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

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Running title: Selection of urease-negative *S. thermophilus*

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Abstract

Streptococcus thermophilus strains are used in fermented dairy products for their capacity to metabolize lactose into lactic acid. The rate of lactic acid production in milk is of major economic importance, as rapid acidification prevents growth of undesirable microorganisms. It is also of paramount significance for aroma, texture and flavor of the end product. Besides achieving customer satisfaction, improvement of production rate and operational costs incite industrials into selecting fast acidifying strains. Another important trait of *S. thermophilus* influencing acidification is the urease, which catabolizes urea into ammonia and has a detrimental effect on acidification. Unfortunately, most of the *S. thermophilus* strains possess the urease, and the urease-negative ones are necessary for industrial applications. Urease activity is a widely distributed activity in *S. thermophilus* species, and urease-negative strains are rare. The later are however interesting from an industrial point of view, as they may give faster acidification in dairy applications, because lactic acid is not buffered by urea-derived ammonia. Nowadays, the efforts to improve the characteristics of strains for industrial applications are based on natural strategies such as random mutagenesis. This implies the need of a screening method that is efficient in terms of time and success. In this context, the aim of this study was the development of a new medium that allows selection of urease-defective mutants based on *S. thermophilus* colony morphology. Discrimination capacity of the new medium was verified using previously characterized urease-negative recombinant strains. The new milk-based medium, applied to industrial *S. thermophilus* strains subjected to UV mutagenesis, allowed the selection of 3 mutants, partially or completely defective in urease activity. Genetic characterization of urease-defective mutants highlighted the presence of nonsense or missense mutations in the *ureA*, *ureC* and *ureG* genes, thus supporting their phenotype. Evaluation of milk acidification revealed increased performance for one out of three urease-defective mutants compared to wild-type strains.

Keywords: *S. thermophilus*, urease, mutant, selective-medium

1. Introduction

Streptococcus thermophilus is one of the most widely used lactic acid bacteria in dairy applications such as yogurt, other fermented milk and cheese production, 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 rapid acidification of milk producing lactic acid from lactose. Lactic acid contributes to milk coagulation and curd draining, imparts a fresh acid flavor and helps to restrain development of pathogens and spoilage microorganisms (Pernoud et al., 2004). The rate of acidification is an important technological trait, because delay in acidification may have severe effects on quality of a product or economic consequences in the industrial process (Mora et al., 2004).

The rate of acidification is a strain-dependent metabolic feature. Several factors influence it, and the urease activity is one of the most important ones. *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 into 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 the urea, naturally present in milk, slows down the desired acidification, sometimes inducing a temporary increase of pH, thereby prolonging fermentation time. This delays the acidification time and can have detrimental effects on texture and percentage of moisture of fermented products (Martin et al., 1997). It could further lead to the development of contaminants, especially when 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 at 37–42°C. Furthermore, since milk contains different amounts of urea from one batch to another (ranging from 3 to 6 mM), a further negative consequence of urease activity is an unpredictable rate

of acidification during the fermentation processes (Mora et al., 2004). Considering these aspects, 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. Indeed, it is well known that urease activity is a phenotypic trait widely distributed among *S. thermophilus* strains (Rasmussen et al., 2008; Spinnler and Corrieu, 1989; Tinson et al., 1982), and that urease-negative strains have been rarely described (Louaileche and Bracquart, 2001; Mora et al., 2002).For this purpose, the recombinant DNA technology would be an ideal method to eliminate urease as an unwanted property, due to its precision and versatility. However, the restricted food legislation and the doubtful consumer acceptance for genetically modified food ingredients discourage use of this technique (Derkx et al., 2014). In this context, currently, most 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 screening 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. Therefore, 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*.

92 ## 2. Material and Methods

93 ### 2.1. Bacterial strains and growth conditions

94 All *S. thermophilus* strains used in this work and all urease-defective mutants selected and
95 characterized are listed in Table 1. All strains were cultivated in M17 broth (Difco™, Sacco System,
96 Cadorago, Italy) containing 20 g/L of lactose at 37 °C and maintained as cultures supplemented with
97 glycerol (25% v/v final concentration) at –80 °C. Strains MIM13 and MIM772 were provided by
98 Sacco System.

99

100 ### 2.2. Measurement of temperature-dependent urease activity and homolactic fermentation

101 To measure the temperature-dependent urease activity, *S. thermophilus* cells, cultivated in M17 at 37
102 °C, were collected by centrifugation in the late exponential phase ($O.D._{600nm}$ 1.0), washed twice and
103 suspended in saline solution (9 g/L NaCl). Cell were quantified by flow cytometry (Accuri C6, BD
104 Biosciences, Milan, Italy) as described in Arioli et al. (2017). The phenol red assay (Lanyi, 1987)
105 was carried out by mixing 30 µL of solution A (2 g of urea dissolved in 2 mL of ethanol and 4 mL of
106 sterilized deionized water) with 470 µL of solution B (1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 5 g/L NaCl, 20
107 µg/mL phenol red) and 10^8 *S. thermophilus* cells. Cell suspension was aliquoted (100 µL) in PCR
108 tubes and incubated for 6 h at the following temperatures: 25.0, 30.0, 30.5, 31.7, 33.6, 36.2, 38.8,
109 41.2, 43.8, 46.4, 48.3, 49.5 and 50.0 °C in a thermal-cycler (Mastercycler Nexus Gradient, Eppendorf,
110 Milan, Italy) with a gradient temperature ranging from 25 °C to 50 °C. After incubation, the
111 development of a red-violet color due to the release of ammonia by urease was measured using a
112 spectrophotometer ($O.D._{555nm}$). Urease activity was expressed as percentage of the maximum activity
113 using as a reference the maximum $O.D._{555nm}$ measured, *i.e.* those measured at 48.3 and 49.5 °C. The
114 urease activity was expressed as the average of three determinations ± SEM.
115 To measure the temperature-dependent homolactic fermentation, *S. thermophilus* was inoculated in
116 liquid milk-based medium avoiding the addition of urea. Cell suspension was aliquoted (100 µl) in
117 PCR tubes and incubated as described above for the evaluation of the 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 using a photo camera (Nikon 1 J4, Tokyo, Japan) and visually evaluated.

2.3. Determination of urease activity

Urease activity of *S. thermophilus* was evaluated by the phenol red assay described by Lanyi (1987) with some modifications. In detail, *S. thermophilus* cells were collected and suspended in solutions A and B, as previously described. Cell suspension, prepared as described in chapter 2.2, was aliquoted (150 µL) in 96 well microtiter plates and incubated at 37 °C for 6 h. Development of a red-violet color due to the release of ammonia by urease was monitored using a spectrophotometer EON (Biotek, Winoosky, VT) that was programmed for readings ($O.D._{555nm}$) 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 Gene5 (Biotek, Winoosky, VT). 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.

2.4. 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 in order to distinguish urease-positive and urease-defective strains on the basis of the colony morphology and color of the medium surrounding the colonies, as a consequence of its pH. To prepare the medium, skimmed milk (Difco™, Sacco System) (90 g/L), sucrose (Sigma-Aldrich, Milan, Italy) (10 g/L), yeast extract (Difco™, Sacco System) (1 g/L) and the mix of pH sensitive dyes (50 mL/L) (1 g/L bromocresol green, pKa 4.7; 1 g/L bromocresol purple, pKa 6.3, ; 4 mM NaOH) (Sigma-Aldrich) were dissolved in half of the final volume of deionized water and sterilized at 110 °C for 15 min. The agar was

144 dissolved in the remaining volume (15 g/L) and sterilized at 110 °C for 30 min. After sterilization,
145 the components were mixed in sterile conditions, urea was added at a final concentration of 20 mM,
146 and the medium was poured into Petri dishes. To identify the urease phenotype, overnight cultures of
147 *S. thermophilus* grown in M17 (containing 2% lactose) were diluted in sterile saline solution and
148 plated on the milk-based medium. After incubation for 18 h at 37 °C, plates were further incubated
149 at 25 °C for at least 5 h to allow the slowing-down of the homolactic fermentation and the appearance
150 of the urease activity, if present. The later determined the color change of the mix of pH indicators,
151 around the colonies, from yellow to blue, due to the ammonia released from urea.
152
153 2.5. UV mutagenesis and screening of urease-defective strains of *S. thermophilus*
154 *Streptococcus thermophilus* MIM13 and MIM772 were cultured in M17 broth containing 20 g/L of
155 lactose at 37 °C, until the culture reached an $O.D._{600nm}$ of 0.2. Cells were collected by centrifugation
156 and washed twice in sterile saline solution. One mL of cell suspension was poured in a Petri dish and
157 exposed to UV light (UV-C lamp, 30 Watt, wavelength 254 nm, intensity at a distance of 15 cm 1780
158 $\mu W/cm^2$, exposure time 30 s). After UV treatment cells were diluted to obtain theoretically 1 CFU/µL,
159 plated (200 µL) on milk-based medium and incubated at 37 °C for 24–36 h in anaerobic conditions,
160 followed by at least 5 h of incubation at room temperature (25 °C) in aerobic conditions. Based on
161 colony morphology, the potential urease-defective mutants were isolated, cultivated in M17
162 (containing 2 % lactose), tested for their urease activity using the phenol red assay, and screened for
163 their ability to coagulate milk. To this aim, M17 cultures, obtained after 24 h of incubation at 37 °C,
164 were used to inoculate (1% v/v) 10 mL of reconstituted skimmed milk (Difco™, Sacco System).
165 After incubation at 37 °C for 12 h, milk coagulation was quantified visually.
166
167 2.6. Milk acidification
168 Acidification rates of the mutants were evaluated in sterilized reconstituted skimmed milk and
169 compared to their wild type in absence or presence of 20 mM of urea (filter-sterilized and added after

milk sterilization). Cells after the overnight growth in M17 broth were used to inoculat (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 the addition of filter-sterilized urea. The pH was measured continuously and recorded every 30 min for 24 h at 37 °C using an iCINAC system (AMS, Guidonia, Rome, Italy). Data were reported as the average of three replicates.

For *S. thermophilus/L. delbrueckii* subsp. *bulgaricus* yogurt association, the acidification rates were measured in pasteurized skimmed milk (85 °C, 15 min). *S. thermophilus* wild-type and urease-defective mutants and *L. delbrueckii* subsp. *bulgaricus* MIMLB08 were inoculated in M17 and MRS broth, respectively, and incubated at 37 °C for 18 h. Subsequently, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* strain cells were counted by flow cytometry as previously described (Arioli et al., 2017). Two hundred mL of pre-heated pasteurized skimmed milk, supplemented with 4 mM urea, were inoculated with 10^7 events/mL of single strain or with 5×10^6 events/mL of *L. delbrueckii* subsp. *bulgaricus* MIMLB08 and 5×10^6 events/mL of *S. thermophilus* wild-type or urease-defective mutant. Milk pH was measured continuously as described above. Data were reported as the average of three replicates.

2.7. PCR protocols and DNA sequencing

Total bacterial DNA was extracted starting from 100 µl of M17 broth culture as previously described (Mora et al., 2004). The amplification of DNA regions encompassing the whole urease cluster was performed as recommended by the supplier (Takara Bio Europe, Saint-Germain-en-Laye, France) using 0.5 µM of the following primers: UreF 5'- GAGTGTCCAGGCTCCGATAA -3', UreR 5'-CTAAGATACGTAACACCAGA -3', NICKF1 5'- TCCTTAGATATCTCAGGTTTG -3', NICKR1 – 5' TTGTAACAGAATTCACTAAGC – 3' and 2 U of ExTaq DNA polymerase (Takara Bio Europe). 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

196 CFX96 instrument (Bio-Rad Laboratories, Milan, Italy). The PCR product was purified (QIAquick
197 PCR Purification Kit, Qiagen, Hilden, Germany) and sequenced using the above-mentioned primers
198 followed by primer walking (Microsynth AG, Balgach, Switzerland). The obtained sequences were
199 analyzed with BLAST services at the National Center for Biotechnology Information and
200 subsequently manually aligned. To distinguish between *ureC* and the deleted version *ΔureC3*, a DNA
201 region encompassing *ureC* gene was amplified on DNA extracted from M17 cultures of strain DSM
202 20617^{T}, A16(*ΔureC3*), and from colonies grown in milk-based medium. PCR was carried out using
203 the primer set (UreCd0f 5' – CTGTTCATGATCCTATTCAG – 3'; UreCd0r 5' –
204 CAACACCAATAGCTAGGACA – 3'), which allowed the amplification of a 2439 bp and 1800 bp
205 fragments in the wild-type and in the urease-negative mutant A16(*ΔureC3*), respectively. PCR
206 reactions were performed in a 25 μL reaction mixture containing 1 colony (picked up with a sterile
207 wooden stick) or 50 ng of DNA, 2.5 μL 10× Dream Taq^{TM} reaction buffer, 200 μM of each dNTP,
208 0.5 mM $MgCl_2$, 0.5 μM each primer and 0.5 U Dream Taq^{TM} DNA polymerase (Thermo Fisher
209 Scientific, Monza, Italy). Amplifications were carried out using a PCR-Mastercycler 96 (Eppendorf,
210 Milan, Italy). The PCR mixtures were subjected to the following thermal cycling: initial hold at 95
211 °C for 3 min and 39 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 50 s and a single final
212 extension at 72 °C for 10 min. Amplification products were electrophoresed in 1.5 % (w/v) agarose
213 gel (with 0.2 μg/mL of ethidium bromide) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH
214 8.0) and photographed.
215

3. Results and Discussion

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. Monnet and colleagues (Monnet et al., 2004) had already proposed a screening method for *S. thermophilus* urease-defective mutants after a mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (NTG). The screening method was based on the formulation of a modified M17 agar on which the mutants were plated and incubated for 2 days. Subsequently, 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 screening for urease-defective mutants of *S. thermophilus* directly on the plates after the proper incubation time. Milk contains lactose, and *S. thermophilus* catabolizes the glucose moiety through the glycolytic pathway and the homolactic fermentation producing lactic acid. Lactic acid production decreases the pH, and in presence of the pH-sensitive dyes bromocresol green and bromocresol purple, the medium changes color from blue/green 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 (40 mM when all urea present in the medium is hydrolyzed) overcomes the lactic acid produced, which is approximately 23 mM at pH 5.2 (Mac Bean et al, 1979), resulting in medium alkalization, which changes the medium color from yellow to blue/dark blue (Figure 1). After 18 h of incubation at 37 °C, milk-based Petri plates containing colonies were further incubated at room temperature (25 °C) for at least 5 h to allow the medium alkalization resulting from urea hydrolysis. At room temperature (25 °C) glycolysis and homolactic fermentation strongly slow down, whereas the urease activity is still present, maintaining 74% of its maximum (Figure 2). The use of sucrose as an additional carbon source in the milk-based medium was necessary to obtain larger colonies compared to just having the milk lactose. The increase of colony dimension in presence of sucrose could be due to a higher cellular production of exopolysaccharides, as previously observed in *S.*

242 *thermophilus* by Shene et al. (2008). The use of an opaque milk-based medium instead of other
243 laboratory media was suggested by the need to have a strong contrast between the color of bacterial
244 colony and the color generated by the pH-sensitive dyes in the medium. Further, the use of a milk-
245 based medium instead of a laboratory medium, reduces the probability of isolating mutants with
246 secondary mutations that are not able to grow well in case of industrial dairy applications.
247 The efficacy of the developed medium, denominated by us the Blue-Green-Milk-based (BGM)
248 medium, to discriminate between urease-positive and urease-defective strains was tested using
249 urease-positive strain *S. thermophilus* DSM 20617^T and its derivative, urease-negative mutant
250 A16(*ΔureC3*) (Mora et al., 2004) (Table 1). On the BMG medium, DSM 20617^T showed white
251 smooth colonies against a blue background. On the other hand, the urease-negative derivative, strain
252 A16(*ΔureC3*) showed yellow smooth colonies on a yellow background (Figure 3a, b).
253
254 3.2. Effectiveness of BGM medium in selecting urease-defective mutants
255 To confirm the actual screening procedure to distinguish urease-positive and urease-negative colonies
256 on the same plate, a mixed culture of *S. thermophilus* DSM 20617^T and A16(*ΔureC3*) was prepared
257 and plated. As shown in Figure 3c, the two colony morphotypes were easily identified. The urease
258 phenotype of each colony morphotype was assessed using the colorimetric assay, whereas the genetic
259 identity of the strains was confirmed by PCR, designed to distinguish the wild-type and the
260 A16(*ΔureC3*), based on an in-frame deletion of 649 bp in *ureC* gene (Table 1).
261 The discriminatory power of the BMG medium was also tested on the urease positive nickel-
262 dependent *S. thermophilus* MIMO1 isolated from commercial yogurt (Mora et al., 2002) and
263 previously characterized. Strain MIMO1 shows urease activity only if cultivated in presence of
264 minimum of 1 μM $NiCl_2$, due to a defective nickel transport system (Table 1, Table 2). When strain
265 MIMO1 was cultivated on the BMG medium supplemented with $NiCl_2$, it showed the urease-positive
266 morphology of the colonies, whereas it showed a urease-negative morphotype when nickel was not
267 added to the BMG medium. Due to the high level of urease activity of strain MIMO1 when cultivated

268 in presence of $NiCl_2$, that determined a high ammonia release, its colonies appeared smaller than
269 those of the strain DSM 20617^T, probably as a consequence of the excessive environmental
270 alkalization, as evidenced by the deep blue color of the BMG medium (Figure 4).
271
272 3.3. Selection and genetic characterization of *S. thermophilus* urease-defective mutants
273 Random mutagenesis with e.g. UV, as a strain-improvement strategy, was already carried out with
274 industrially relevant lactic acid bacterial species. UV mutagenesis was successfully applied for the
275 selection of *L. delbrueckii* mutants with an enhanced lactic acid production (Kadam et al., 2016) or
276 with an ameliorated utilization of cellobiose and cellotriose (Adsul et al., 2007). Moreover, UV
277 mutagenesis was used to improve *L. rhamnosus* acid tolerance and lactic acid production (Wang et
278 al., 2007), for the selection of a *Bifidobacterium animalis* subsp. *lactis* strain with a decreased ability
279 to produce acetic acid (Margolles and Sánchez, 2012), and to improved *Bifidobacterium breve*
280 viability at low pH (Saarela et al., 2011). The recombinant DNA technology would be a more
281 powerful tool for these purposes. However, the tight food regulation and the reluctance of consumers
282 towards genetically modified microorganisms in foods have kept the random mutagenesis methods
283 in use (Šeme et al., 2011).
284 Two industrially relevant *S. thermophilus* strains, MIM13 and MIM772, were subjected to the UV
285 mutagenesis to screen for urease-defective mutants using the above-mentioned assay. After the
286 mutagenesis process, the UV treated cultures were plated on the BMG medium, and approximately
287 30 potential urease negative clones for each mutagenized strain were isolated based on colony
288 morphology and color as described above. The potential urease negative colonies were further
289 investigated through the evaluation of urease activity by the phenol red assay. Only three mutants,
290 showing absence or weak urease activity, maintained also the ability to acidify milk after 12 h of
291 incubation at 37 °C. Two mutants, MIM22 and MIM10, derived from MIM13 and MIM772,
292 respectively, were found to completely lack urease activity. Meanwhile, mutant MIM12 had a weaker
293 urease activity than the corresponding wild-type MIM772 (Table 2). The urease-defective phenotype

294 of each selected mutant was assessed after 10 consecutive subcultures in M17 medium and in
295 reconstituted skimmed milk.
296 The genetic characterization of the genomic locus encompassing the urease operon of wild-type and
297 urease-defective derivatives highlighted mutations that should justified the observed phenotype. In
298 detail, sequence analysis of urease operon of the mutant MIM22 revealed a nonsense mutation in
299 *ureA* gene (coding for urease gamma subunit), which generated a truncated UreA protein. Sequence
300 analysis of the urease operon of mutant MIM10 showed only a single nucleotide substitution, which
301 determined a missense mutation in the *ureC* gene ($Pro_{172} \rightarrow Leu_{172}$) in a non-conserved UreC domain,
302 thus suggesting that the observed urease-negative phenotype could be linked to other mutations
303 occurred outside the urease-operon. A single nucleotide substitution determining a missense mutation
304 in the *ureG* gene (coding for urease accessory protein) was identified in the mutant MIM12
305 characterized by weak but not absent urease activity.
306
307 3.4. Evaluation of milk-acidification performance of wild-type strains and urease-defective
308 mutants
309 The milk acidification assay is the gold standard for industrial characterization of *S. thermophilus*
310 strains for dairy applications. Therefore, all urease-defective mutants selected in this study were tested
311 in absence and in presence of urea (20 mM). The obtained results (Figure 5) clearly showed that
312 mutant MIM10 performed better than the wild-type MIM772, which acidified milk despite of the
313 presence of urea. Meanwhile, the mutant MIM12 maintained the urease activity (even if postponed
314 for 1.5 h) compared to the wild-type MIM772. The shift of urease peak observed for the mutant
315 MIM12 could be related to a delay or an incorrect assembling of the active urease as a consequence
316 of the missense mutation in the accessory *ureG* gene.
317 Mutant MIM22 showed a milk-acidification not affected by the presence of urea even if it did not
318 perform better than the wild type to reach pH 5 used as a reference.

Considering the same parameter, among the three selected mutants, only MIM10 performed better than the wild type, but only in presence of urea. In absence of urea, MIM10 showed a lower acidification rate compared to the wild type (Figure 5b and 5e). The possible reasons of the lower performance of MIM10 compared to the wild type could be related to the urease-negative phenotype of the mutant itself, since urease activity is known to boost homolactic fermentation in *S. thermophilus* (Arioli et al., 2010; 2017). Even in absence of additional urea supplementation, a residual urea could be present in the sterilized skimmed milk. Moreover, in mutant MIM10, we could not exclude the presence of mutation/s outside the *ure* operon that could have affected its milk acidification rate.

3.5. Evaluation of the milk-acidification performances of wild-type strains and urease defective mutants in association with *Lactobacillus delbrueckii* subsp. *bulgaricus* MIMLB08

With the aim of testing the performances of urease-defective strains in a dairy application, the selected mutants have been co-cultured in pasteurized milk with *L. delbrueckii* subsp. *bulgaricus* MIMLB08 to simulate the standard yogurt association. *L. delbrueckii* subsp. *bulgaricus* MIMLB08 was co-cultured with *S. thermophilus* MIM13 or MIM772, and the acidification curves were compared to those obtained substituting the *S. thermophilus* wild-type strains with the urease-defective derivative mutants. The obtained results (Figure 6) highlighted that only the co-culture composed by *L. delbrueckii* subsp. *bulgaricus* MIMLB08 and the urease-defective mutant MIM10 showed a significantly faster milk acidification rate compared to that obtained with the corresponding urease-positive culture association (MIMLB08/MIM772), thus confirming the previous observation in single cultures (Figure 5b and 5e). Interestingly, a slightly faster (even if not significant) acidification was observed also by the association MIMLB08/MIM22 (Figure 6e).

4. Conclusions

344 Development of efficient screening protocols based on strain-dependent metabolic traits is critical for
345 the selection of new strains for dairy applications. In the present study, we aimed to develop a new
346 medium for the identification of urease-defective strains of *S. thermophilus*. Urease activity is one of
347 the most important metabolic factors that influence milk acidification rate, the most relevant
348 parameter to consider when we deal with industrial dairy fermentations. Delays in the acidification
349 rate, indeed, can have effects on product quality and economic consequences. In this context, the
350 selection of urease-defective mutants starting from *S. thermophilus* strains, already chosen for their
351 technological traits and for their industrial relevance, could represent a further improvement of their
352 technological performance.
353
354

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427

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

Strain	Urease phenotype	Urease genotype *	Reference
DSM 20617^T	urease-positive	functional urease operon	(Mora et al., 2004)
A16*(ΔureC3)*	urease-negative	DSM 20617^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 acid 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* (Met_{140}→Leu_{140})	this study (MH700462)

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

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

436

Strain	Urease activity (mO.D.$_{555nm}$/min)
DSM 20617^T	2.7 ± 0.1
A16(*ΔureC3*), a DSM 20617^T derivative	nd
MIMO1	3.4 ± 0.1*
MIM13	4.9 ± 0.3
MIM22, a MIM13 derivative	nd
MIM772	3.9 ± 0.4
MIM10, a MIM772 derivative	nd
MIM12, a MIM772 derivative	1.0 ± 0.1

437

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

439

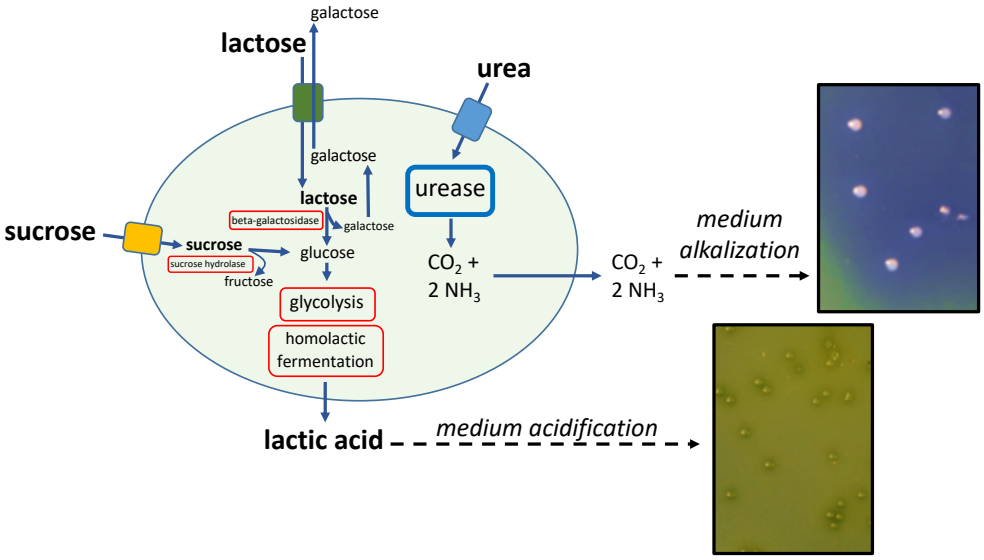

Figure 1. Schematic representation of the physiology of *S. thermophilus* cells cultivated in BMG medium. Pictures of urease-positive and urease-negative *S. thermophilus* colonies grown on BGM medium are also reported.

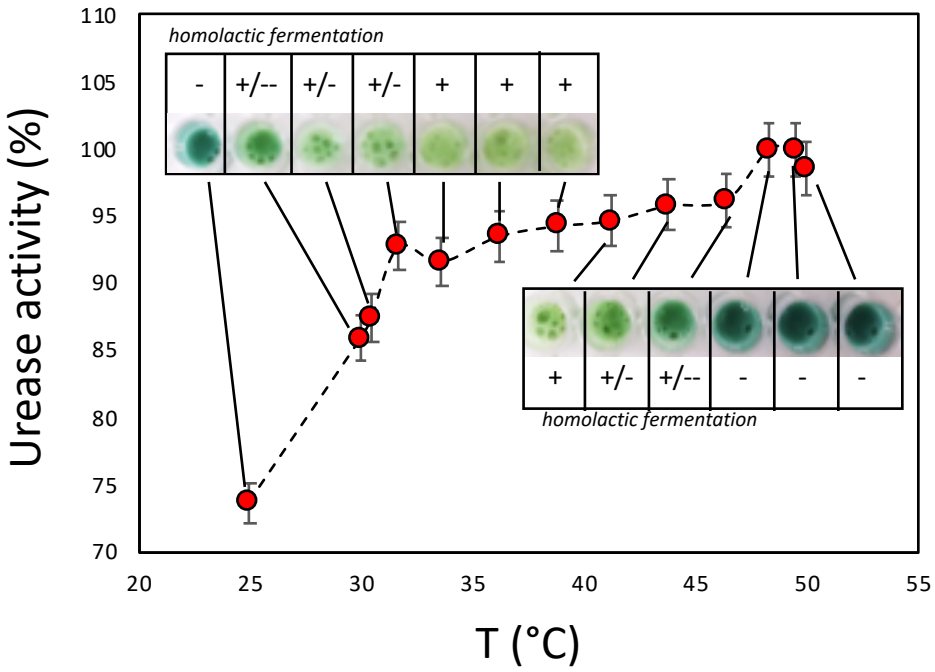

Figure 2. Relative urease activity as a function of the temperature (red circles) and BMG medium acidification through homolactic fermentation (graphical insets) of *S. thermophilus* MIM13. The maximum acidification (+) recorded referred to a yellow/green color of the BGM medium. The absence of acidification (-) referred to a blue/green color of the BMG medium. The urease activity is expressed as the average of three determinations ± SEM as % of the maximum activity using the maximum $O.D._{555nm}$ measured as a reference.

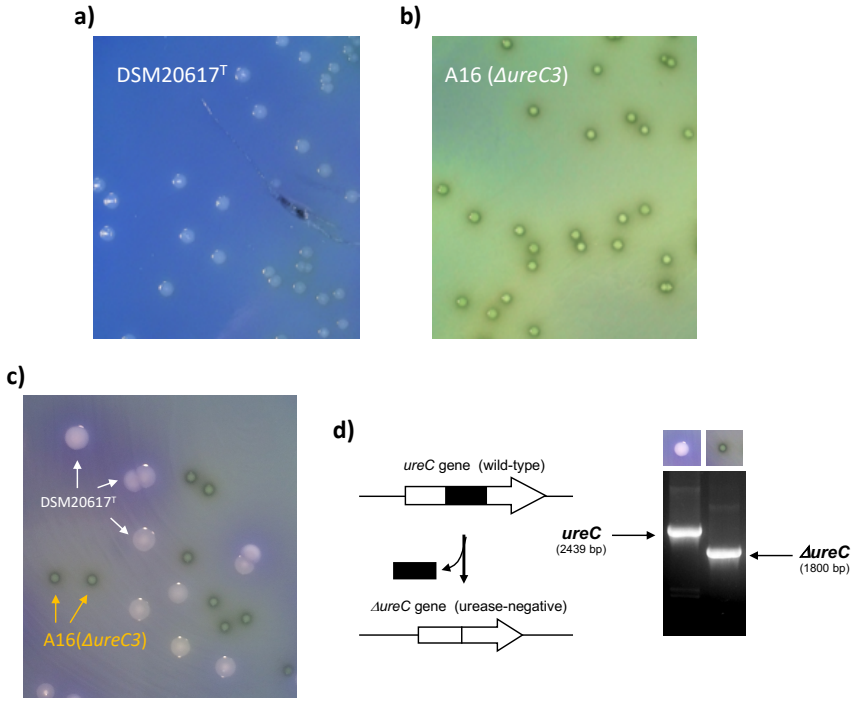

Figure 3. Colony morphology of urease-positive and urease-negative *S. thermophilus* in B;G medium. a) Colony morphology of urease-positive *S. thermophilus* DSM 20617^T. b) Colony morphology of *S. thermophilus* A16(*ΔureC3*), a DSM 20617^T urease-negative recombinant. c) Colony morphology of mixed culture of *S. thermophilus* DSM 20617^T and A16(*ΔureC3*). d) Schematic representation of the genetic strategy adopted to generate the recombinant urease-negative A16(*ΔureC3*) (Mora et al., 2004) and an example of the PCR assay used to confirm the genetic identity of the two colony morphotypes detected in the BMG medium.

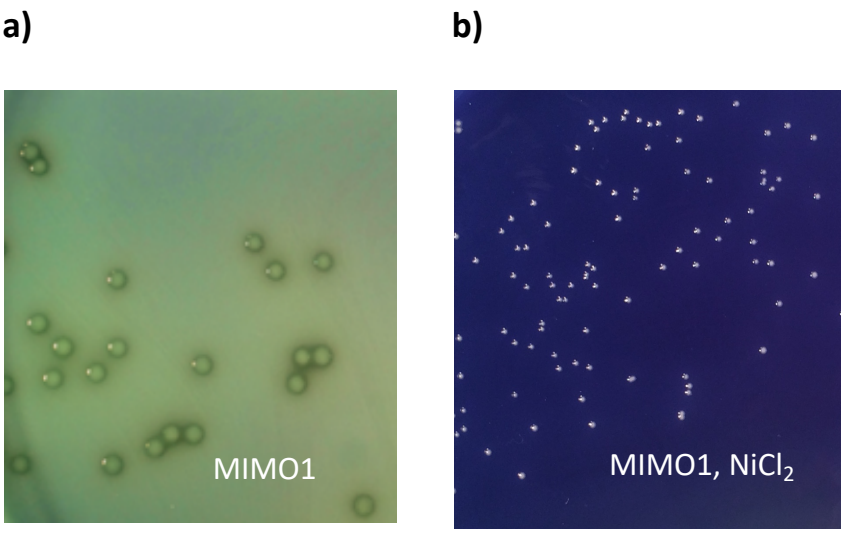

Figure 4. Colony morphology of urease-positive Ni-dependent *S. thermophilus* MIMO1 in BMG medium without (a) and with addition of 5 μM $NiCl_2$ (b).

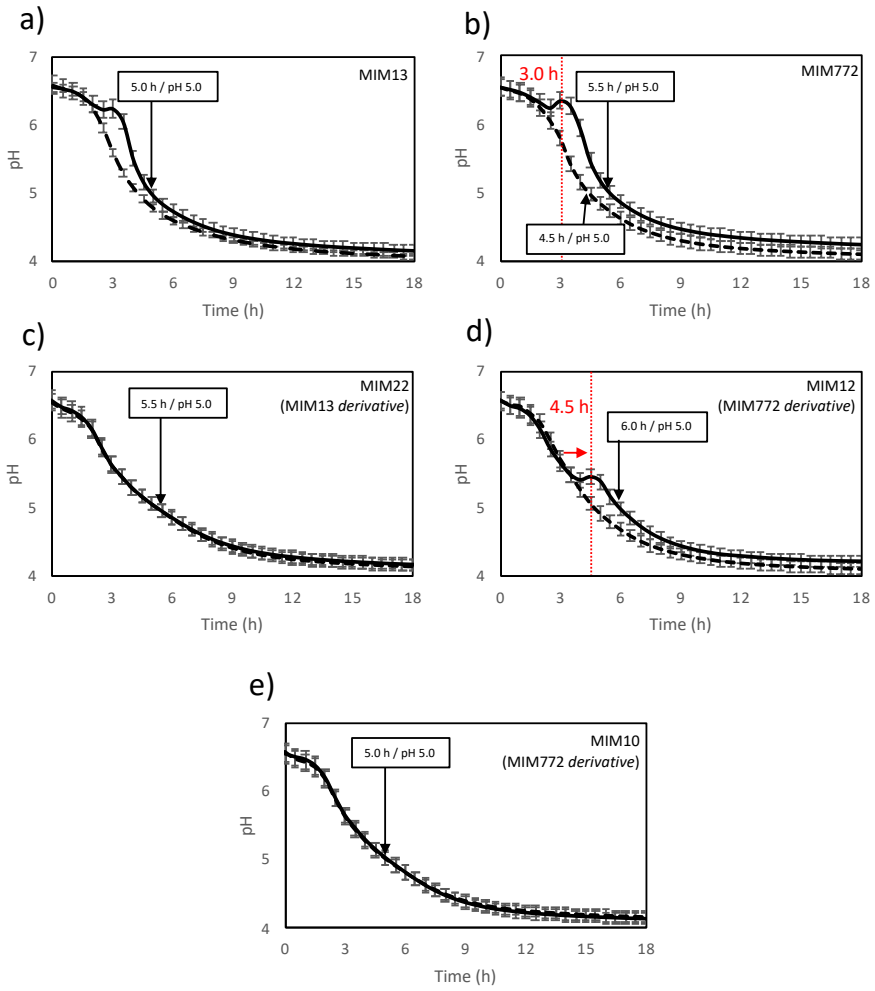

Figure 5. Acidification curves in reconstituted sterilized skimmed milk of *S. thermophilus* MIM13 (a) and MIM772 (b) and their derivative urease-defective mutants MIM22 (c), MIM12 (d), and MIM10 (e) in absence (dashed line) and in presence (solid line) of 20 mM urea . The acidification curves are expressed as the average of three determinations ± SEM. The time to reach pH 5.0 is indicated. Red vertical dashed line refers to the time when the maximum alkalization due to the urea hydrolysis was detected.

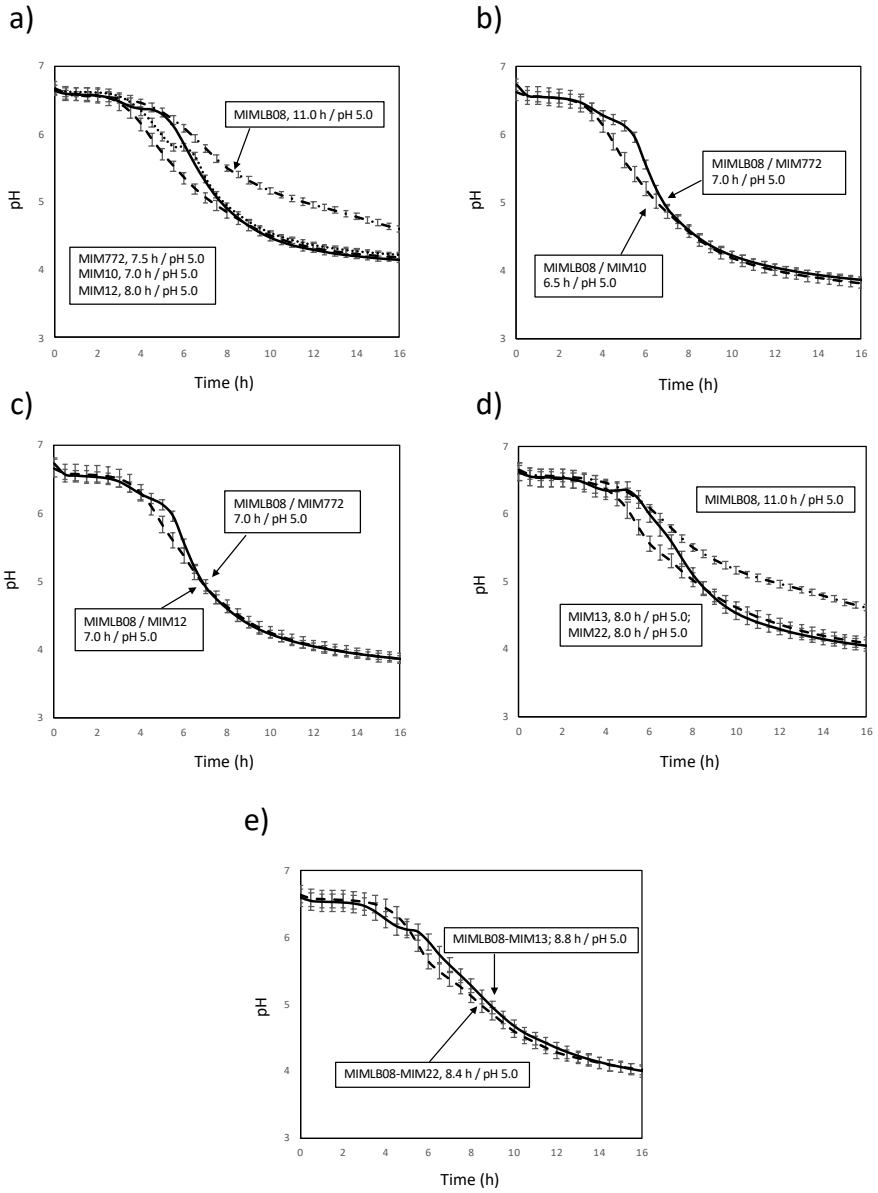

Figure 6. Acidification curves in reconstituted pasteurized skimmed milk supplemented with 4 mM urea of *L. delbrueckii* subsp. *bulgaricus* MIMLB08 (dotted and segmented line), *S. thermophilus* wild-type strains (continuous line) and their urease-defective derivatives (segmented lines, dotted line only for MIM12) as single cultures (a, d) and culture associations (b, c, e). The time to reach pH 5.0 is indicated. The acidification curves are expressed as the average of three determinations ± SEM.