gold-standard for industrial characterization of *S. thermophilus* strains for dairy application. Therefore, all urease-defective mutants selected in this study were tested in absence and in presence of urea (20 mM). The results obtained (Figure 5) clearly show that mutant MIM10 (Figure 5d) performs better than the wild-type MIM772 showing a milk acidification irrespective of the presence of urea, whereas mutant MIM12 (Figure 5e) maintained the urease activity even if postponed of 1.5 h compared to the wild-type MIM772. Mutant MIM22 showed a milk-acidification not affected by the presence of urea even if it did not perform better than the wild-type to reach pH 5 used as reference.

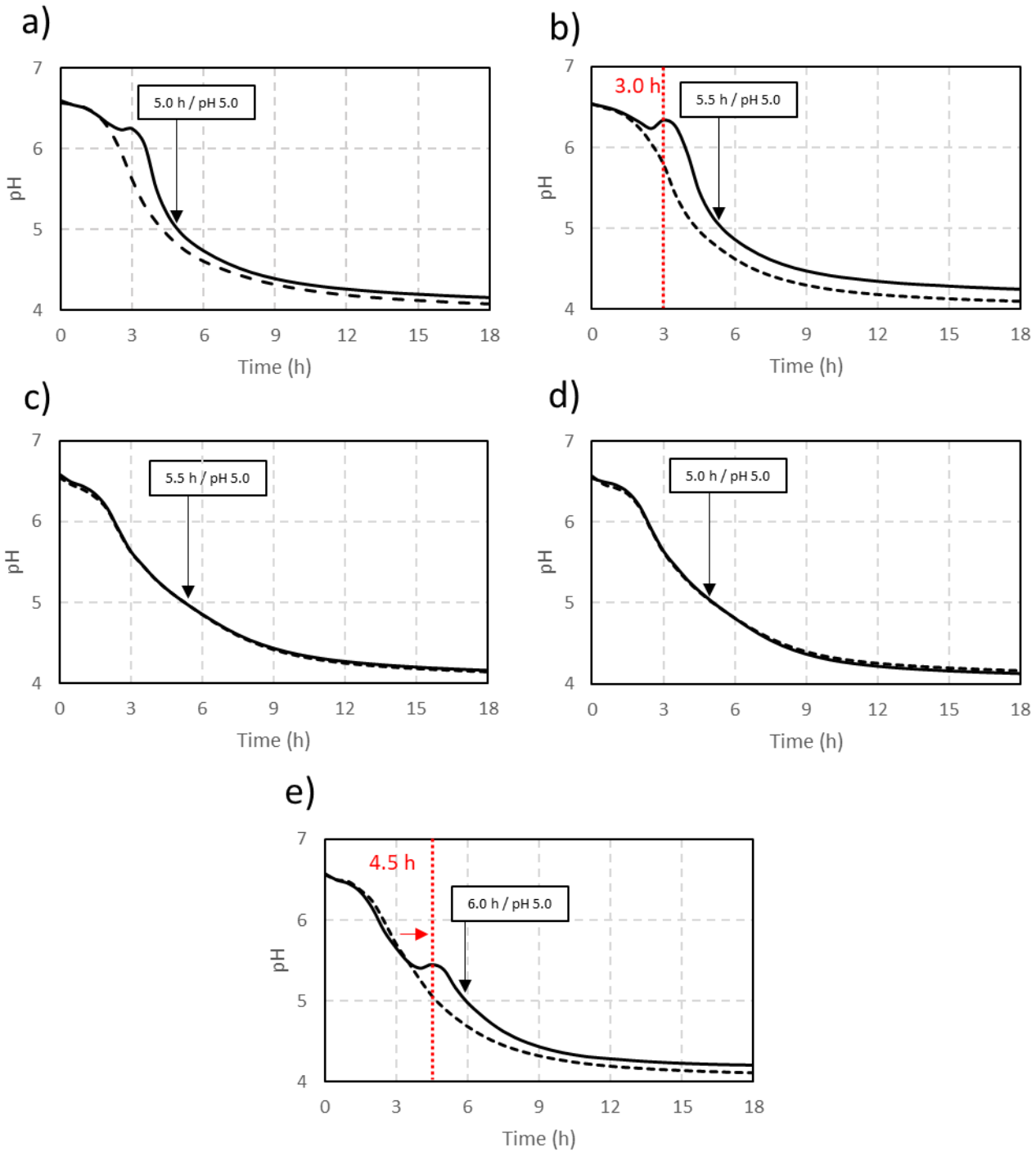

Figure 5. Acidification curves in reconstituted skimmed milk of *S. thermophilus* MIM13 (a) and MIM772 (b) and their derivative urease-defective mutants MIM22 (c), MIM10 (d), and MIM12 (e) in absence (dashed line) and in presence of urea 20 mM (solid line).

4.4 Discussion

The development of efficient screening protocols based on strain-dependent metabolic traits are critical for the selection of new strains for dairy application. In the present study, we aimed to develop a new medium for the identification of urease-defective strains of *S. thermophilus*. Urease activity is one of the most important metabolic factors that influence the milk acidification rate, the most relevant parameter to consider when we deal with industrial dairy fermentations; delays in the acidification rate, indeed, can have effects on product quality and economic consequences. In this context, the selection of urease-defective mutants starting from *S. thermophilus* strains, already chosen for their technological traits and for their industrial relevance, could represent a further improvement of their technological performance. Random mutagenesis, with for example UV, as strain-improvement strategy, was already carried out with industrial lactic acid bacterial species. UV mutagenesis was successfully applied for the selection of *L. delbrueckii* mutants with an enhanced lactic acid production (Kadam *et al.*, 2005) or with an ameliorated utilization of cellobiose and cellotriose (Adsul *et al.*, 2007). Moreover, UV mutagenesis was used to improve *L. rhamnosus* acid tolerance and lactic acid production (Wang *et al.*, 2006), for the selection of a *B. animalis* subsp. *lactis* strain with a decreased ability to produce acetic acid (Margolles and Sánchez, 2012), and to improved *B. breve* viability at low pH (Saarela *et al.*, 2010). The recombinant DNA technology would be a more powerful tool for these purposes but the tight food regulation and the reluctance of consumers toward genetically modified microorganism in foods have kept the random mutagenesis methods in use (Šeme *et al.*, 2017). Monnet and colleagues (Monnet *et al.*, 2004) had already proposed a screening method for *S. thermophilus* urease-defective mutants after a mutagenesis method that expected the use of N-methyl-N′-nitro-N-nitrosoguanidine (NTG). The screening method was based on the formulation of a modified M17 broth on which the mutants were plated and incubated for 2 days; then, a top agar solution had to be prepared and a further incubation was needed. Here we propose a new simpler milk-based medium that allows to screen urease-defective mutants of *S. thermophilus* directly on the plates after the proper incubation time. Moreover, the method has been validated, using genetically characterized urease-positive and urease-negative *S. thermophilus* strains and it was applied for the selection of urease-defective mutants derived from industrially relevant strains. In this case, the application of UV mutagenesis with the new developed selection process allowed to obtain three urease-defective mutants, one of which showed a milk acidification rate faster than the urease-positive wild-type.

4.5 References

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5. EFFECT OF UREASE ACTIVITY ON *STREPTOCOCCUS THERMOPHILUS* PHYSIOLOGY DURING MILK ACIDIFICATION

5.1 Introduction

Streptococcus thermophilus is one of the most widely used lactic acid bacteria in dairy applications such as yogurt, other fermented milk, and cheeses; its main roles in milk fermentations are to provide rapid acidification and the development of texture and flavor. *S. thermophilus* is a "Generally Recognized as Safe" (GRAS) microorganism, even if it is so closely related to harmful human pathogens, such as *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae*. *S. thermophilus* evolved from those closely phylogenetically related pathogenic streptococci through loss-of-function events counterbalanced by the acquisition of relevant traits, such as lactose and urea utilization (Bolotin *et al.*, 2004), that have allowed the assembly of new genomic organization suitable for the colonization of the dairy niche (Mora *et al.*, 2013). Regarding the lactose utilization, it is interesting to notice that a specific symporter for lactose, the main milk carbohydrate, is present in the *S. thermophilus* genome but absent in other streptococci (Bolotin *et al.*, 2004). Urease is a complex enzyme coded by a 11 genes operon that accounts for 0.9% of the estimated core genome size of *S. thermophilus* (Mora *et al.*, 2004; Rasmussen *et al.*, 2008) and it has been found in all *S. thermophilus* characterized. If we consider that *S. thermophilus* genome has evolved mainly from close phylogenetically related pathogenic streptococci following a divergent evolutionary path through loss-of-function events that have allowed the assembling of a new "regressed" genome suitable for the colonization of dairy niche, the urease operon should display an essential physiological function (Mora *et al.*, 2005). In the light of these considerations, the maintenance of urease activity within the species *S. thermophilus* is certainly dependent on how it contributes to the fitness of the microorganism in its environment. Urease is a multi-subunit urea amidohydrolase (EC 3.5.1.5) that catalyzes the hydrolysis of urea to yield ammonia and carbamate. It has been previously observed that urea hydrolysis increases the catabolic efficiency of *S. thermophilus* by modulating the intracellular pH and increasing the activity of β-galactosidase, glycolytic enzymes and lactate dehydrogenase. Moreover, the transient local pH increase due to the ammonia release positively affects the cell bioenergetics of urease-negative microorganisms sharing the same environment of a urease-positive *S. thermophilus* (Arioli *et al.*, 2010). In particular, this is true also for the species *Lactobacillus delbrueckii* subsp. *bulgaricus*, which composes the yogurt consortium together with *S. thermophilus* (Arioli *et al.*, 2017). The previous observations regarding the metabolic role of urease activity in *S. thermophilus* and in mixed bacterial communities have been conducted working with model systems or with cells in saline solution;

in this study, we aim to corroborate those studies analyzing the effect of urease activity on *S. thermophilus* cells growing in milk.

5.2 Materials and methods

5.2.1 Effect of urease activity on *S. thermophilus* metabolism during milk acidification

S. thermophilus strain MIM13 was inoculated (1%) in skimmed milk with 4 mM ^{13}C-urea, with or without the addition of flurofamide, a urease inhibitor (Pernoud *et al.*, 2004), at final concentration of 5 μM. The milk fermentation was monitored recording the pH every 15 minutes for 6 hours. A focus on the first 3 hours of fermentation has been made: samples have been collected every 15 minutes and maintained at -80 °C waiting for further investigation. On each sample, the total cell count by flow cytometry and the NMR quantification of urea, lactose and lactic acid were performed.

5.2.1.1 Flow cytometry total cell count

Total cell count of *S. thermophilus* samples were measured by flow cytometry. 500 μl of milk culture was subjected to a clarification procedure by adding an equal volume of Tris–HCl (2 M) EDTA (0.2 M) buffer (pH 8). After 10 min of incubation at 50 °C, the cell suspension was labelled with SYBR green I (Sigma-Aldrich, Milan, Italy) at 37 °C for 15 min. The labelled cell suspension was diluted to reach approximately 10^6 events per ml and analyzed by flow cytometry. The obtained data were analyzed using BD Accuri™ C6 software (BD Biosciences, Milan, Italy). The SYBR green I fluorescence intensity of stained cells was recovered in the FL1 channel. Density plots of green fluorescence (FL1) vs. FSC (forward scatter) allowed for optimal distinction between the SYBR green I stained microbial cells and instrument noise or sample background. An electronic gate on the green fluorescence/FSC density plot was used to select and measure the total bacterial concentration (events per ml).

5.2.1.2 ^{13}C Nuclear Magnetic Resonance Spectroscopy.

The ^{13}C NMR spectra were recorded on a Bruker AV600 spectrometer operating at a frequency of 150.93 MHz, equipped with a 5 mm TXI inverse probe and z -axis gradients at 25°C. The NMR experiments were performed using a 5-mm NMR tube containing 600 μl of solution. All ^{13}C NMR spectra were referenced to a 5M D_2O external solution of ^{13}C sodium formiate set to 172 ppm. The deuterium in the capillary was enough for the lock system without changes in the concentration of the considered samples. ^{13}C NMR acquisition parameters for all samples were: 10 μs acquisition pulse 60°, 14 s relaxation delay, 37879 Hz spectral width; 0.9 s acquisition time, 400 scans, 64 K time domain. Proton broad-band decoupling was achieved by a waltz-type pulse sequence. Chemical shifts (δ) were measured in ppm.

The NMR spectra were transformed with a line broadening (LB: 6.0 Hz and GB: 0.02) by TOPSPIN software and the baseline was corrected using a polynomial function. Standard solutions of different concentrations of lactose (30, 40 and 50 g/l), urea (1, 2, 4, 8 g/l) and lactic acid (3.05, 6, 12 g/l) were prepared for the quantitative measurements. Selected signals were deconvoluted by Lorentzian lineshape in order to calculate their areas. In details, the signals lying at 104 ppm and 97 ppm for the lactose solutions and lying at 164 ppm and 21.7 ppm for the urea and lactic acid solutions respectively, were considered. Different calibration lines were obtained by plotting the ratio of the areas of these ^{13}C signals of standard solutions and the area of the reference signal *vs* the concentration expressed in g/l. The complete assignment of all carbon signals was performed following the literature (Lu *et al.*, 2016).

5.2.2 Effect of urease activity on energetically discharged *S. thermophilus* cells (EdCs)

Energetically discharged *S. thermophilus* cells (EdCs) have been prepared as described by Arioli *et al.*, (2010): MIM13 was cultivated in 500 ml of M17 broth containing 20 g/l of lactose until O.D. $_{600\ nm}$ of 1.0 was reached. The cells were washed and concentrated 10-fold in 9 g/l of NaCl and 100 μg/ml of chloramphenicol: chloramphenicol at the concentration used here was expected to block translation, so that only the enzymes already present within the cells are supposed to participate in the experiment (Lopez *et al.*, 1998). The cells suspension was deenergized through an incubation at 37 °C for 30 min in presence of 10 mM urea; after incubation the cell suspension was washed four times with the saline solution previously described and called energetically discharged cells (EdCs). With the aim of verifying how urease activity affects the energetic metabolism, EdCs were divided in three batches and the pH was monitored for 20 minutes in consequence of the addition of: i) lactose 14 mM; ii) urea 1 mM; iii) lactose 14 mM and urea 1 mM.

5.2.3 The role of urease activity in the competition between a urease-positive *S. thermophilus* strain and its urease-negative mutant

The wild-type urease-positive MIM13 and its urease-negative mutant MIM22 were mixed with ratio 1:1 in sterilized reconstituted skimmed milk supplemented with 4 mM urea, with and without the addition of 5 μM of flurofamide. After overnight growths at 37 °C, each day, for 10 days, milk subcultures were prepared. The relative abundance of the urease-positive and the urease-negative strain was assessed, every day, by dilution and plating on the milk-based medium previously described (Chapter 4) that allowed the discrimination of the two types of strains based on the colony morphology. Figure 1 shows the experimental setting.

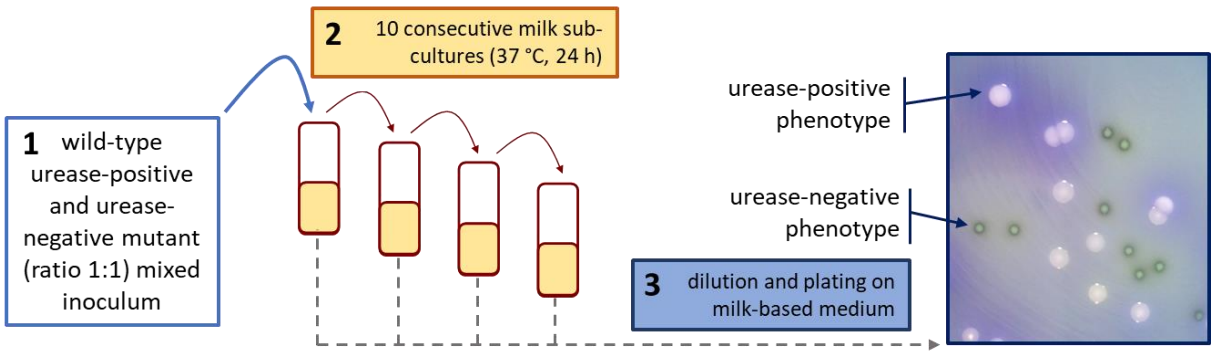

Figure 1. Experimental design of the competition experiment.

5.3 Results

5.3.1 Urease activity stimulates *S. thermophilus* metabolism

The role of urease activity in *S. thermophilus* energetic metabolism has been previously investigated but always working in model systems or with cells in saline solution (e.g. Arioli *et al.*, 2010; Arioli *et al.*, 2017). Our goal was to investigate the physiological role of urease of *S. thermophilus* growing in milk. The addition of 4 mM of urea to the milk simulated the average amount of the urea naturally present in milk; the use of flurofamide allowed us to compare the metabolic effect consequence of the presence of an active urease (no flurofamide added) and the absence of an active urease (5 μM of flurofamide added). Flurofamide has been previously described to inhibit urease, since one of its moiety has a structure like the urea molecule, it binds to the combining site of urease, substituting to urea itself (Kenny, 1983). Panel A of Figure 2 shows the acidification curves built by recording the pH every 15 minutes, with focus on the first 3 hours of fermentation; as expected, when urease is active, the ammonia released by urea determined a slower decrease of the pH, if compared to the condition in which flurofamide is present. The samples collected during the acidification allowed us to evaluate the effect of urease activity on the total cell count, measured by flow cytometry after labelling the cells with SYBR green I and on the metabolites consumption and production, with focus on the amount of urea and lactose consumed and lactic acid produced, measured by NMR quantification. It is interesting to notice that concomitantly with the decrease of milk acidification and the beginning of urea hydrolysis (Figure 2B), we measured a significant increase in cell count (Figure 2C), supported by the data of the doubling time of the cells, that resulted higher when urease activity was present, together with the increase of lactose consumption (Figure 2E) and lactic acid production (Figure 2D).

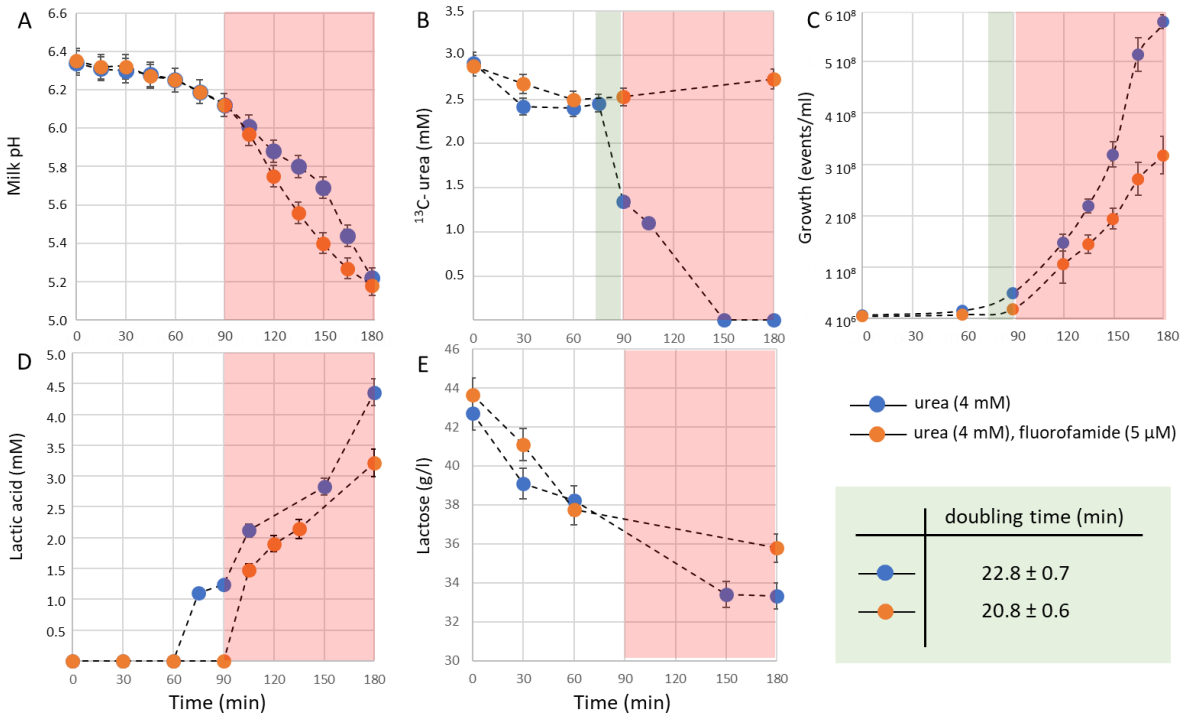

Figure 2. *S. thermophilus* MIM13 growth in milk supplemented with ^{13}C-urea, with and without the addition of the flurofamide. The shaded pink area referred to the time interval where the buffer effect due to urea hydrolysis is detectable by measuring the milk pH. The green shaded area referred to the start of urea hydrolysis as detected by NMR measurement of ^{13}C-urea. The doubling time has been calculated in the time interval 90 - 165 min.

Urea hydrolysis started approximately before 90 min of incubation when pH is above 6. All urea in milk was consumed within the first 150 min of incubation, therefore we speculate that the physiological role of urease is restricted to the first 150 min of growth in milk. Previously, it was demonstrated (Arioli *et al.*, 2010) that urea hydrolysis positively increases the activity of β-galactosidase and lactate dehydrogenase in *S. thermophilus*. Moreover, by measuring the intracellular ATP production it was observed a positive effect, exerted by urea hydrolysis and/or ammonia supplementation, to the overall glycolytic activity. We therefore hypothesize that ammonia generation by urease activity, and the consequent increase of intracellular pH, could positively affect the activity of all the enzymes involved in the energetic metabolism, thus resulting in the effect recorded in our experiment.

5.3.2 Urease stimulation of metabolism is independent of *S. thermophilus* growth

Although urease activity has been historically classified as a stress response that is activated to counteract acidic environmental pH in several bacteria (Cotter and Hill, 2003), here we propose that urease should be considered a metabolic regulatory mechanism for energetic metabolism in the dairy bacterium *S. thermophilus*. To corroborate this consideration, we prepared energetically discharged *S. thermophilus* cells (EdCs) in non-growing status and with gene translation blocked by high chloramphenicol

concentration and we exposed them to urea, lactose and the mixture of the two compounds: we observed that urease activity can dramatically boost the homolactic fermentation when both urea and lactose were supplemented. As it is shown in Figure 3, while urea hydrolysis by EdCs determined only a dramatic pH increase (from pH 6.67 to pH 8.72 in 12 minutes), when EdCs were supplemented with lactose the homolactic fermentation was not efficient and determined a minimal acidification (ΔpH of 0.03). Meanwhile, when EdCs were supplemented with both lactose and urea, we assisted, after an initial alkalization, to a rapid pH drop with a ΔpH of 1.78, thereby indicating that in presence of urea hydrolysis the homolactic fermentation was dramatically boosted.

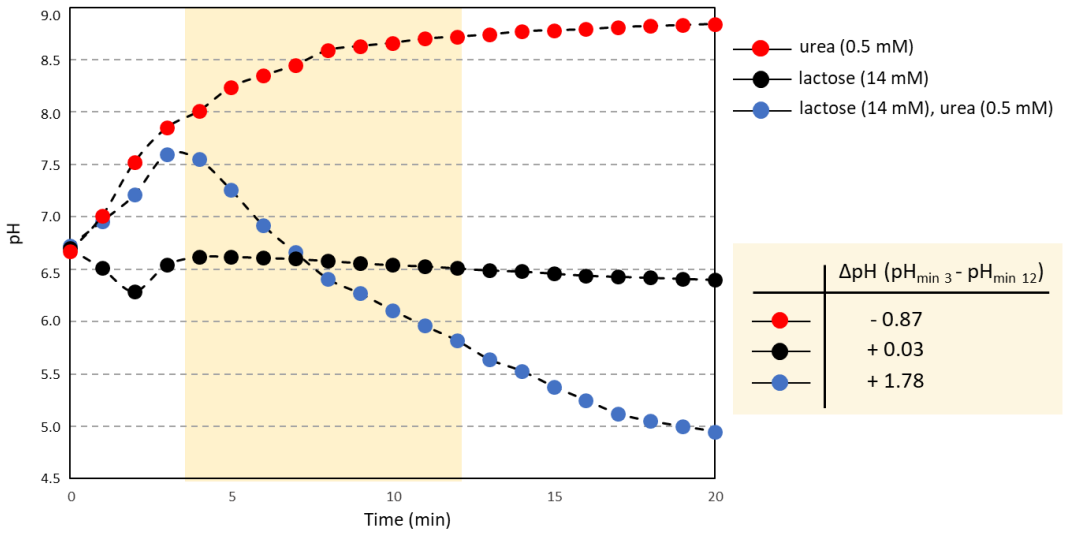

Figure 3. pH kinetics of EdCs in presence of urea, lactose or urea and lactose together.

5.3.3 Urea hydrolysis in mixed bacterial community

To investigate the role of urease activity when a urease-positive strain is co-cultured with a urease-negative strain we performed a competition experiment in milk: we inoculated a urease-positive *S. thermophilus* strain together with its urease-negative mutant, assessing, every day for 10 consecutive subcultures, the relative abundant of each population. The use of 4 mM urea and the addition/no addition of 5 µM of flurofamide replicates the condition previously described (Paragraph 5.3.1). The cooperative role of urease was previously demonstrated for the yogurt consortium (Arioli *et al.*, 2017). Figure 4 shows that both in presence or in absence of flurofamide, the urease-negative strain took the lead after few days, but with a higher relative abundance when flurofamide was added to milk and urease activity was inhibited. These results underline that: i) the ammonia released positively affects the metabolism of

the urease-negative mutant which is sharing the same environment of the urease-positive strain, ii) when urea hydrolysis is inhibited by flurofamide, we can speculate that both the wild-type and the mutant lose the advantage of ammonia released by an active urease, but the former suffers from the energetic costs of urease biosynthesis, since eleven genes are involved for the biosynthesis of the active enzyme.

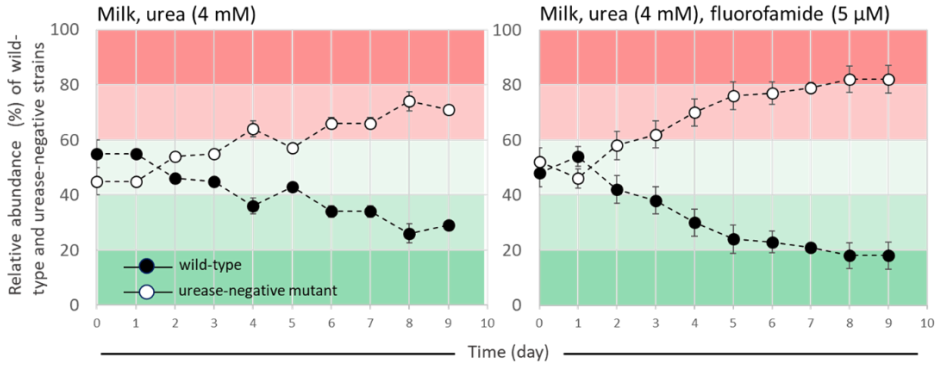

Figure 4. Competition between *S. thermophilus* MIM13 and its urease-negative mutant MIM22 in milk during 9 subcultures.

5.4 Discussion

In the present study we investigated the metabolic role of urease activity, with focus on the energetic metabolism, in particular when *S. thermophilus* is growing in milk. Urease has been always considered a stress response to acidic environmental pH in several bacteria species (Cotter and Hill, 2003); this observation could be confirmed in *S. thermophilus* by the strong induction of urease biogenesis at pH 6 (Mora *et al.*, 2005). Lately, the studies on the urease activity on *S. thermophilus* had been focused, instead, on the proposal of urease as contribution to the environmental fitness of this microorganism (Arioli *et al.*, 2010; Arioli *et al.*, 2017), supported by the genomic analysis on *S. thermophilus* that highlighted that the 11-genes urease operon, that accounts for the 0.9% of the estimated core genome size of the species (Rasmussen *et al.*, 2008), is not common in close phylogenetically related pathogenic streptococci (Bolotin *et al.*, 2004), so its acquisition and maintenance within the *S. thermophilus* genome is likely dependent upon its involvement in some essential physiological function. Our results let us support the concept that urease activity should not be considered a stress response to low environmental pH in *S. thermophilus*: indeed, as it is shown in Figure 2A and 2B urea hydrolysis starts when pH above 6, approximately after 75 minutes of fermentation. The observed increases in the lactose consumption and lactic acid production suggested that urease activity contributes to the fitness of the species, by

modulating the intracellular pH toward alkaline values, that are optimal to the efficiency of the glycolytic enzymes (Arioli *et al.*, 2010). Since all the urea is consumed within the first 150 minutes of fermentation, we speculated that the physiological role of urease is restricted to the first 150 min of growth in milk, but further investigation would be necessary to verify whether, despite all urea has already been consumed, the ammonia released exerts its beneficial effects for longer time. The contribution of urease to the energetic metabolism has been demonstrated also by the pH kinetics of EdCs treated with lactose, urea or lactose and urea together. We observed that cells which do not contain any metabolic intermediate, in consequence of their energetic discharge, have their homolactic fermentation boosted only in presence of the mixture of the molecule, meanwhile when they were supplemented only with lactose we assisted to a very slight acidification (Figure 3). The pH drop obtained activating the energetic metabolism of EdCs with lactose and urea strongly suggested that the positive effect of urea hydrolysis on the glycolytic pathway and homolactic fermentation is strictly associated to the intracellular alkalization generated by the release of ammonium ions in the cytoplasm. It has been previously demonstrated, with a similar approach, that if sodium oxamate is added to EdCs, together with lactose and urea, no ATP generation is recorded, confirming that the urea-dependent ATP synthesis, that in our experiment is observed as the acidification produced by EdCs, is generated by the activity of glycolytic enzymes (Arioli *et al.*, 2010). Sodium oxamate is an analog of pyruvate that competitively inhibits lactate dehydrogenase and it is therefore considered a glycolytic inhibitor in a homofermentative bacteria (Liu *et al.*, 2001). *S. thermophilus* urease activity has been previously proposed to be considered an altruistic cooperative trait, which is costly for urease-positive species but provides a local benefit allowing other species taking advantage of the release of ammonia (Arioli *et al.*, 2017); here we investigate the role of urease activity in the colonization of milk when a urease-positive *S. thermophilus* strain is co-cultured together with a urease-negative one, after the cooperative role of this enzymatic activity has already been established for the species of the yogurt consortium (Arioli *et al.*, 2017). The obtained results supported the concept of a costly enzymatic activity to harbor, since we speculated that the advantage obtained by the release of ammonia has a major impact on the urease-negative strain, since it does not have to produce an active enzyme, in which assembling 11 genes are involved. In conclusion, the data presented here support the concept, previously proposed that urease activity should be taken in consideration for the optimal growth of lactic acid bacteria in milk, for its role of metabolic booster for the species sharing the same environment of the urease-positive one (Arioli *et al.*, 2017).

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6. UREASE ACTIVITY AS A QUALITY CONTROL PARAMETER FOR STARTER CULTURES AND PROBIOTICS

6.1 Introduction

Urease activity plays a key role in the pathogenesis of several bacteria, i.e. *Helicobacter pylori* (Weeks *et al.*, 2000), *Klebsiella pneumoniae* and *Ureoplasma urealyticum* (Burne and Chen, 2000), *Proteus mirabilis*, *Yersinia enterocolitica* and *Salmonella* spp. (Mobley *et al.*, 1995) and *Staphylococcus saprophyticus* (Gatermann *et al.*, 1989), and it has been reported to be involved in diseases such as urolithiasis, pyelonephritis, ammonia encephalopathy, hepatic encephalopathy, hepatic coma, and gastroduodenal infections (Mora and Arioli, 2014); moreover, more recently, urease has also been described as an emerging pathogenic factor during infection of the fungal species *Cryptococcus neoformans*, a basidiomycete, and *Coccidioides posadasii*, an ascomycete (Rutherford, 2014). However, even probiotics can be urease-positive, i.e. *Lactobacillus reuteri* (Wilson *et al.*, 2014), *Streptococcus salivarius* strain K12 (Power *et al.*, 2008) and *Streptococcus thermophilus* (Douillard *et al.*, 2018). *S. thermophilus*, besides the probiotic features, is also one of the most widely used lactic acid bacteria in dairy application (Hols *et al.*, 2005) and the detrimental effects of urease activity in milk acidification processes had been largely described (Martin *et al.* 1997; Mora *et al.*, 2004; Sepulchre *et al.*, 2005). In this context, the present study proposes a cytofluorimetric method for the determination of urease activity of probiotic products and starter cultures biomasses containing/composed of *S. thermophilus* cells. The method should be seen as an innovative tool to put besides the standard ones to evaluate the quality of the product, in terms of enumeration of the cells population in the case of the probiotic product, and in terms of intensity of a relevant technological enzymatic activity in the case of the starter culture biomasses.

6.2 Materials and methods

6.2.1 Evaluation of urease activity by flow cytometry with cFSE staining

A cytofluorimetric protocol for the evaluation of urease activity of various samples containing *S. thermophilus* cells was set up. It expects the use of the pH-sensitive fluorescence probe 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE): the fluorescence intensity of this probe increases at alkaline pH and decreases at acidic pH. The intracellular pH of *S. thermophilus* cells increases in consequence of the release of ammonia from urea by urease activity, so the fluorescence shift recorded after the addition of urea to the cells suspension can be linked to the intensity of urease activity. The samples analyzed have to be washed in saline solution and supplemented with 4 μM cFDASE, which is

a precursor molecule of cFSE. The suspensions were then incubated for 1 hour at 37 °C. During this incubation, the membrane-permeating cFDASE was cleaved by intracellular esterases and the resultant cFSE molecules were conjugated to the aliphatic amines of intracellular proteins. At the end of the incubation time the cells were washed and resuspended in saline solution. The stained cells were then analyzed using an Accuri C6 flow cytometer; the obtained data were analyzed using BD Accuri™C6 software. The cFSE fluorescence intensity of stained cells was recovered in the FL1 channel; density plots of green fluorescence (FL1) and FSC allowed for optimal distinction between the cFSE-stained microbial cells and instrument noise or sample background. The same cell suspensions previously analyzed were exposed to 20 mM of urea and incubated at 37 °C for 10 minutes and then the change of intracellular pH, consequence of the release of ammonia from urea caused by urease activity, was determined by measuring again the fluorescence intensities in the FL1 channel via flow cytometry. Control experiments to verify the operation of the procedure were performed adding, together with urea, 5 µM of flurofamide, a urease inhibitor (Pernoud *et al.*, 2004) or 200 µM of m-chlorophenylhydrazone (CCCP) an uncoupler of the proton gradient of the cells membrane.

6.2.2 Urease activity as quality parameter of a multi-strain probiotic product

The procedure previously set up was applied as method to evaluate the quality of a multi-strain probiotic product. Among the mix of strains composing the probiotic product, *S. thermophilus* is the only displaying urease activity, breaking down urea releasing ammonia, thus increasing its intracellular pH. In consequence of the cFSE staining and the addition of urea, the fluorescence shift that is recorded belong to the *S. thermophilus* population, allowing the evidence of similarity/differences among products/lots of the same products in the amount of the *S. thermophilus* itself and its urease activity. In the specific case of the analysis of a lyophilized probiotic product, the procedure expects a 10-fold dilution of the powder in Mitsuoka buffer (cysteine hydrochloride monohydrate 0.5 g/l; monobasic potassium phosphate 4.5 g/l; sodium phosphate dibasic 6 g/l; Tween® 80 0.5 g/l), homogenization in a Stomacher 400 Circulator (Seward, Worthing, UK) for 2 min at 200 rpm, washing two times in saline solution and then following the procedure previously presented.

6.2.3 FACS analysis, cell sorting and quantification of bacterial species in the cytometric populations

The quality parameter obtained with the cytofluorimetric method was coupled with the quantification of bacterial species composing the probiotic product; the experimental design is shown in Figure 1. First the samples underwent a dual nucleic acid staining with the cell permeant SYTO®24 and cell impermeant

propidium iodide (PI) according to the International Standard ISO19344:2015 IDF232:2015. SYTO®24 permeates the membranes of total cells and stains nucleic acids with green fluorescence meanwhile PI penetrates only bacteria with damaged membranes, causing a reduction in SYTO®24 fluorescence when both dyes are present. Thus, live bacteria with intact cell membranes fluoresce bright green (defined as active fluorescent cells), bacteria with slightly damaged membranes exhibit both green and red fluorescence (defined as slightly membrane damaged cells) and cells with broken membranes fluoresce red (defined as non-active fluorescent cells). Then the different populations underwent a Fluorescence-Activated Cell Sorting (FACS) analysis, performed on a BD FACSJazz™ cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a blue (488 nm, 80 mW) laser. Sorting was performed using a 100 μm nozzle, pressure of 27 PSI and a frequency of 39.2 kHz. PBS (prepared according the ISO 19344/IDF 132) was used as the sheath fluid. The instrument was configured for logarithmic signal amplification for forward scatter light (FSC), for side scatter light (SSC) and for fluorescence detectors. Fluorescence was measured on two channels: FL1 with a 530/40-nm emission filter (SYTO®24) and FL3 with a 692/40-nm emission filter (PI). The rate of sample injection was maintained at <5000 events/s, because high event rates greatly raise the possibility of having double events and cell coincidence. Data were collected for 100 s or for 5,000 (post-sort)–10,000 (pre-sort) counts within the gated population, whichever came first. For analysis of the sorted fraction's purity, collected samples were analyzed under the same conditions. All data were analyzed using BD FACS™ Software v1.2 (BD Biosciences). Sorted cells have been subjected to ultracentrifugation (25000 x g, 30 min) using a OptimaTM Max (Beekman Coulter Milan, Italy), and the biomass obtained was subjected to total DNA extraction as described by (Arioli *et al.*, 2007). Quantification of the species of the probiotic product was carried out by qPCR using primer sets targeted to the single copy gene pyk coding for pyruvate kinase; the primer sequences used to identify the species *S. thermophilus* are GTGGTTACCATGTGAGAAGTTGAAAC (primer forward) and TTGAAATCCGTGGTGGCAAGAAATT (primer reverse). qPCR was performed using 5 ng of DNA in a total volume of 20 μl, by using the EvaGreen™ kit (BioRad, Laboratoires, Milano, Italy) and following manufacturer's recommendations. PCRs were performed in triplicate and run on a CFX96 instrument (BioRad, Laboratoires, Milano, Italy). Data were recorded as threshold cycles (CT), expressed as the mean ± standard deviations, and analyzed using BioRad CFX Manager™ software. A calibration curve that reported the CT vs. number of cells, was obtained and expressed as the Log_{10} Fluorescent Units (FU). To this aim a quantified suspension of each product species (ranging from 10 to 10^6 FU) was subjected to DNA extraction as described before, and the DNA was used as a template in qPCR assays with the appropriate species-specific primer set. To establish the number of viable cells of each species

per g of product the relative abundance of each species (obtained by qPCR assays) was referred to the overall number of viable cells measured using SYTO®24/PI flow cytometry counting.

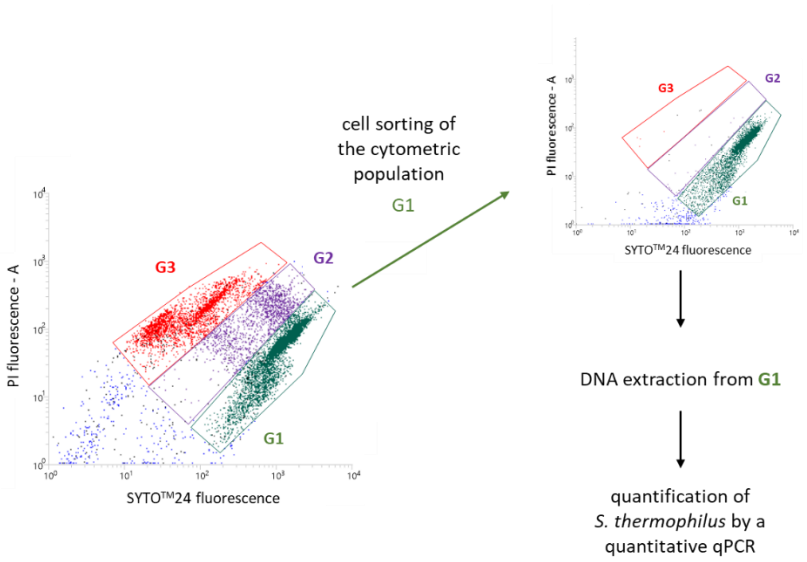

Figure 1. Experimental design of the FACS analysis, cell sorting and quantification of bacterial species in the cytometric populations for the evaluation of the quality of a multi-strain probiotic product.

6.2.4 Urease activity as quality parameter of *S. thermophilus* starter culture biomasses

The procedure previously set up was applied as method to evaluate the quality of two *S. thermophilus* starter culture biomasses, produced following two different production processes, by quantifying their urease activity, which is a metabolic activity that is known to severely affects the acidification capacity of the biomasses themselves. The analysis can be applied both to fresh broth cultures, dry ice pellets or lyophilized powder; prior to the staining, in the first case the cells suspension needs to be washed and resuspended in saline solution, in the other cases the procedure previously explained for the probiotic product can be followed.

6.2.5 Evaluation of urease activity by phenol red assay

The urease activity of the *S. thermophilus* starter culture biomass was evaluated also by the phenol red assay as previously described in Paragraph 4.2.5.

6.3 Results

6.3.1 Cytofluorimetric method to evaluate the urease activity with cFSE staining

Figure 2 reports the validation of the cytofluorimetric method to evaluate the urease activity with cFSE staining of various samples containing the species *S. thermophilus*. We can observe that after the urea addition (4B), part of the population of the sample, increases its intracellular pH, shifting toward higher

fluorescence values, if compared to the cells only stained with cFSE (4A). This is consequence of the release of ammonia by the urease activity of the species contained in the sample and, since *S. thermophilus* is the only harboring this enzymatic activity, we can conclude that we are evaluating the intensity of the urease activity of this species. When flurofamide, a urease inhibitor, is added together with urea (4C), no fluorescence shift is recorded, according to the block of the enzymatic activity caused by this compound. Similarly, if we add both urea and CCCP (4D), which causes the uncoupling of the proton gradient of the cells membrane, no fluorescence shift is recorded, since urea is carried inside the cells by a Urea H^+ permease.

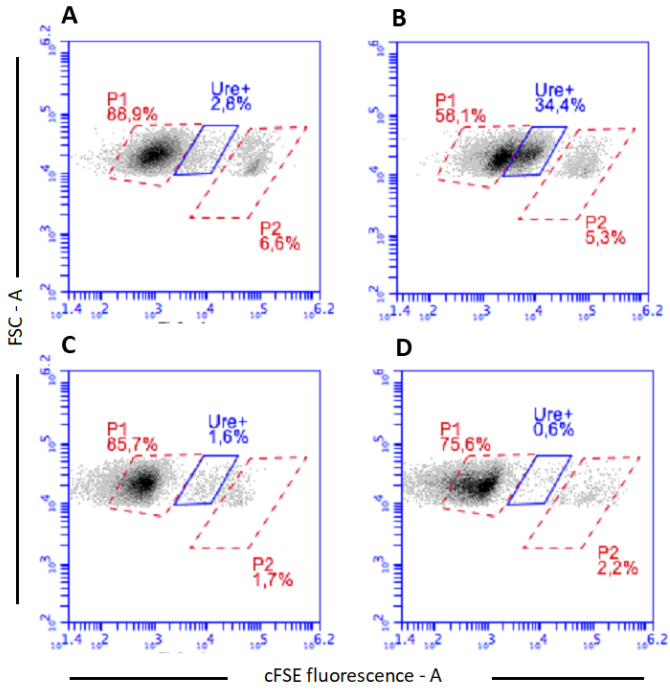

Figure 2. The density diagrams show the green (cFSE) fluorescence levels vs. the FSC of cells. A) The sample stained with cFSE; B) After 10 minutes of incubation with 20 mM of urea; C) After 10 minutes of incubation with 20 mM of urea in presence of 5 µM of flurofamide; D) After 10 minutes of incubation with 20 mM of urea in presence of 200 µM of m-chlorophenylhydrazone (CCCP).

6.3.2 Urease activity as quality parameter of a multi-strain probiotic product

The cytofluorimetric method with the cFSE staining was used to evaluate the quality of a multi-strain probiotic product; specifically, different lots of the same product have been analyzed in terms of amount of the different bacterial species contained in the mixture. In particular, the method previously presented allows to determine the amount of *S. thermophilus* cells in the product, the only one capable of breaking down urea through urease. It was expected to obtain always the same amount of population that increases its fluorescence in consequence of the release of ammonia, since the amount of *S. thermophilus* cells in

the product should be the same between different lots. Figure 3 shows the analysis on four different lots of the same product and it is interesting to notice that between lots 1, 2 and 3 there is a certain correspondence in the amount of *S. thermophilus* population (47.0, 43.8, 43.6 % of the total respectively, calculated as the percentage of the population that increases its fluorescence after the addition of urea), meanwhile lot 4 strongly differs from the previous, and only the 9.1% of the population is represented by *S. thermophilus* cells. This represent a huge quality control manner, since all the lots of the same product should be equally composed. These data were confirmed by the quantification of each species, with focus on *S. thermophilus* population, in the four lots of the product by qPCR after cell sorting of the green populations. As Table 1 shows, the amount of *S. thermophilus* cells in the lot 4 in significant lower compared to the other three lots, thus confirming the failure in the quality production of lot 4.

6.3.3 Urease activity as quality parameter of *S. thermophilus* starter culture biomasses

The urease activity of two *S. thermophilus* starter culture biomasses produced following two different production processes was assessed by the cytofluorimetric method with cFSE staining and supported by the spectrophotometric monitoring of the red phenol assay. The two production processes differed for one parameter in the handling of the bioreactor: the standard (STD) procedure is the one commonly applied to produce *S. thermophilus* biomasses meanwhile the new (NEW) one is the modified one. Figure 4 shows that the standard procedure brought to a biomass with high level of urease activity, since a significant fluorescence increase is recorded after the addition of urea (4B), meanwhile the new procedure determined a biomass insensitive to the addition of urea, since almost no fluorescence shift is recorded (4D). The cytofluorimetric data were supported by the spectrophotometric monitoring of urease activity of the biomasses with the red phenol assay. The data of the maximum velocity of the two biomasses are 20.32±0.09 $m_{O.D.555\ nm}$/min for the one produced with the STD procedure and 1.95±0.07 $m_{O.D.555\ nm}$/min for the NEW one, supporting what was previously observed, that the new production protocol determined a biomass that carries a very little amount of urease activity.

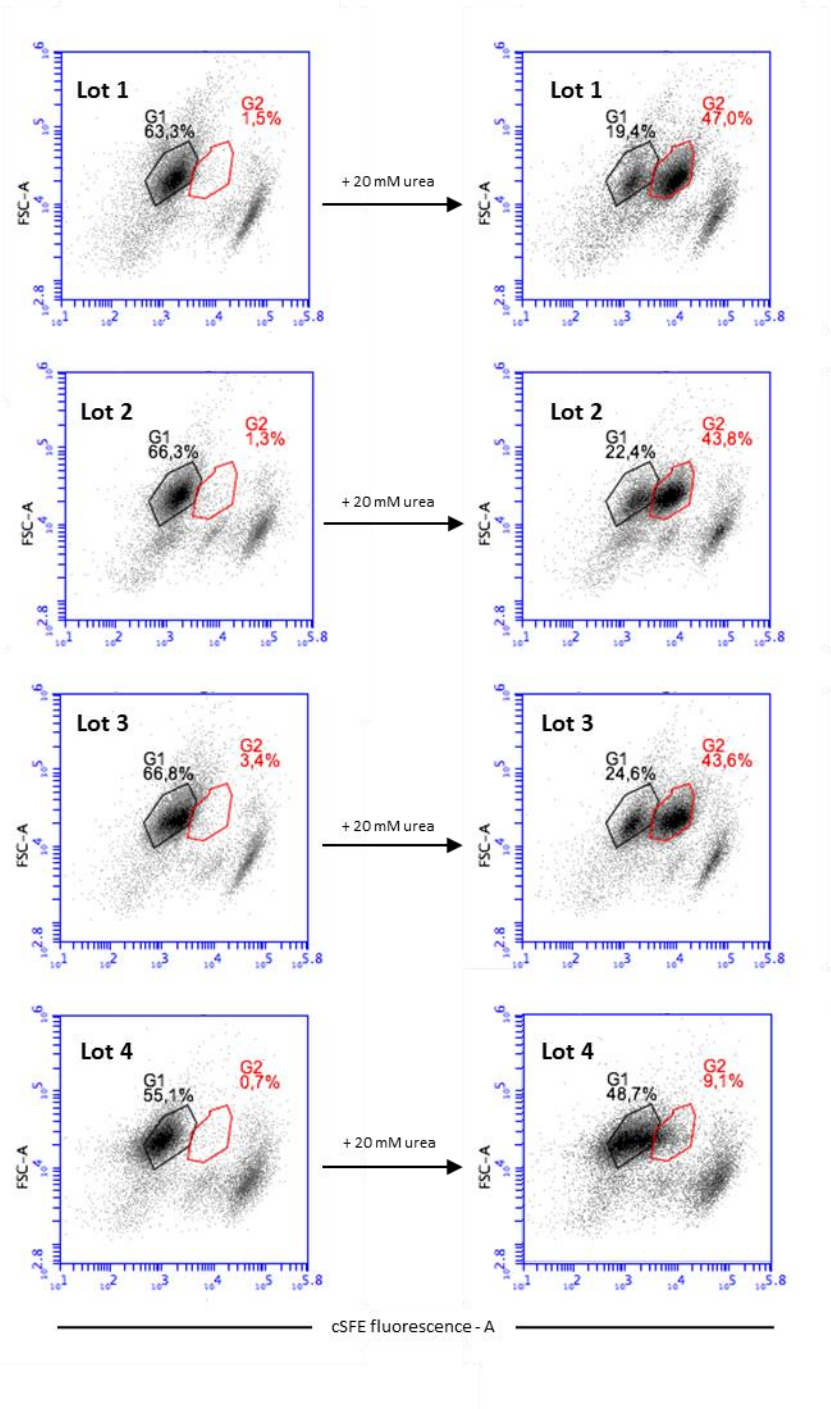

Figure 3. The density diagrams show the green (cFSE) fluorescence levels vs. the FSC of cells of four different lots of the same multi-strain probiotic product stained with cFSE (left panel) and after the addition of 20 mM of urea (right panel).

Table 1. Quantification of *S. thermophilus* population in the four lots of the product by qPCR assay after cell sorting of the green populations.

S. thermophilus	Lot 1	Lot 2	Lot 3	Lot 4
cells/g	1.59E+11	1.75E+11	1.72E+11	4.79E+10

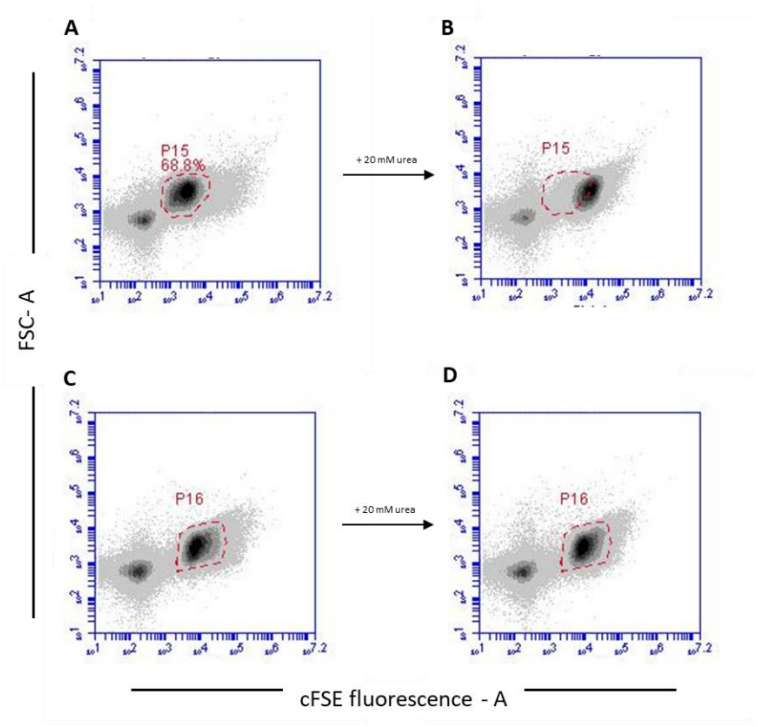

Figure 4. The density diagrams show the green (cFSE) fluorescence levels vs. the FSC of cells. A) The biomass produced with the STD protocol stained with cFSE; B) After 10 minutes of incubation with 20 mM of urea. C) The biomass produced with the NEW protocol stained with cFSE; D) After 10 minutes of incubation with 20 mM

6.4 Discussion

The set up of the cytofluorimetric method to evaluate the urease activity of *S. thermophilus* population, either it is part of a multi-strain probiotic product, either it is a starter culture biomass, represents an innovative tool to characterize the microbial product; it represents a faster and ready-to-use analysis to evaluate the quality of the specific product that can be coupled to standard, but more time-wasteful, protocols. The use of flurofamide and CCCP represents a validation of the method developed and the corroboration of the results by independent analysis strengthen the obtained data. The novelty of the method has to be seen in the possibility to apply it as a routine analysis, which requires a specific instrument, a cytofluorimeter, but provides results within few hours, allowing a real on-line quality monitoring, compared to the days needed in case of other procedures. This led to a better control over the production process, reducing to a minimum the risk that products, like those belonging to the lot 4 (Figure 3), enter the market, despite their inadequacy. In the perspective of the starter culture biomasses, an on-line monitoring of urease activity should be useful to establish range of this enzymatic activity that should not be overcome if a controlled milk acidification is desired, allowing to discard those biomasses that, in consequence of unpredictable problems during the production, results with a too high urease activity.

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7. MODULATION OF UREASE ACTIVITY THROUGH THE CONTROL OF THE FERMENTATION pH

7.1 Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea into ammonia and carbamate, which spontaneously decomposes to yield a second molecule of ammonia and carbonic acid; the net effect of these reactions is an increase in pH of the environment that surrounds the urease-positive microorganisms. *S. thermophilus* is the only lactic acid bacterium displaying urease activity; it is a phenotypic trait widely distributed between the species (Spinnler and Corrieu, 1989) and only a few urease-slow or -negative strains have been described (Mora *et al.*, 2002). Urea is present in milk in concentration ranging from 0.2 to 0.4 g per liter, so, during the milk acidification process, the *S. thermophilus* urease cleaves urea releasing ammonia: these reactions lead to a slower decrease in pH, buffering the lactic acid production during the fermentation process (Mora *et al.*, 2004). The influence of urease activity on acidification rate is relevant to various milk fermentation processes: the variations in acidifying activity caused by urea hydrolysis lead to high variability in the cheeses and defect in their texture (Martin *et al.*, 1997), percentage of moisture, ripening (Sepulchre *et al.*, 2005). Moreover, delays in the acidification process determine the increase of the heating costs of the production, since it is carried out at around 40 °C, the optimal growth temperatures of the microbial starters used. The longer fermentation time and the higher pH could also lead to the development of contaminants, especially when the fermentation is carried out using unpasteurized raw milk (Mora *et al.*, 2004). In this context, this study aims to verify whether change in the production fermentation process of *S. thermophilus* biomasses, at industrial level, could influence the urease activity expressed in milk of the biomasses themselves. To this purpose, the bioreactor's parameter that has been modified was the pH value at which the fermentation is normally carried out. It was previously demonstrated that urease biogenesis strongly increases at pH 6, meanwhile when *S. thermophilus* is cultivated at neutral pH, a reduction in active urease biogenesis, even if not complete repression, was observed (Mora *et al.*, 2005). It is interesting to point out that the pH value at which the *S. thermophilus* fermentations are normally produced is 6: this led us to propose to increase the pH toward an alkaline value, to verify whether the biomasses produced could express a lower urease activity when used to ferment milk.

7.2 Materials and methods

7.2.1 Batch fermentations of *S. thermophilus*

Batch experiments were performed in a 10-l bioreactor filled with 6-l of culture medium. *S. thermophilus* MIM13 was inoculated (3%) as clotted milk. Temperature and rotation speed were fixed at 40 °C and 200 rpm, respectively. The standard procedure of production of *S. thermophilus* biomasses expects that the pH is maintained at 6.0 (STD), meanwhile a further pH has been chosen as modified parameter: pH 6.8 (NEW); the pH is controlled by automatic addition of 15% NH_3. At fixed points, samples are collected and subjected to the online monitoring of the growth by optical density at 600 nm ($O.D._{600\ nm}$) and flow cytometry quantification of live, damaged and dead cells. At the end of the fermentation, established based on the amount of NH_3 consumed, the lyophilized powder of each biomasses had been produced, which represents the starter culture biomass used for dairy applications. The products were evaluated for their urease activity by red phenol assay and by flow-cytometry with cFSE staining and milk acidification performances were also performed.

7.2.2 Monitoring of cells growth by $O.D._{600\ nm}$ and flow cytometry count

The cell growth was monitored online by measuring spectrophotometrically the optical density at 600 nm ($O.D._{600\ nm}$), meanwhile the collected samples underwent the determination of the amount of live, damaged and dead cells by flow cytometry. The samples were subjected to dual nucleic acid staining with cell permeant SYTO®24 and cell impermeant propidium iodide (PI) according to the International Standard ISO19344:2015 IDF232:2015. SYTO®24 permeates the membrane of total cells and stains nucleic acids with green fluorescence meanwhile PI penetrates only bacteria with damaged membranes, causing a reduction in SYTO®24 fluorescence when both dyes are present. Thus, live bacteria with intact cell membranes fluoresce bright green (defined as active fluorescent cells), bacteria with slightly damaged membranes exhibit both green and red fluorescence (defined as slightly membrane damaged cells) and cells with broken membranes fluoresce red (defined as non-active fluorescent cells). After incubation at room temperature for 15 min, the labeled cell suspensions were diluted to approximately 10^6 events per ml and analyzed by an Accuri C6 flow cytometer (BD Biosciences, Milan, Italy). The obtained data were analyzed using BD Accuri™ C6 software (BD Biosciences, Milan, Italy). The SYTO®24 fluorescence intensity of stained cells was recovered in the FL1 channel; the PI fluorescence was recovered in the FL3 channel. Active fluorescent cells, damaged cells and nonactive fluorescent cells were electronically gated in density plots of green fluorescence (FL1) versus red (FL3) fluorescence.

7.2.3 Evaluation of urease activity

Urease activity of the lyophilized powders from the batch fermentations was evaluated by flow cytometry with cFSE staining as described in Paragraph 6.2.1 and by the phenol red assay as described in Paragraph 6.2.5.

7.2.4 Milk acidification

The acidification rates of the lyophilized powder were evaluated in reconstituted skim milk. The pH was measured continuously using a CINAC system that recorded every 30 minutes for 24 hours.

7.3 Results

7.3.1 Monitoring of cells growth by O.D. $_{600\ nm}$ and flow cytometry count of the bioreactors

Figure 1 shows the growth curves of *S. thermophilus* produced at pH 6 (STD), following the standard procedure, and at pH 6.8 (NEW), following the modified one; both the determination of the optical density at 600 nm and the total cells count are reported. The total cells count is calculated as the sum of the amount of live, damaged and dead cells after dual nucleic acid staining.

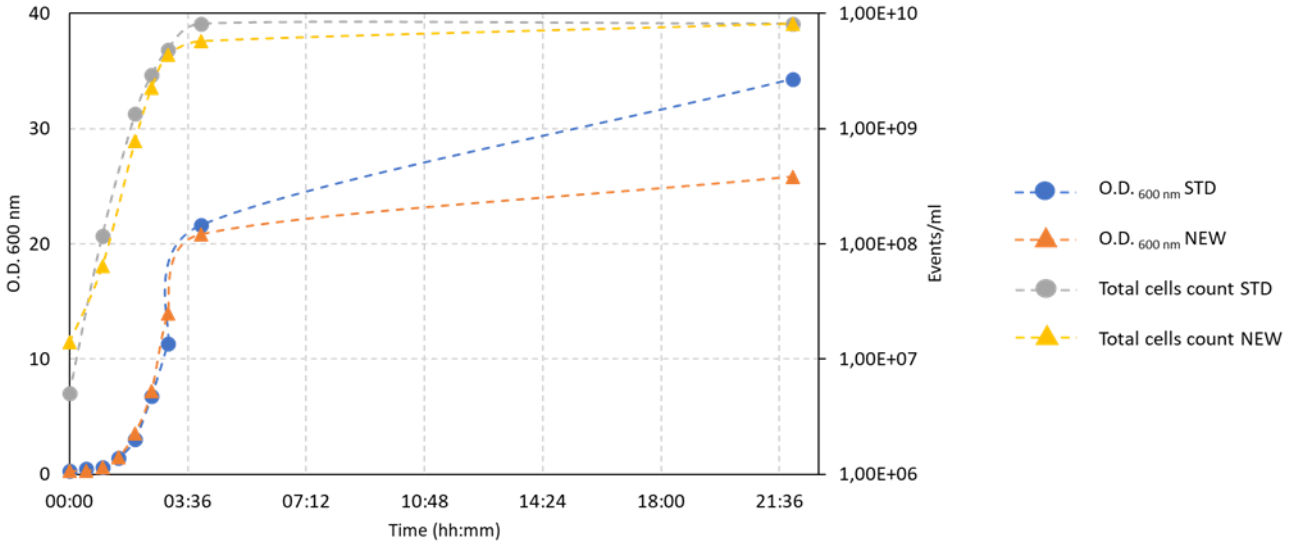

Figure 1. Growth curves of the biomasses produced at pH 6 (STD) and at pH 6.8 (NEW) by monitoring the optical density at 600 nm and the total cells count by flow-cytometry.

It is interesting to observe that no differences in the cells amount are recorded by flow cytometry during the fermentation. The use of optical density revealed differences in the last samples, which are probably related to differences in cell morphology. In fact, the FSC parameter in the flow-cytometry analysis, which is proportional to cell-surface area or size, resulted 43.8% higher for the STD biomass compared

to the NEW one, thus according to the percentage of increase of the optical density of the STD biomass (42.9%) in the last sample.

The dual nucleic acid staining with SYTO®24 and PI performed on the lyophilized powders produced with the biomasses of the bioreactors (Figure 2) revealed that the standard procedure, which means the acidic pH 6, exerts a protective effect toward the cells that will be submitted to the lyophilization procedure, allowing the production of a powder that account for the 65.9% of live cells, 20.3% of damaged cells and 11.4% of dead cells (Figure 2A), meanwhile the one produced from the biomass cultivated at pH 6.8 is composed of only 26.9% of live cells, 39.8% of damaged cells and 30.7% of dead cells (Figure 2B).

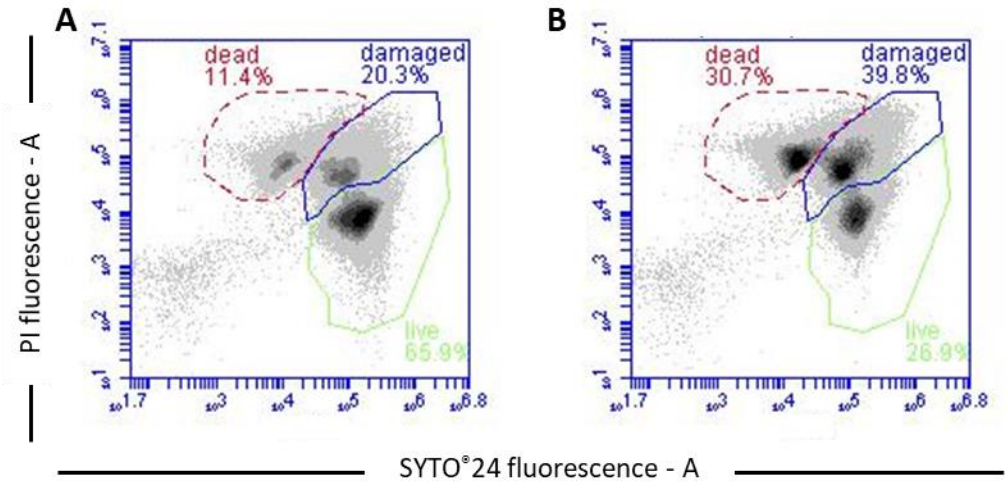

Figure 2. Flow cytometry density diagrams show SYTO®24 vs PI fluorescence of the lyophilized powders derivative from the biomasses produced with the STD production protocol (A) and the NEW production protocol (B). Viable cells are gated in green; dead cells with damaged membranes are gated in red; the transition of cell populations from the green gate to the red gate (blue gate) is correlated to cell membrane damage.

7.3.2 The pH of production of the biomass affects its urease activity

The lyophilized powders were evaluated for their urease activity by flow-cytometry after cFSE staining and by phenol red assay. Figure 3 shows that the *S. thermophilus* population produced at pH 6 (3A-3B) strongly increases its intracellular pH after the urea addition, thus increasing the fluorescence intensity of the staining. Conversely, when *S. thermophilus* is cultivated at pH 6.8 (3C-3D), the resulting biomass is least affected by the urea addition, with a very slight fluorescence shift. The red phenol assay confirms what previously observed: the data of the maximum velocity of the two biomasses are 20.32±0.09

$m_{O.D.555nm}$/min for the one produced with the STD procedure and 1.95±0.07 $m_{O.D.555nm}$/min for the NEW one. These data support our first supposition that moving the pH of production toward alkaline values could affect the urease activity of the biomass itself, resulting in a lower activity of the enzyme, compared to an acidic pH like pH 6.

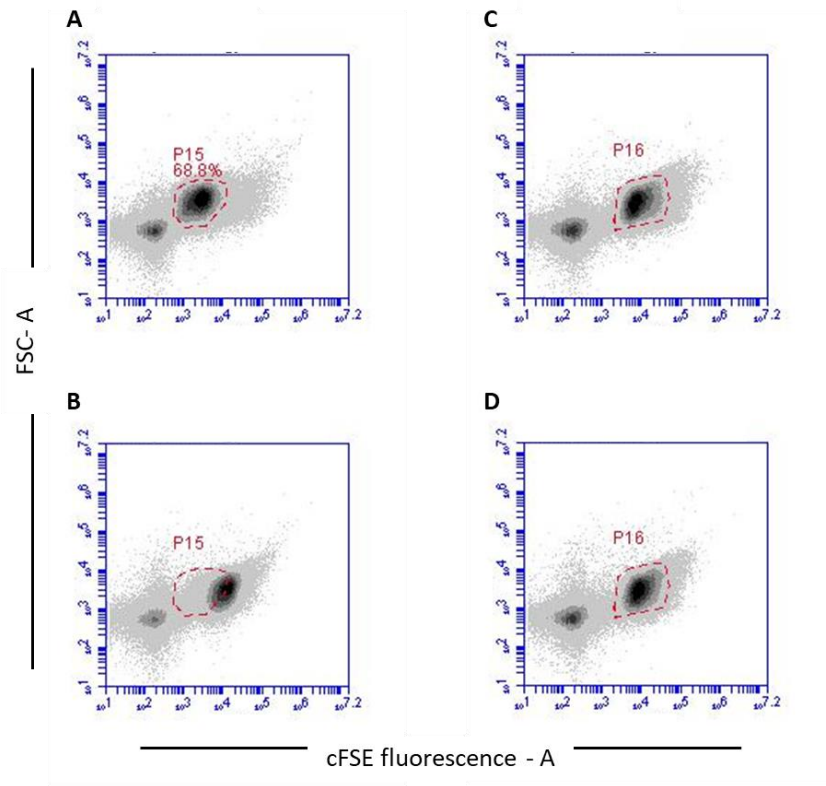

Figure 3. Density diagrams that represent the cytofluorimetric protocol for the evaluation of urease activity: they show the green (cFSE) fluorescence levels vs. the FSC of cells. A) The biomass produced with the STD protocol stained with cFSE; B) After 10 minutes of incubation with 20 mM of urea. C) The biomass produced with the NEW protocol stained with cFSE; D) After 10 minutes of incubation with 20 mM.

7.3.3 Decrease in urease activity of biomasses is not accompanied by a higher acidification rate

Despite the lower urease activity of the biomass produced with the NEW protocol, when it is used to ferment milk, it is the one produced with the STD protocol showed the higher milk acidification rate, as shown in Figure 4.

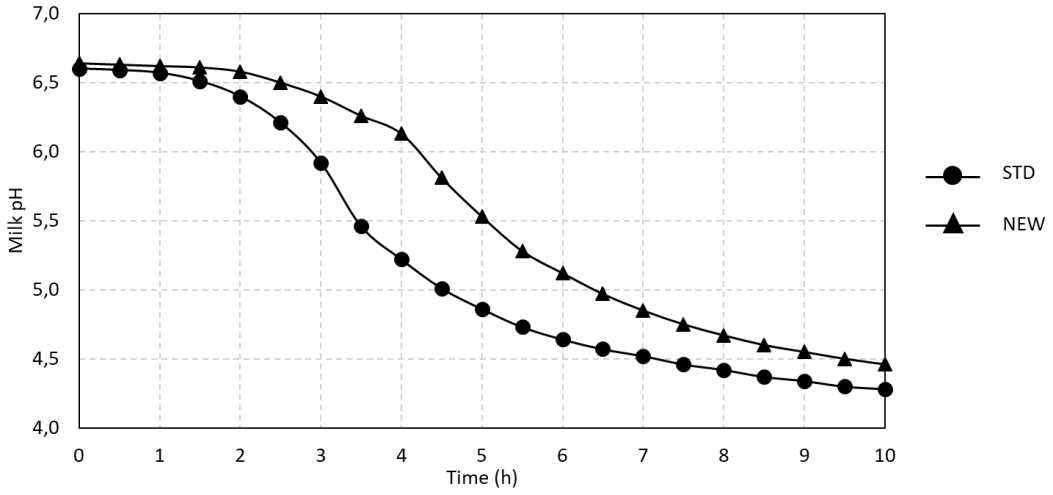

Figure 4. Milk acidification performed by the biomasses produced with at pH 6 (STD) and at pH 6.8 (NEW).

Two possible explanations can interpret this fact. As observed in the previous Chapters, urea hydrolysis, that supposedly happened when the STD biomass is growing in milk, and not for the NEW one, as we can speculate from the urease activity tests, positively affects the activity of all the enzymes involved in the energetic metabolism, thus resulting in a faster milk acidification process. Secondly, the higher % of damaged and dead cells composing the lyophilized powder coming from the production at pH 6.8, affects the acidification rate, having a lower amount of active cells in the milk, at parity weight of product used to ferment milk.

7.4 Discussion

Despite the well-known positive effects exert by urease activity in *S. thermophilus* metabolism and in the consortium with other relevant lactic acid bacteria (Arioli *et al.*, 2010; Arioli *et al.*, 2017; Chapter 5 of this thesis), at industrial level it is still considered a detrimental feature, especially for the delays in the acidification time caused by the buffering effect of ammonia on the lactic acid produced. Hence, the demand of a solution to this "problem" arises. In the present work we propose, instead of substituting the strains that show the urease activity, since they are well characterized and already inserted into the dynamics of a company that produces starter culture biomasses, to modify the production process of the biomasses with the aim of obtaining cells which carry a lower urease activity compared to those currently produced. The knowledge about the urease biogenesis led us to modify the pH at which the bioreactors are set up during the production processes of *S. thermophilus* biomasses, moving from 6, the standard pH of production, to 6.8. The monitoring of the growth in the bioreactors revealed that the pH of

production did not affect the growth, except for the size of the cells, which appeared to be smaller when the set point pH is fixed at pH 6.8. Interestingly, an effect of the pH of production resulted in the cells viability of the lyophilized powder produced from the biomass: as shown in Figure 2, the pH 6.8 biomass is characterized by a higher amount of damaged and dead cells. The effects of the pH of production on the cells viability has been previously reported for *Lactobacillus acidophilus* (Wang *et al.*, 2005), *L. reuteri* (Palmfeldt and Hahn-Hägerdal, 2000), *L. delbrueckii* subsp. *bulgaricus* (Shao *et al.*, 2014) and *S. thermophilus* (Béal *et al.*, 2001) and demonstrated that an acidic fermentation pH was better for the preservation of lactic acid bacteria undergone freeze drying or lyophilization, confirming our observations. Regarding the urease activity of the biomasses, the new pH of production allowed the obtaining of cells that carried a very low amount of urease activity, as observed with the red phenol assay and by flow cytometry with the cFSE staining. These results were very promising but, when the biomasses are used to ferment milk, the biomass produced with the standard pH showed a higher acidification rate compared to the one produced at pH 6.8. We proposed that this effect is consequence of two possible reasons: the first is the differences in the amount of live cells in the two lyophilized powders, secondly the urease activity of the biomasses itself. The urea hydrolysis positively affects the activity of all the enzymes involved in the energetic metabolism (Arioli *et al.*, 2010, Arioli *et al.*, 2017, Chapter 5 of this thesis), thus resulting in a faster milk acidification process of the biomass produced at pH 6, meanwhile, the pH 6.8 biomass did not benefit of the positive effects exerted by urease activity, since its urease activity is really low. However, since we demonstrated that the pH of production of the biomass has actually an effect on the modulation of the urease activity, future perspectives of the present work could regard the testing of an intermediate pH, between pH 6 and pH 6.8, with the aim of a substantial reduction of the urease activity but such that the residual urease activity is enough to stimulate the metabolism, allowing a higher acidification rate, if compared to the pH 6 biomass.

7.5 References

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8. CONCLUSIONS

The aim of this Ph.D. work was to investigate the physiological role and the technological relevance of the urease activity of the dairy bacterium *Streptococcus thermophilus*. We achieved a deeper comprehension of this peculiar enzymatic activity following different approaches.

The development of innovative procedures to evaluate the urease activity and its effect on the milk acidification, the overall metabolism and quality aspect of microbial products, related to urease, represented a substantial part of the present work. In particular, we developed an automatized fluorescent-based method for the characterization of *S. thermophilus* milk acidification profiles, that allowed us to have a rapid method to use whenever we had the need of comparing the acidification performances of several strains in different conditions, allowing also a focus on the characteristics of the urease peaks. Then, we developed a milk-based medium that allows the discrimination between urease-positive *S. thermophilus* strains and urease-negative ones based on the colonies morphology: the study of the physiology of the species represented a crucial step for the formulation of the medium and the key point of its effectiveness. It was used primarily as screening method looking for urease-defective mutants after UV mutagenesis of urease-positive strains of interest, but also as counting method when there was the need of distinguish between urease-positive and urease-negative strains, having no genetic information that would have allowed the use of molecular protocols. Moreover, we developed a cytofluorimetric protocol for the evaluation of urease activity of various samples containing *S. thermophilus*; the proposed applications are related to the evaluation of the urease activity of starter culture biomasses of *S. thermophilus* and to the enumeration of the *S. thermophilus* population in probiotic products containing this species, among others. We propose that the cytofluorimetric method should be seen as an innovative tool to put besides the standard ones to evaluate the quality of the products previously mentioned.

The investigation of the physiological role of urease of *S. thermophilus* cells growing in milk highlighted that in presence of this enzymatic activity the overall metabolism of the species is boosted, as the analysis of the total cells count and the metabolites production and consumption during the milk acidification demonstrated together with the experiment with the EdCs. Moreover, the cooperative role of urease, previously described for the yogurt consortium, has been extended also to the cooperation between a urease-positive *S. thermophilus* strain and a urease-negative one, supporting the proposal of the urease activity as an altruistic cooperative trait, which is costly for urease-positive species but provides a local benefit to the urease-negative species sharing the same environment, which can take advantage of the release of ammonia.

At industrial level, urease activity is still considered more for its detrimental effects than for the positive effects exerted on *S. thermophilus*: we proposed different strategies to overcome this industrial problem. Firstly, after the development of the screening method for the urease-defective mutants, we produced mutants of urease-positive strains of industrial interest, but, despite we actually obtained two urease-negative and one urease-weak mutants, they showed lower acidification rate compared to their wild types. So, we proposed, instead of substituting well characterized strains, already inserted into the dynamics of a company that produces starter culture biomasses, to modify the production process of the biomasses with the aim of obtaining cells which carry a lower urease activity compared to those currently produced. Despite the promising results of the quantification of the urease activity of the biomass produced with the new production protocol, that was significantly lower to the one produced with the standard one, when it was used to ferment milk, it was the standard one to show the higher milk acidification rate. This suggested that the urease activity expressed in milk is affected by several complex aspects and the attempt to remove it from the acidification courses is a hurdle option to cross.

In conclusion, the present work gives new insight in the comprehension of the urease activity of the dairy bacterium *S. thermophilus*, on how it can be exploited to improve the acidification performances of the strains and how it cannot, so far, be controlled during the milk acidification processes.

9. PRODUCTS

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Streptococcus thermophilus urease activity boosts *Lactobacillus delbrueckii* subsp. *bulgaricus* homolactic fermentation

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ABSTRACT

The proto-cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in the yogurt consortium enhances the growth rate and size of each population. In contrast, the independent growth of the two species in milk leads to a slower growth rate and a smaller population size. In this study, we report the first evidence that the urease activity of *S. thermophilus* increases the intracellular pH of *L. delbrueckii* in the absence of carbon source. However, in milk, in the presence of lactose the alkalizing effect of urea-derived ammonia was not detectable. Nevertheless, based on glucose consumption and lactic acid production at different pH_{in}, *L. delbrueckii* showed an optimum of glycolysis and homolactic fermentation at alkaline pH values. In milk, we observed that ammonia provided by urea hydrolysis boosted lactic acid production in *S. thermophilus* and in *L. delbrueckii* when the species were grown alone or in combination. Therefore, we propose that urease activity acts as an altruistic cooperative trait, which is costly for urease-positive individuals but provides a local benefit because other individuals can take advantage of urease-dependent ammonia release.

1. Introduction

Most food fermentation processes involve mixed cultures in which different microbial species interact with each other. These interactions may have neutral, positive or negative effects on the fitness of the strains performing the fermentation. Microbial interactions that exert positive effects on the micro-organisms participating in the process are classified as mutualistic interactions (Boucher et al., 1982). The best example of mutualism in a food process is represented by the yogurt consortium, which consists of the lactic acid bacteria (LAB) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Sieuwerts et al., 2008). In the yogurt consortium, the interactions that occur between the two LAB species in milk have been described as a proto-cooperative process, which has positive effects on the growth rate and size of each population; in contrast, the independent growth of the two species in milk led to a slower growth rate and a smaller population size (Zourari et al., 1992; Sieuwerts et al., 2010). The seemingly simplistic metabolism of these organisms, the availability of complete genomic information for the two species involved in the yogurt consortium (Bolotin et al., 2004; van de Guchte et al., 2006), and the availability of molecular tools suitable for the design of appropriate recombinant strains have made this microbial consortium an attractive model system for studies of species–species interactions. The molecular interactions that play key roles in the mutualistic behaviour of the yogurt consortium have been investigated using several approaches (Sieuwerts et al., 2010; Courtin et al., 2002; Liu et al., 2009; Herve-Jimenez et al., 2009) and have been reviewed by several authors (Sieuwerts et al., 2008; Smid and Lacroix, 2013). Validated interactions between *S. thermophilus* and *L. delbrueckii* during the growth of these species in milk have been described in relation to: i) the availability of nitrogen in milk (Courtin et al., 2002), ii) the exchange of formic acid, pyruvic acid and folic acid (Crittenden et al., 2003), iii) the production and utilisation of carbon dioxide (Driessen et al., 1982), iv) and the metabolism of purine, amino acid, long-chain fatty acids (Sieuwerts et al., 2010) and iron (Herve-Jimenez et al., 2009). A schematic summary of these metabolic interactions is presented in Fig. 1. As expected, most of the previously described interactions are trophic interactions in which the species feed each other with pyruvate, folate, long chain fatty acids, ornithine, carbon dioxide, peptides, amino acids and putrescine (Sieuwerts et al., 2010). Among the LAB involved in dairy fermentation processes, urease activity is present and widely distributed in only *S. thermophilus*, for which urease-negative strains are rare in nature (Driessen et al., 1982; Mora et al., 2002, 2005; Zourari et al., 1991). Urease is a multi-subunit urea amidohydrolase (EC 3.5.1.5) that catalyses the hydrolysis of urea to yield ammonia and carbamate. Urease is encoded by an 11-gene operon that accounts for 0.9% of the estimated core genome of *S. thermophilus* (Mora et al., 2004; Rasmussen et al., 2008). The urease activity of *S. thermophilus* shares metabolic

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Viromes As Genetic Reservoir for the Microbial Communities in Aquatic Environments: A Focus on Antimicrobial-Resistance Genes

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Despite studies of viromes isolated from aquatic environments are becoming increasingly frequent, most of them are limited to the characterization of viral taxonomy. Bacterial reads in viromes are abundant but the extent to which this genetic material is playing a role in the ecology of aquatic microbiology remains unclear. To this aim, we developed of a useful approach for the characterization of viral and microbial communities of aquatic environments with a particular focus on the identification of microbial genes harbored in the viromes. Virus-like particles were isolated from water samples collected across the Lambro River, from the spring to the high urbanized Milan area. The derived viromes were analyzed by shotgun metagenomic sequencing looking for the presence, relative abundance of bacterial genes with particular focus on those genes involved in antimicrobial resistance mechanisms. Antibiotic and heavy metal resistance genes have been identified in all virome samples together with a high abundance of reads assigned to cellular processes and signaling. Virome data compared to those identified in the microbiome isolated from the same sample revealed differences in terms of functional categories and their relative abundance. To verify the role of aquatic viral population in bacterial gene transfer, water-based mesocosms were perturbed or not perturbed with a low dose of tetracycline. The results obtained by qPCR assays revealed variation in abundance of *tet* genes in the virome and microbiome highlighting a relevant role of viral populations in microbial gene mobilization.

Keywords: virome, microbiome, antimicrobial resistance genes, mesocosms, Lambro River

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INTRODUCTION

Studies of the environments through metagenomics analysis are becoming increasingly frequent and the development of instruments, techniques and databases makes the study and the characterization of viral communities with a shotgun metagenomics approach more and more informative. Water has been described as the major environment for bacteria on earth (Taylor et al., 2011), and bacteriophages outnumber bacteria by a factor ranging from 1 to 10 (Muniesa et al., 2013a). Water is an ideal medium for bacterial life, characterized by accessible dissolved nutrients, as well as protection from desiccation and UV light. In this context, the high frequency of encounters between bacteria and bacteriophages strongly favors, through transduction, the

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Promysalin is a salicylate-containing antimicrobial with a cell-membrane-disrupting mechanism of action on Gram-positive bacteria

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Promysalin was previously described as a narrow spectrum molecule with a unique species-specific activity against *Pseudomonas aeruginosa*. Here we demonstrate that promysalin is active against Gram-positive and Gram-negative bacteria using a microdilution assay. Promysalin acts on Gram-positive bacteria with a mechanism of action involving cell membrane damage with leakage of intracellular components. The evaluation of MICs and MBCs on 11 promysalin analogs, synthesized utilizing diverted total synthesis, allowed the identification of the structural moieties potentially involved in cell membrane interaction and damage. The mechanism of action of promysalin against Gram-negative bacteria is still not clarified, even if a synergistic effect with the bisguanidine chlorhexidine on cell membrane disruption has been observed.

The recent studies that highlight the long term effects on human physiology and health of the early exposure to antibiotics[1], have prompted researchers to increase their efforts towards the search of new molecules with a narrow spectrum of activity. Antibiotics do not have species-specific activity, but are characterized by a wide spectrum of activity against several bacterial species and therefore they severely impact on microbiota composition[2]. It is well known that perturbations to the microbiota, such as those caused by the use of oral antibiotics, deplete the commensal microbiota, allowing pathogens to proliferate, thus determining gastrointestinal inflammations[3]. In this context, promysalin, a salicylate-containing *Pseudomonas putida* antibiotic, was described to selectively target members of the genus *Pseudomonas*, without affecting the viability of other Gram-negative and Gram-positive bacteria[4,5]. More recently[6], diverted total synthesis of promisalyn analogs demonstrates that the bioactivity of the molecule is sensitive to changes within its hydrogen bond network. Furthermore, it was reported that promysalin inhibits the production of the siderophore pyoverdine which is often linked to virulence. Therefore, the inhibition of pyoverdine by promysalin was considered an interesting point in order to develop novel antivirulence therapy[6].

The amphiphilic nature of promysalin, prompted us to assume a mechanism of action based on cell membrane interaction. However, the interaction of promysalin with the membrane phospholipid bilayer was never investigated. The narrow spectrum of activity of promysalin against only bacteria of the genus *Pseudomonas*[4], was described by the authors as a novelty. To the best of our knowledge, there are no other molecules of microbial origin known to be active in a genus-specific way with the exception of a genus-specific molecule which was obtained modifying the original amino acid sequence of the antimicrobial peptide protegrin I through cycles of peptidomimetic synthesis[7].

The basic purpose of the work outlined here was to investigate the activity of promysalin and analogues, prepared by a recently developed synthesis protocol[8], against several Gram-negative and Gram-positive reference species using a validated standard microdilution method[9]. Additionally, a further aim was to get insights into the mechanism of action of promysalin.

Results

Promysalin is a broad spectrum antimicrobial. The data obtained (Table 1), highlight that promysalin is active against Gram-positive and Gram-negative bacteria including several human pathogens. The Minimal

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ORIGINAL ARTICLE

Characterization of airborne viromes in cheese production plants

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Keywords
antibiotic resistance gene, cheese, metagenomics, virome, virus-like particles.

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Abstract

Aims: To characterize airborne virus-like particles isolated from two cheese production plants in order to reveal their complexity in terms of viral communities and microbial genes potentially mobilized by viruses.

Methods and Results: Airborne virus-like particles have been isolated from Grana Padano and Gorgonzola PDO cheese production plants and ripening cellars. A shotgun metagenomics analysis of the isolated viromes highlighted a high complexity of the viral communities both in terms of viral taxonomy and phage–host associations. Bacterial reads in each of the viromes were confirmed to be abundant and their taxonomy appeared to be associated with the environmental parameters and the technological processes that characterize the sampling area. Antibiotic resistance genes have been identified in each virome thus confirming that phages could be involved in the mobilization of antimicrobial resistances among bacterial populations. Interestingly human viruses were also identified even if the contamination source was not revealed.

Conclusions: The environmental conditions, which are imposed by the technology of the dairy process, seam to shape the viral populations as a consequence of the adaptation of microbial taxa to those environments. The identification of sequences belonging to *Legionella pneumophila* and to the human papillomavirus, raised some considerations about the safety of cheese-ripening cellars.

Significance and Impact of the study: In conclusion, the analysis of the dairy airborne viromes, has revealed a high complexity of the viral communities even if the environments where the samples were collected were confined environments. Metagenomics of airborne viral population could be a promising monitoring tool for the biological characterization of dairy environments.

Introduction

In many cheese-making activities, micro-organisms in the air could represent a hidden and dangerous risk factor. The air present in the processing areas can be a source of pathogenic or spoilage micro-organisms that could contaminate foods causing problems both on the safety and the quality of the foods themselves. The greatest aerosol sources in dairy plants are personnel, floor drains, ventilation system and water when applied under pressure in the cleaning and sanitizing procedures (Andersen 1958; Vickers 1986; Kang and Frank 1990; Salustiano *et al.* 2003). Air can easily move micro-organisms, spores and dust to reach food in preparation. Identification of micro-organisms present in the air cannot be limited to bacteria and fungi but should consider also viral populations. Viruses, and specifically phages, have a meaningful impact due to their role in modulating the abundance of microbial populations (Heldal and Bratbak 1991; Breitbart and Rohwer 2005; Danovaro *et al.* 2016), and because they represent a tool for horizontal gene transfer

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Development of an automatized fluorescent-based method for the monitoring of milk acidification

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State of the art. The selection of lactic acid bacteria for dairy applications is mainly based on their ability to rapidly acidify milk. Milk acidification by lactic acid bacteria can be affected by several metabolic properties that are usually strain-dependent features (Mora et al., 2002). Among them, the most relevant are the presence of cell envelope proteinases (Letort et al., 2002), the auxotrophy towards amino acids (Arioli et al., 2008), the lactose transport, the efficiency of the glycolytic/homolactic fermentation pathway and the urease activity (Mora et al., 2002; Monnet et al., 2004). Milk composition, specifically urea content, and milk heat treatment can also significantly affect milk acidification. Due to the high number of factors that can affect milk acidification of lactic acid bacteria, the measurement of this parameter often requires the simultaneous comparison of several strains or the simultaneous comparison of several milk treatments or milk formulations using one or few strains. In this context, despite the relative simplicity of pH measurement, quite often milk acidification is required to be tested in several samples and in their related replicates using a multichannel pH analyzer which is currently commercially available up to a 32 measurement channels, therefore allowing the analysis of a 16 samples in duplicate at maximum (AMS France, Frepillon, France). Probes such as the 6-carboxyfluorescein have been used in several applications due to the pH-dependent fluorescence of the molecule; here we present a revised and simplified version of the methods already presented in literature, that allow a complete automation through a liquid handling system because 6-carboxyfluorescein is added directly in milk. The new protocol is applied to *Streptococcus thermophilus*.

Procedure. The 96-well-plates are automatically charged by EPMotion 5070 (Eppendorf, Amburg, Germany) with 200 µl of sterile milk supplemented with 6-carboxyfluorescein, and 2 µl of inoculum from an overnight growth. The pH-dependent carboxyfluorescein fluorescence was than recorded every 10 min for 24 h using a fluorometer (Perkin-Elmer, Waltham, MA). The optimal concentration of 6-carboxyfluorescein to be used was 0,8 µM, since lower and higher amount did not allow to detect efficiently the milk pH variations. The addition of urea to milk at a final concentration of 10-20 mM was necessary to detect pH variation related to *S. thermophilus* urease activity.

1° application: screening of S. thermophilus strains based on their milk acidification rate.

Once the method was set up, it was used to screen *S. thermophilus* strains based on their milk acidification rate focusing the attention on urease activity. As control, a urease-negative *S. thermophilus* strain A16 (a DSM20617 derivative), was used (Fig. 1). Figure 2 and 3 show the acidification curves of two different *S. thermophilus* strains characterized by different levels of urease activity, strongest in strain ST2903. The buffering effect of ammonia, released by urease activity from urea, on lactic acid production was showed by the reduced decrease of carboxyfluorescein fluorescence (see arrows in Fig. 2 and Fig. 3). The decrease of carboxyfluorescein fluorescence, during the growth in milk of *S. thermophilus*, is therefore a balance between the lactic acid produced by the homolactic fermentation and the efficiency of urease activity.

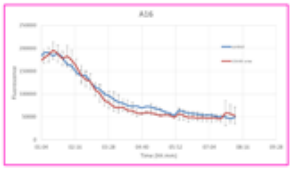

Fig 1: Acidification curves of *S. thermophilus* A16 in presence and absence of 20 mM urea.

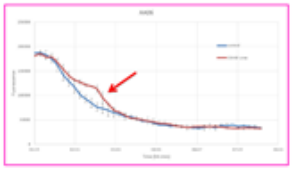

Fig 2: Acidification curves of *S. thermophilus* AA06 in presence and absence of 20 mM urea.

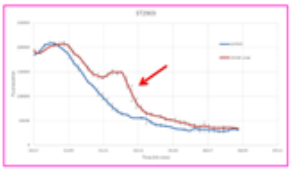

Fig 3: Acidification curves of *S. thermophilus* ST2903 in presence and absence of 20 mM urea.

2° application: screening of S. thermophilus mutants with decreased urease activity.

The method has also been used to screen *S. thermophilus* mutants with decreased urease activity. The mutants have been produced by UV-mutagenesis of the AA06 strain, which showed already weak urease activity. About 1000 mutants were produced and after some preliminary screening, about 50 mutants were selected to be analyzed through the fluorimetric method. Figure 4 shows the milk acidification of mutant 3/M17 which showed an improved urease activity compared to the wild-type, meanwhile Figure 5 shows the milk acidification of mutant 1/M08, which appeared urease-negative, since the acidification curves in presence and in absence of urea are almost perfectly overlapped.

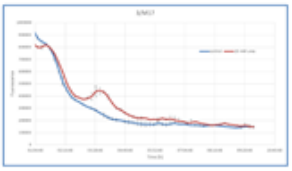

Fig 4: Acidification curves of *S. thermophilus* mutant 3/M17 in presence and absence of 20 mM urea.

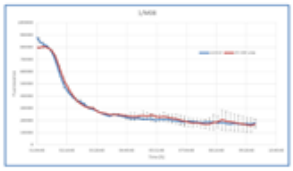

Fig 5: Acidification curves of *S. thermophilus* mutant 1/M08 in presence and absence of 20 mM urea.

Characterization of the mutant 1/M08.

Mutant 1/M08 was subjected to further investigation. Figure 6 shows the milk acidification curves of wild-type and its derivative mutant 1/M08, recorded with a standard pH-meter. Mutant 1/M08 shows a weaker and a posticipated urease activity compared to the wild-type (see arrow in Fig. 6). Interestingly, galactose and lactic acid quantifications, (carried out enzymatically and by HPLC, Figures 7-8) show that mutant 1/M08 produces a higher amount of lactic acid and galactose compared to the wild-type. Further characterization of mutant 1/M08 are ongoing to understand whether this new improved strain could successfully find applications in dairy fermentations.

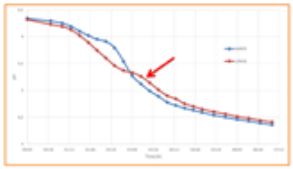
Fig 6: Acidification curves of *S. thermophilus* wild type strain AA06 and its mutant 1/M08.


Fig 7: Galactose released by *S. thermophilus* wild type strain AA06 and its mutant 1/M08.

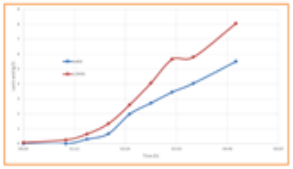
Fig 8: Lactic acid produced by *S. thermophilus* wild type strain AA06 and its mutant 1/M08.

Conclusions. An automatized-fluorescence based method has been developed and successfully applied for the screening of *S. thermophilus* strains on the basis of their acidification curves and was sensitive enough to highlight differences in the intensity of their urease activity. The method was successfully applied for the screening of new improved strains which can find application in the dairy fermentation processes due to their new characteristics, such as a decreased urease activity or a faster milk acidification rate.

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Full pH Control in Dairy Process (FullpHD)

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State of the art

One of the main role of *S. thermophilus* during milk fermentation is to provide rapid acidification by producing lactic acid from disaccharide lactose. Lactic acid plays an important role in milk coagulation and curd draining, imparting a fresh acidic flavor to fermented milk while helping to suppress the growth of pathogens and spoilage microorganisms. The rate of acidification is an important technological feature because delay in the time course of acidification may have dramatic effects on the quality of the product or economic consequences. The rate of acidification is a strain-dependent metabolic trait that may be influenced by several factors such as the lactose-galactose metabolism, the proteolytic system and urease activity [1].

Urease (Fig. 1) is a urea amidohydrolase that catalyzes the hydrolysis of urea to yield ammonia and carbamate that spontaneously decomposes to yield a second molecule of ammonia and carbonic acid. These reactions lead to a slower decrease in pH, buffering the lactic acid production during the fermentation process. As milk contains different amounts of urea from one batch to another, the consequence is an unpredictable rate of acidification during fermentation processes.

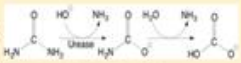

Figure 1: Reactions of the hydrolysis of urea by urease

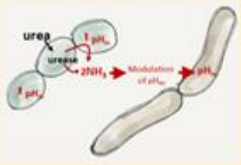

Figure 2: Schematic representation of the modulation of the intracellular pH of *L. delbrueckii* in consequence of the urea hydrolysis in *S. thermophilus* (Adapted from [8]).

Urease is a complex enzyme that is encoded by a 11-gene operon that accounts for 0.9% of the estimated core genome of *S. thermophilus* [2]. This enzyme has been found in all previously characterized *S. thermophilus* strains, and urease-negative mutants are not common in nature [3]. The urease biogenesis is affected by pH, nickel ions availability, the presence of the enzyme substrate but not by the carbohydrate availability, as observed instead in *S. salivarius*, its closest neighbor [4]. Carbon dioxide, generated by urea hydrolysis, might be used as a substrate in several anaplerotic reactions mainly involved in amino acid and nucleic acid biosynthesis [5], while ammonia has been previously reported to be involved in glutamine biosynthesis [6]. Moreover, the urease expression is sensitive to aspartate, glutamate, glutamine, and ammonia availability [7].

Regarding the physiological role of urease activity, it has been reported that urea and ammonia activate the cells metabolism accelerating the glycolytic flux and homolactic fermentation by an intracellular alkalization that is generated by the release of ammonia into the cytoplasm [8]. Furthermore, it has been demonstrated that urea hydrolysis increases the catabolic efficiency not only of *S. thermophilus* by modulating the intracellular pH but also of *L. delbrueckii*, the other specie involved in the yogurt consortium (Fig. 2). These findings put a new light in the interactions that occur between a urease-positive species and other urease-negative lactic acid bacteria in milk environment; in this context, urease activity should be taken into account as a metabolic booster for the optimal growth of lactic acid bacteria in milk, not only as a negative feature of the dairy *S. thermophilus* strains [9].

Aim of the project

This PhD research project is aimed to better understand how urease activity can modulate milk acidification. Should urease activity be considered a negative trait in milk fermentation processes? Is the selection of urease-negative strains a possible solution, or mutants showing a reduced but not absent activity could be more performant? Is it possible to control the post-acidification processes during cold storage through the modulation of urease activity? This PhD project is aimed to answer to the previous questions in order to develop new technological strategies, to select and to genetically improve *S. thermophilus* strains for dairy applications at industrial level.

PhD Thesis Objectives and Milestones

The project activities will be organized in four main work packages (WPs) and deliverables (A1-11):

- **WP1 - Development of a fluorimetric assay for monitoring milk acidification**
  - A1: Development of an automatized fluorescent-based method for the monitoring of milk acidification
  - A2: Screening of *S. thermophilus* strains based on their milk acidification rate
- **WP2 - Selection of *S. thermophilus* mutants with decreased urease-activity**
  - A3: set up of the UV-based mutagenesis
  - A4: Screening of mutants based on milk acidification rate using a fluorimetric assay
  - A5: Genomic characterization of the "most performant" mutants
- **WP3 - Detailed description of *S. thermophilus* metabolism and urease activity during milk fermentations**
  - A6: Monitoring of urea metabolism using a ^{13}C-urea NMR approach
  - A7: Monitoring of lactose consumption, galactose release and lactic acid production by HPLC and enzymatic analysis
  - A8: Transcriptional analysis of *ure* genes and genes involved in carbon and nitrogen metabolism
  - A9: Monitoring total cells count, alive, damaged and dead cells with flow cytometry approach
- **WP4 - Evaluation of industrial performance of selected mutants and yogurt/cheese productions**
  - A10: Biomass yield and activity of selected mutants in a pilot scale industrial production
  - A11: Yogurt/cheese productions using a starter culture blend enriched with the selected mutants

Gantt Diagram

Activity \ Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Development of a fluorimetric assay for monitoring milk acidification (WP1)	■	■	■	■	■	■	■	■																												
Selection of *S. thermophilus* mutants with decreased urease-activity (WP2)						■	■	■	■	■	■	■	■	■	■	■	■	■																		
Detailed description of *S. thermophilus* metabolism and urease activity during milk fermentations (WP3)																	■	■	■	■	■	■	■	■	■	■										
Evaluation of industrial performance of selected mutants and yogurt/cheese productions (WP4)																											■	■	■	■	■	■	■	■	■	■
Preparation of manuscripts and thesis	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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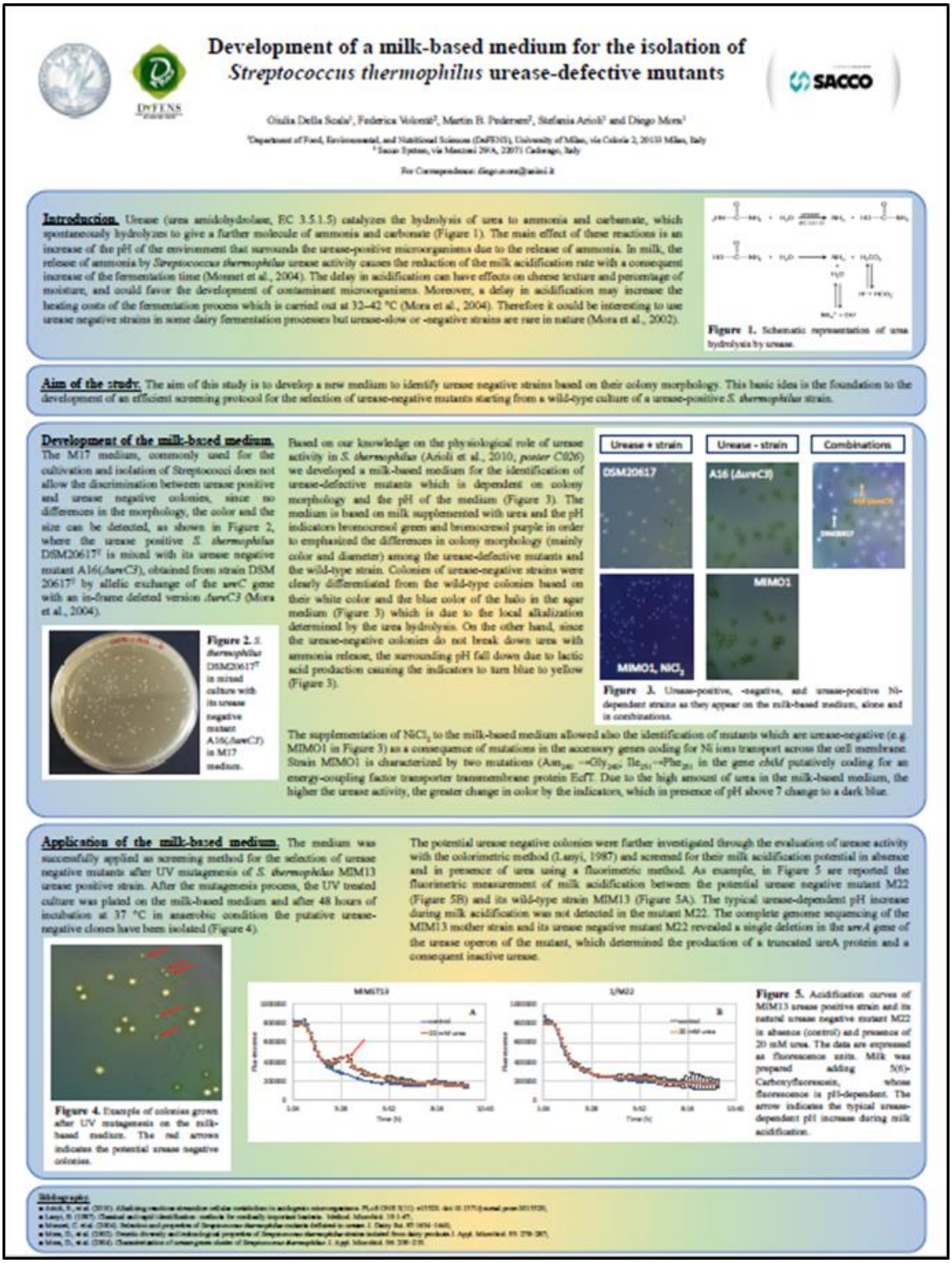

Development of a milk-based medium for the isolation of *Streptococcus thermophilus* urease-defective mutants

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Introduction. Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbamate, which spontaneously hydrolyzes to give a further molecule of ammonia and carbonate (Figure 1). The main effect of these reactions is an increase of the pH of the environment that surrounds the urease-positive microorganisms due to the release of ammonia. In milk, the release of ammonia by *Streptococcus thermophilus* urease activity causes the reduction of the milk acidification rate with a consequent increase of the fermentation time (Morinet et al., 2004). The delay in acidification can have effects on cheese texture and percentage of moisture, and could favor the development of contaminant microorganisms. Moreover, a delay in acidification may increase the heating costs of the fermentation process which is carried out at 32–42 °C (Mora et al., 2004). Therefore it could be interesting to use urease negative strains in some dairy fermentation processes but urease-slow or -negative strains are rare in nature (Mora et al., 2002).

Figure 1. Schematic representation of urea hydrolysis by urease.

Aim of the study. The aim of this study is to develop a new medium to identify urease negative strains based on their colony morphology. This basic idea is the foundation to the development of an efficient screening protocol for the selection of urease-negative mutants starting from a wild-type culture of a urease-positive *S. thermophilus* strain.

Development of the milk-based medium. The M17 medium, commonly used for the cultivation and isolation of Streptococci does not allow the discrimination between urease positive and urease negative colonies, since no differences in the morphology, the color and the size can be detected, as shown in Figure 2, where the urease positive *S. thermophilus* DSM20617[T] is mixed with its urease negative mutant A16(*ΔureC3*), obtained from strain DSM 20617[T] by allelic exchange of the *ureC* gene with an in-frame deleted version *ΔureC3* (Mora et al., 2004).

Figure 2. *S. thermophilus* DSM20617[T] in mixed culture with its urease negative mutant A16(*ΔureC3*) in M17 medium.

Based on our knowledge on the physiological role of urease activity in *S. thermophilus* (Arioli et al., 2010; *poster C026*) we developed a milk-based medium for the identification of urease-defective mutants which is dependent on colony morphology and the pH of the medium (Figure 3). The medium is based on milk supplemented with urea and the pH indicators bromocresol green and bromocresol purple in order to emphasized the differences in colony morphology (mainly color and diameter) among the urease-defective mutants and the wild-type strain. Colonies of urease-negative strains were clearly differentiated from the wild-type colonies based on their white color and the blue color of the halo in the agar medium (Figure 3) which is due to the local alkalization determined by the urea hydrolysis. On the other hand, since the urease-negative colonies do not break down urea with ammonia release, the surrounding pH fall down due to lactic acid production causing the indicators to turn blue to yellow (Figure 3).

Figure 3. Urease-positive, -negative, and urease-positive Ni-dependent strains as they appear on the milk-based medium, alone and in combinations.

The supplementation of $NiCl_2$ to the milk-based medium allowed also the identification of mutants which are urease-negative (e.g. MIMO1 in Figure 3) as a consequence of mutations in the accessory genes coding for Ni ions transport across the cell membrane. Strain MIMO1 is characterized by two mutations (Asn_{240}→Gly_{240}; Ile_{251}→Phe_{251} in the gene *cbiM* putatively coding for an energy-coupling factor transporter transmembrane protein EcfT. Due to the high amount of urea in the milk-based medium, the higher the urease activity, the greater change in color by the indicators, which in presence of pH above 7 change to a dark blue.

Application of the milk-based medium. The medium was successfully applied as screening method for the selection of urease negative mutants after UV mutagenesis of *S. thermophilus* MIM13 urease positive strain. After the mutagenesis process, the UV treated culture was plated on the milk-based medium and after 48 hours of incubation at 37 °C in anaerobic condition the putative urease-negative clones have been isolated (Figure 4).

Figure 4. Example of colonies grown after UV mutagenesis on the milk-based medium. The red arrows indicates the potential urease negative colonies.

The potential urease negative colonies were farther investigated through the evaluation of urease activity with the colorimetric method (Lanyi, 1987) and screened for their milk acidification potential in absence and in presence of urea using a fluorimetric method. As example, in Figure 5 are reported the fluorimetric measurement of milk acidification between the potential urease negative mutant M22 (Figure 5B) and its wild-type strain MIM13 (Figure 5A). The typical urease-dependent pH increase during milk acidification was not detected in the mutant M22. The complete genome sequencing of the MIM13 mother strain and its urease negative mutant M22 revealed a single deletion in the *ureA* gene of the urease operon of the mutant, which determined the production of a truncated ureA protein and a consequent inactive urease.

Figure 5. Acidification curves of MIM13 urease positive strain and its natural urease negative mutant M22 in absence (control) and presence of 20 mM urea. The data are expressed as fluorescence units. Milk was prepared adding 5(6)-Carboxyfluorescein, whose fluorescence is pH-dependent. The arrow indicates the typical urease-dependent pH increase during milk acidification.

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Poster presented at "12th International Symposium on Lactic Acid Bacteria", 27th-31st August 2017, Egmond am Zee, the Netherlands.

Optimization of milk acidification through milk alkalization: the paradox of *Streptococcus thermophilus* metabolism

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Streptococcus thermophilus is one of the most widely used lactic acid bacteria in dairy applications. Among the lactic acid bacteria involved in dairy fermentation processes *S. thermophilus* is characterized by a urease activity which is a species-specific trait and urease-negative strains are extremely rare in nature. Urease is a multi-subunit urea amidohydrolase (EC 3.5.1.5) that catalyses the hydrolysis of urea to yield ammonia and carbamate which spontaneously generates another ammonia molecule and carbon dioxide. During the milk fermentation performed by *S. thermophilus*, the production of ammonia from urea slows down the pH decrease, with resulting delays in the milk acidification process (Fig. 1). **To understand the physiological role of urease the first 180 min of *S. thermophilus* growth in milk have been carefully investigated** evaluating: milk pH, urea and lactose consumption, lactic acid production by NMR (Fig. 2).

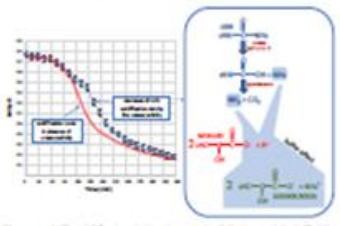

Figure 1 Milk acidification during the growth of *S. thermophilus* MM03 in sterile milk supplemented with urea (3 mM). The modelling of *S. thermophilus* milk acidification in absence of urea is shown as a orange line. The buffering effect on lactic acid due to urease activity is shown in the right panel of the figure.

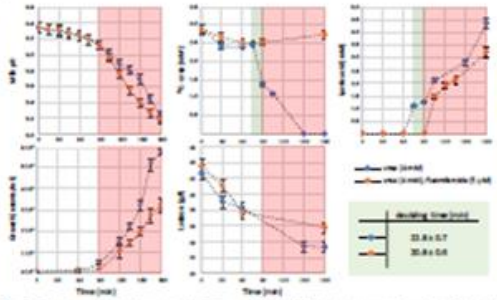

Figure 2 *S. thermophilus* MM03 growth in milk supplemented with ^{15}N-urea (3 mM), with and without the addition of the potent urease-inhibitor fluorofamide (5 μM). The shaded pink area referred to the time interval where the buffer effect due to urea hydrolysis is detectable by measuring the milk pH. The green shaded area referred to the start of urea hydrolysis as detected by NMR measurement of ^{15}N-urea. *S. thermophilus* was cultivated in sterilized reconstituted skimmed milk (DiRo) at 37 °C.
Data are reported as the average of three replicates. Bars are referred to the standard deviation. The doubling time has been calculated in the time interval 90 - 165 min. NMR quantification of ^{15}N-urea, lactose and lactic acid have been carried out as previously described (1). Cell count was carried out by flow cytometry (2).

Urease activity stimulate *S. thermophilus* metabolism The data collected reported in Figure 2, highlighted that concomitantly with the decrease of milk acidification and the start of urea hydrolysis we measured a significant increase in cell count together with the increase of lactose consumption and lactic acid production. Urea hydrolysis starts approximately around 90 min of incubation at a pH above 6. All urea in milk is consumed within the first 150 min of incubation, therefore we speculate that **the physiological role of urease is restricted to the first 150 min of growth in milk**. In a previous study (1) we demonstrated that urea hydrolysis positively increases the activity of β-galactosidase and lactate dehydrogenase. Moreover, by measuring the intracellular ATP production we observed a positive effect, exerted by urea hydrolysis and/or ammonia supplementation, to the overall glycolytic activity (1). We therefore hypothesize that ammonia generation by urease activity, and the consequent increase of intracellular pH could positively act on the activity of all the enzymes involved in the energetic metabolism (Figure 3).

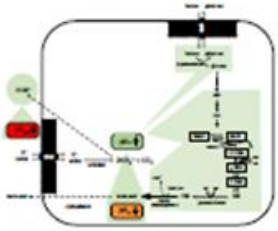

Figure 3 Graphical representation of *S. thermophilus* main metabolism together with urease activity. The green shaded areas represent the known effect of the urease-dependent ammonia release on the intra- and extra-cellular pH, and on the activity of glycolytic enzymes, β-galactosidase and lactate dehydrogenase.

Urease stimulation of metabolism is independent of *S. thermophilus* growth While the positive role of culture pH-control during the growth of *S. thermophilus* is well known, and industrially applied in order to achieve the highest yield as a tradeoff between biomass and its activity, the real effect of such pH control is not completely understood. Most of the data available in the literature are focused on the detrimental effect of the exposure to low pH for a long period of time which causes arrest of growth, dramatic reduction of glycolytic fluxes and a progressive loss of viability (3). Although urease activity has been historically classified as a stress response that is activated to counteract acidic environmental pH in a number of bacteria, here we propose that urease should be considered a metabolic regulatory mechanism for energetic metabolism in the dairy bacterium *S. thermophilus*. Working with energetically discharged *S. thermophilus* cells (EdCs) in non-growing status and with gene translation blocked by high chloramphenicol concentration, we showed that urease activity can dramatically boost the homolactic fermentation when urea and lactose were supplemented (Figure 4). While urea hydrolysis by EdCs determined a dramatic pH increase (from pH 7 to pH 8.3) in 5 min, when EdCs were supplemented with lactose the homolactic fermentation was not efficient and determined a minimal acidification with a ΔpH of 0.07. However, when EdCs were supplemented with lactose and urea, after a first alkalization (from pH 7 to pH 8.1 in 1 min), we then assisted to a rapid pH drop with a ΔpH of 1.25, thereby indicating that **in presence of urea hydrolysis the homolactic fermentation was dramatically boosted (Figure 4)**.

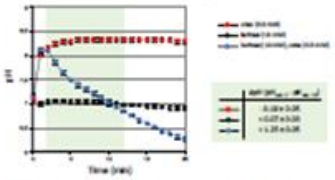

Figure 4 pH change by *S. thermophilus* metabolism in saline solution supplemented with chloramphenicol (100 μg/ml) at 37 °C. *S. thermophilus* cells were prepared as energetically discharged cells as previously described (1). The ΔpH was calculated within the 10 min time interval highlighted in the figure by the green shaded area. Urea and or lactose were added at time 0.

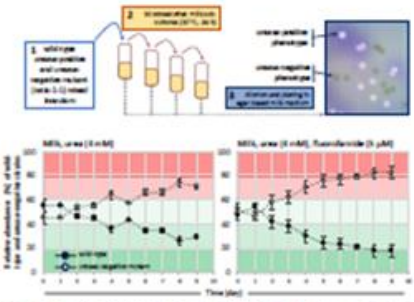

Figure 5 Competition between *S. thermophilus* MM03 and its urease-negative mutant MM22 in milk during 9 subcultures. After each milk subculture the relative abundance of the wild-type and the urease-negative mutant were estimated by plating on the selective agar-based milk medium (*for detail see poster* C025).

Urea hydrolysis has a cooperative behaviour in a mixed bacterial community but urease biosynthesis is energetically costly for the wild-type In order to investigate the role of urease activity when a urease positive strain is co-cultured with a urease negative strain we performed a competition experiment. The cooperative role of urease was previously demonstrated for the yogurt consortium (2). Here we show that both in presence of in absence of fluorofamide, the urease-negative strain took the lead after few days, but with a higher relative abundance when fluorofamide was added to milk and urease activity was inhibited. These results (Figure 5) underlined that: i) the ammonia released positively affects the metabolism of the urease-negative mutant which is sharing the same environment, ii) **when urea hydrolysis is inhibited by fluorofamide, both the wild-type and the mutant lose the advantage of ammonia released by an active urease, but the former suffers from the energy costs of urease biosynthesis**. Urease biosynthesis is energetically costly for the wild-type strain because eleven genes are involved for the biosynthesis of an active urease.

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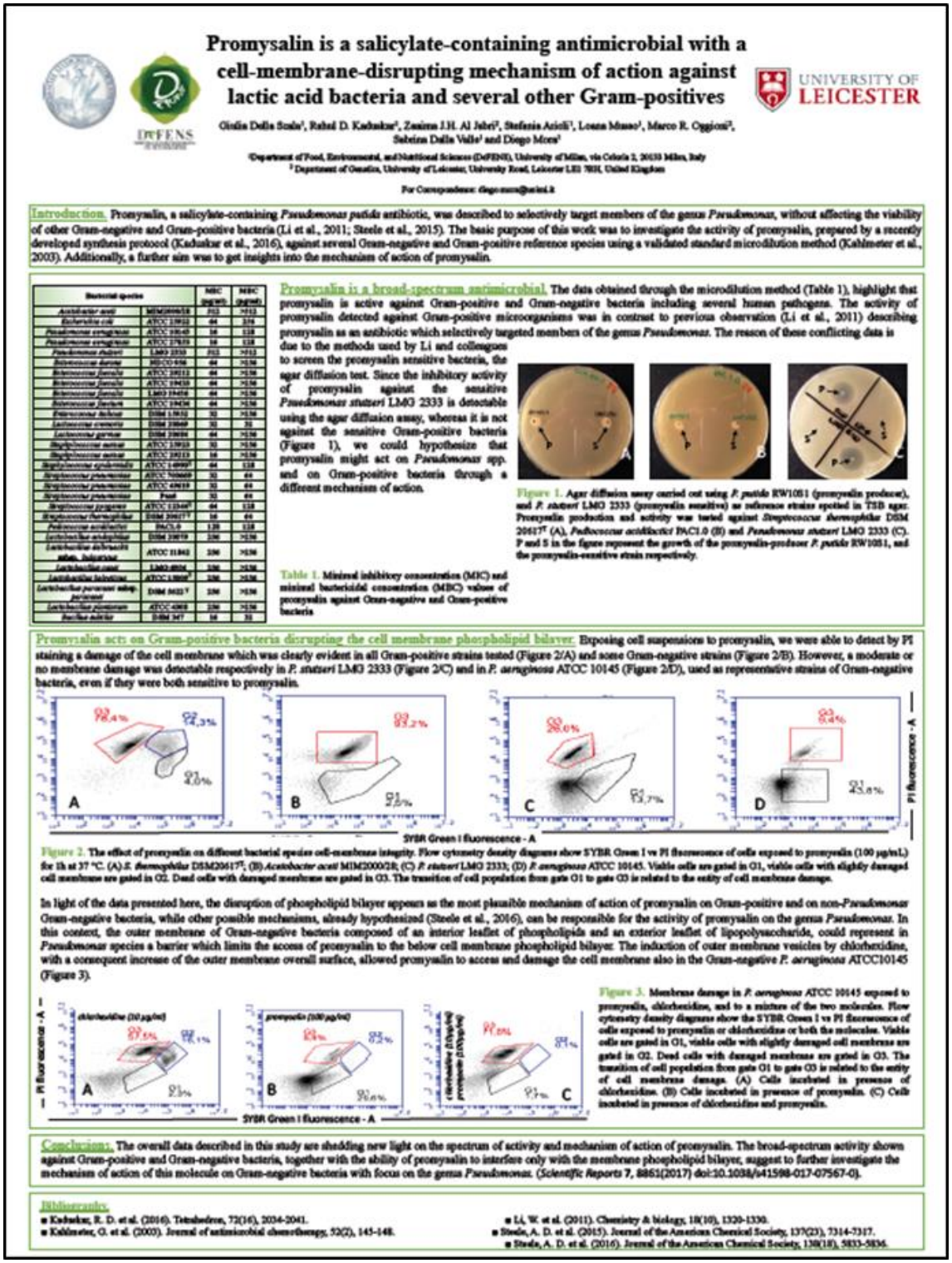
Poster presented at "12th International Symposium on Lactic Acid Bacteria", 27th-31st August 2017, Egmond am Zee, the Netherlands.



Promysalin is a salicylate-containing antimicrobial with a cell-membrane-disrupting mechanism of action against lactic acid bacteria and several other Gram-positives

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Introduction. Promysalin, a salicylate-containing *Pseudomonas putida* antibiotic, was described to selectively target members of the genus *Pseudomonas*, without affecting the viability of other Gram-negative and Gram-positive bacteria (Li et al., 2011; Steele et al., 2015). The basic purpose of this work was to investigate the activity of promysalin, prepared by a recently developed synthesis protocol (Kadukar et al., 2016), against several Gram-negative and Gram-positive reference species using a validated standard microdilution method (Kahlmeter et al., 2003). Additionally, a further aim was to get insights into the mechanism of action of promysalin.

Bacterial species		MIC (µg/ml)	MBC (µg/ml)
Acetobacter aceti	MIM2000/28	512	>512
Escherichia coli	ATCC 25922	64	256
Pseudomonas aeruginosa	ATCC 10145	16	128
Pseudomonas aeruginosa	ATCC 27853	16	128
Pseudomonas stutzeri	LMG 2333	512	>512
Enterococcus durans	MIMO 936	64	>256
Enterococcus faecalis	ATCC 29212	64	>256
Enterococcus faecalis	ATCC 19433	64	>256
Enterococcus faecalis	LMG 16458	64	>256
Enterococcus faecium	ATCC 19434	64	>256
Enterococcus hirae	DSM 13032	32	>256
Lactococcus cremoris	DSM 20069	32	32
Lactococcus garvieae	DSM 20684	64	>256
Staphylococcus aureus	ATCC 25923	32	>256
Staphylococcus aureus	ATCC 29213	16	>256
Staphylococcus epidermidis	ATCC 14990[T]	64	128
Streptococcus pneumoniae	ATCC 700669	32	64
Streptococcus pneumoniae	ATCC 49619	32	64
Streptococcus pneumoniae	Penf	32	64
Streptococcus pyogenes	ATCC 12344[T]	64	128
Streptococcus thermophilus	DSM 20617[T]	16	64
Pediococcus acidilactici	PACI.0	128	128
Lactobacillus acidophilus	DSM 20079	256	>256
Lactobacillus delbrueckii subsp. bulgaricus	ATCC 11842	256	>256
Lactobacillus casei	LMG 6904	256	>256
Lactobacillus helveticus	ATCC 15009[T]	256	>256
Lactobacillus paracasei subsp. paracasei	DSM 5622 Y	256	>256
Lactobacillus plantarum	ATCC 4008	256	>256
Bacillus subtilis	DSM 347	16	32

Table 1. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of promysalin against Gram-negative and Gram-positive bacteria

Promysalin is a broad-spectrum antimicrobial. The data obtained through the microdilution method (Table 1), highlight that promysalin is active against Gram-positive and Gram-negative bacteria including several human pathogens. The activity of promysalin detected against Gram-positive microorganisms was in contrast to previous observation (Li et al., 2011) describing promysalin as an antibiotic which selectively targeted members of the genus *Pseudomonas*. The reason of these conflicting data is due to the methods used by Li and colleagues to screen the promysalin sensitive bacteria, the agar diffusion test. Since the inhibitory activity of promysalin against the sensitive *Pseudomonas stutzeri* LMG 2333 is detectable using the agar diffusion assay, whereas it is not against the sensitive Gram-positive bacteria (Figure 1), we could hypothesize that promysalin might act on *Pseudomonas* spp. and on Gram-positive bacteria through a different mechanism of action.

Figure 1. Agar diffusion assay carried out using *P. putida* RW1051 (promysalin producer), and *P. stutzeri* LMG 2333 (promysalin sensitive) as reference strains spotted in TSB agar. Promysalin production and activity was tested against *Streptococcus thermophilus* DSM 20617[T] (A), *Pediococcus acidilactici* PACI.0 (B) and *Pseudomonas stutzeri* LMG 2333 (C). P and S in the figure represent the growth of the promysalin-producer *P. putida* RW1051, and the promysalin-sensitive strain respectively.

Promysalin acts on Gram-positive bacteria disrupting the cell membrane phospholipid bilayer. Exposing cell suspensions to promysalin, we were able to detect by PI staining a damage of the cell membrane which was clearly evident in all Gram-positive strains tested (Figure 2/A) and some Gram-negative strains (Figure 2/B). However, a moderate or no membrane damage was detectable respectively in *P. stutzeri* LMG 2333 (Figure 2/C) and in *P. aeruginosa* ATCC 10145 (Figure 2/D), used as representative strains of Gram-negative bacteria, even if they were both sensitive to promysalin.

Figure 2. The effect of promysalin on different bacterial species cell-membrane integrity. Flow cytometry density diagrams show SYBR Green I vs PI fluorescence of cells exposed to promysalin (100 µg/mL) for 1h at 37 °C. (A) *S. thermophilus* DSM20617[T]; (B) *Acetobacter aceti* MIM2000/28; (C) *P. stutzeri* LMG 2333; (D) *P. aeruginosa* ATCC 10145. Viable cells are gated in G1, viable cells with slightly damaged cell membrane are gated in G2. Dead cells with damaged membrane are gated in G3. The transition of cell population from gate G1 to gate G3 is related to the entity of cell membrane damage.

In light of the data presented here, the disruption of phospholipid bilayer appears as the most plausible mechanism of action of promysalin on Gram-positive and on non-*Pseudomonas* Gram-negative bacteria, while other possible mechanisms, already hypothesized (Steele et al., 2016), can be responsible for the activity of promysalin on the genus *Pseudomonas*. In this context, the outer membrane of Gram-negative bacteria composed of an interior leaflet of phospholipids and an exterior leaflet of lipopolysaccharide, could represent in *Pseudomonas* species a barrier which limits the access of promysalin to the below cell membrane phospholipid bilayer. The induction of outer membrane vesicles by chlorhexidine, with a consequent increase of the outer membrane overall surface, allowed promysalin to access and damage the cell membrane also in the Gram-negative *P. aeruginosa* ATCC10145 (Figure 3).

Figure 3. Membrane damage in *P. aeruginosa* ATCC 10145 exposed to promysalin, chlorhexidine, and to a mixture of the two molecules. Flow cytometry density diagrams show the SYBR Green I vs PI fluorescence of cells exposed to promysalin or chlorhexidine or both the molecules. Viable cells are gated in G1, viable cells with slightly damaged cell membrane are gated in G2. Dead cells with damaged membrane are gated in G3. The transition of cell population from gate G1 to gate G3 is related to the entity of cell membrane damage. (A) Cells incubated in presence of chlorhexidine. (B) Cells incubated in presence of promysalin. (C) Cells incubated in presence of chlorhexidine and promysalin.

Conclusions. The overall data described in this study are shedding new light on the spectrum of activity and mechanism of action of promysalin. The broad-spectrum activity shown against Gram-positive and Gram-negative bacteria, together with the ability of promysalin to interfere only with the membrane phospholipid bilayer, suggest to further investigate the mechanism of action of this molecule on Gram-negative bacteria with focus on the genus *Pseudomonas*. (*Scientific Reports* 7, 8861(2017) doi:10.1038/s41598-017-07567-0).

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