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Evaluation of a new culture medium for the enumeration and isolation of *Streptococcus salivarius* subsp. *thermophilus* from cheese

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ABSTRACT

Enumeration and isolation of *Streptococcus salivarius* subsp. *thermophilus* from cheese is challenging, due to the relatively high number of species it may host.

We describe medium SPY9.3 for the cultivation of *S. salivarius* subsp. *thermophilus* from cheese. The medium and related incubation conditions (SPY) was compared with 2 other protocols, M17 and ST: sensitivity was assessed by parallel cultivation of 55 strains of *S. salivarius* subsp. *thermophilus*, and selectivity by (i) parallel cultivation of 60 strains belonging to 20 different non-target species and sub-species and (ii) isolating bacteria from 3 raw-milk cheeses. Colony counts were similar on SPY9.3 and M17 (mean difference 0.07 log(cfu/mL), $p > 0.001$) and significantly higher on ST than on M17 and SPY9.3 (mean differences 0.42 and 0.48 log(cfu/mL), respectively, $p < 0.001$). SPY was more specific than ST and M17, with respectively 20%, 40%, and 50% of the investigated non-target species able to grow. *S. salivarius* subsp. *thermophilus*, *Enterococcus* spp., and *Staphylococcus aureus* were indistinguishable using all 3 protocols. Only SPY avoided growth of *Lactobacillus delbrueckii* subsp. *lactis*. Finally, ST and SPY displayed higher recoveries of *S. salivarius* subsp. *thermophilus* colonies from cheese than M17 (5.6, 5.5, and 3.0 adjusted log(cfu/mL), respectively) and the lowest proportion of non-specific isolates.

The protocol described here and based on SPY9.3 presents a promising alternative to existing protocols for the enumeration and isolation of *S salivarius* subsp. *thermophilus* from cheese or other complex fermented products.

1. Introduction

Acidification in industrial and artisanal cheese production relies on a reduced number of lactic acid bacteria (LAB) species, which ferment lactose into lactic acid and are often added to milk in the form of starter cultures. Strains of *Streptococcus salivarius* subsp. *thermophilus* are mainly used in thermophilic starter cultures for the production of hard-cooked cheeses but also in the production of other cheese types (Hols et al., 2005; Delorme, 2008; Iyer et al., 2010). They are usually metabolically very active at the very beginning of the cheese manufacturing process, quickly fermenting lactose and acidifying their environment (Delorme, 2008). This characteristic, together with the ability to ferment galactose (Mora et al., 2002), make *S. salivarius* subsp. *thermophilus* valuable for the cheese industry, reducing production time and limiting the proliferation of undesired spoilage and pathogenic bacteria (Cui et al., 2016; Gobbetti et al., 2018). Furthermore, some strains participate in the formation of aromas and flavors during the ripening of cheese due to their proteolytic activity (Rodriguez-Serrano et al., 2018), influence the texture of fermented products by the production of exopolysaccharides (Mende et al., 2016), produce health beneficial compounds, such as B-group vitamins (Meucci et al., 2018), and even display probiotic attributes (Burton et al., 2017).

The advent of next-generation techniques has opened an immense field of possibilities for the description of bacterial communities and their functions in the last decade. Nevertheless, traditional microbiology techniques remain essential for the isolation and characterization of particular bacterial strains for research purposes as well as for potential industrial applications, such as their use in starter or adjunct cultures for cheese manufacturing. Cheese often provides shelter to a high number of different species, depending, among other factors, on the cheese variety (Dugat-Bony et al., 2016), the manufacturing process (Yeluri Jonnala et al., 2018), or the ripening stage (Sant'Anna et al., 2019). Besides LAB, shown to constitute at least 21 species in cores of traditional uncooked pressed and ripened cheeses (Montel et al., 2014), species belonging to other groups are also frequently found, such as staphylococci, *Clostridiales*, *Proteobacteria*, and *Actinobacteria* (Delbès et al., 2007;

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Falardeau et al., 2019; Giello et al., 2017; Quigley et al., 2012). Thus, enumerating and isolating *S. salivarius* subsp. *thermophilus* from such a complex food matrix presupposes that it can be differentiated from the other species sharing its habitat. One strategy for isolating and/or enumerating particular bacterial species relies on differential culture media, making use of particular biochemical characteristics of each species to differentiate it from other species on an agar plate by, for example, the formation of colored colonies. Several differential media for *S. salivarius* subsp. *thermophilus* have been developed (for a comprehensive review, see Ashraf and Shah, 2011). Differential cultivation is, however, a complex task when several closely related species with similar characteristics are living in the same environment (Saccaro et al., 2012). Another strategy is based on selective culture media, taking advantage of exclusive properties of the species of interest to favor its growth over the other members of the bacterial community. The 2 most common and recommended culture media for the selective or differential cultivation of *S. salivarius* subsp. *thermophilus* are, respectively, M17 (Terzaghi and Sandine, 1975) and ST agar (Dave and Shah, 1996), both incubated at 37 °C for 24 h under an aerobic atmosphere (Dave and Shah, 1996; Ravula and Shah, 1998). While colonies of *S. salivarius* subsp. *thermophilus* are not colored on M17, they grow yellow on ST and induce a yellow coloration in the medium.

To date, most culture media for the enumeration or isolation of *S. salivarius* subsp. *thermophilus* have been developed with the aim of discriminating it from the few other species present in yogurt, mainly *Lactobacillus delbrueckii* subsp. *bulgaricus* (Aryana and Olson, 2017), but occasionally also a few other species of LAB added to improve the flavors (Chen et al., 2017) or with probiotic properties (Lourens-Hattingh and Viljoen, 2001). Nevertheless, these media are often not adapted for the enumeration/isolation of *S. salivarius* subsp. *thermophilus* from cheese because the versatile and unpredictable microbial composition of this fermented food affects proper selectivity of the media, which are designed for low-diversity environments.

In order to increase the selectivity of the overall isolation conditions of *S. salivarius* subsp. *thermophilus*, a new culture medium named SPY9.3 was developed. The objective of this study was to evaluate the performance of SPY9.3 and related incubation conditions for the enumeration and isolation of *S. salivarius* subsp. *thermophilus* from a multiple species cheese environment.

2. Material and methods

2.1. Bacterial strains

Fifty-five *S. salivarius* subsp. *thermophilus* strains originating from various environments were used to assess the sensitivity of the tested protocols (Supplementary Table S1). Sixty strains belonging to 20 other species potentially found in cheese (non-target species, Supplementary Table S1) were selected to evaluate the specificity of the protocols for the isolation of *S. salivarius* subsp. *thermophilus* from cheese. *S. salivarius* subsp. *thermophilus* strains were stored at −80 °C in M17 (Terzaghi and Sandine, 1975) commercial broth (Biolife Italiana Srl, Milan, Italy) supplemented with 5 g/L D-glucose, whereas the non-target species were stored at −80 °C in De Man, Rogosa, Sharpe (MRS) (De Man et al., 1960) broth with Tween 80 (Biolife Italiana Srl, Milan, Italy). Sterile low-fat milk was used as a cryoprotectant.

2.2. Bacterial identification

The identity of all strains was confirmed using matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) on a MicroFlex™ LT/SH MS (Bruker Daltonics, Bremen, Germany) as described previously (Pfrunder et al., 2016), with the modification that direct smearing of the bacterial cells was performed by transferring cell material from the colonies on the agar plate onto the MALDI-TOF target plate using sterile toothpicks. Data were acquired with the FlexControl version FC 3.4.105.

Table 1
Description of the protocols evaluated in this study.

Protocol	Culture medium	Incubation temperature	Incubation time	Aerobic/ anaerobic
M17	M17	42 °C	48 h	Aerobic
ST	ST	42 °C	48 h	Aerobic
SPY	SPY9.3	42 °C	48 h	Anaerobic

The spectra were analyzed with the MBT Compass software v. 1.4 (Bruker Daltonics, Inc., Billerica, MA, USA) and the Realtime Classification Biotyper MBT RUO 3.1 with the BDAL V5.0 library.

2.3. Design of medium SPY9.3

The selectivity of the new medium was based on pH and sucrose concentration. Two distinct experiments were performed using 10 strains of *S. salivarius* subsp. *thermophilus* (Supplementary Table S1) to evaluate the influence of these 2 parameters on colony counts. First, colonies were enumerated using the spread plate technique at 4 different pH values (9.0, 9.5, 10.0, and 10.5 before autoclaving) with a constant sucrose concentration (10 g/L). Second, the colony counts were compared at constant pH (9.2 before autoclaving) and with 2 sucrose concentrations (5 vs 10 g/L). The plates were incubated at 42 °C for 48 h under anaerobic conditions before the colonies were counted. The various conditions were evaluated by comparing the colony counts, expressed as log(cfu/mL), and their variance. Paired *t*-test was used to evaluate the significance of the differences between the colony count distributions.

2.4. Medium preparation

2.4.1. M17

M17 (Terzaghi and Sandine, 1975) commercial agar (Biolife Italiana Srl, Milan, Italy) was dissolved in distilled water according to manufacturer's instructions, the pH value was adjusted to 7.4, and the medium was sterilized at 121 °C for 15 min before being poured into Petri dishes.

2.4.2. Streptococcus thermophilus agar (ST)

Streptococcus thermophilus (ST) agar (Dave and Shah, 1996) was prepared by dissolving 10 g/L of peptone from casein (Merck, Darmstadt, Germany), 1 g/L of sucrose, 5 g/L of yeast extract, and 2 g/L of K_2HP0_4 (Merck, Darmstadt, Germany) in distilled water. The pH value was adjusted to 6.8, after which 6 mL of 0.5% bromocresol purple solution (Merck, Darmstadt, Germany) and 12 g/L of technical agar (Oxoid, Pratteln, Switzerland) were added. The medium was sterilized at 121 °C for 15 min and then poured into Petri dishes.

2.4.3. SPY9.3

SPY9.3 was prepared by dissolving 10 g/L of peptone from casein (Merck, Darmstadt, Germany), 5 g/L of yeast extract (BD Biosciences, Allschwil, Switzerland), 3.8 g/L of Na_2HCO_3 (Merck, Darmstadt, Germany), 0.5 g/L of Na_2CO_3 (Merck, Darmstadt, Germany), and 12 g/L of technical agar (Oxoid, Pratteln, Switzerland). The pH value was adjusted to 9.2 ± 0.02, and the solution was sterilized at 121 °C for 15 min. After the solution had cooled to 45 °C, a sterile sucrose (Merck, Darmstadt, Germany) solution was added to a final concentration of 10 g/L and the medium was then poured into Petri dishes. The pH value of the medium after autoclaving was 9.3. The name of the medium refers to its main components and final pH value (sucrose, peptone, yeast, pH 9.3).

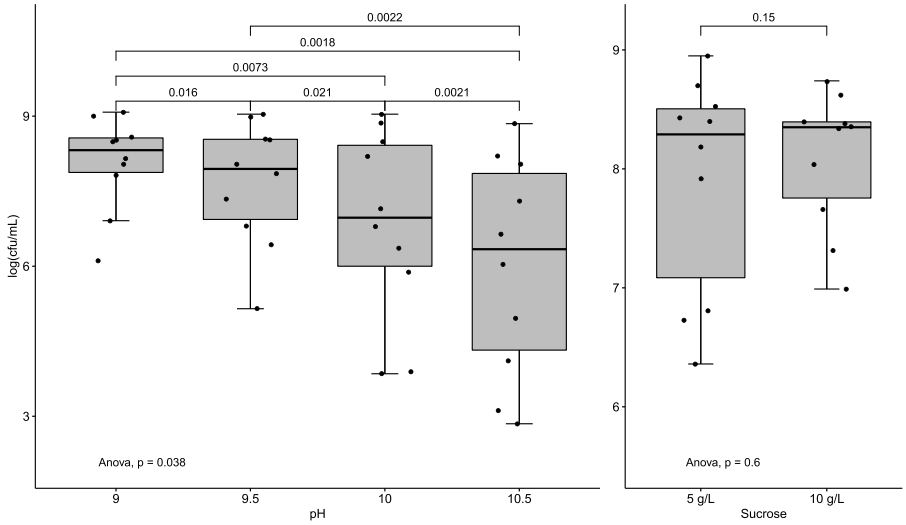

Fig. 1. Box plots showing the colony counts of 10 strains of *Streptococcus salivarius* subsp. *thermophilus* obtained by varying the pH value (left) and the sucrose concentration (right). Paired *t*-test was used to evaluate the significance of mean differences between the distributions (p-values are shown above the box plots). Colony counts of individual strains were superimposed (black dots) on the box plots.

2.5. Protocols for the enumeration and isolation of Streptococcus salivarius subsp. thermophilus

Three protocols for the enumeration and isolation of *S. salivarius* subsp. *thermophilus* were evaluated (Table 1). Protocol M17 was adapted from Ravula and Shah (1998), with M17 as a culture medium and incubation at 42 °C for 48 h under aerobic conditions. Protocol ST was adapted from Dave and Shah (1996), with ST as a culture medium and incubation at 42 °C for 48 h under aerobic conditions. Protocol SPY, which made use of medium SPY9.3, was incubated for 48 h at 42 °C under anaerobic conditions.

2.6. Inoculum preparation for colony count comparisons

Ten-mL fresh M17 broth cultures were prepared by inoculation with 1% overnight cultures of *S. salivarius* subsp. *thermophilus* strains. Each resulting culture was washed twice with 1 x sterile PBS (pH 7.4) at 10,000 rpm for 10 min at 4 °C according to Nwamaioha and Ibrahim (2018). Washed pellets were re-suspended and underwent serial 10-fold dilution in 10 mL of 0.09% NaCl.

2.7. Enumeration of Streptococcus salivarius subsp. thermophilus

Enumeration was carried out using the spread plate technique in duplicates starting from the same dilution tubes. Growth and incubation occurred according to the 3 protocols described above. Plates containing 2 to 200 colonies were enumerated after 48 h. The mean logarithmic colony counts obtained using the 3 protocols were calculated for each strain. In order to compare the colony count distributions obtained with each protocol, a paired *t*-test was calculated (McDonald, 2014) after checking for normality using the Shapiro-Wilk normality test (Royston, 1982) and comparing the distribution variances using the *F*-test of equality of variances (Box, 1953).

2.8. Specificity tests

Two specificity tests were carried out. First, 10 mL of fresh cultures of the selected non-target strains were prepared. The strains were then processed according to the protocols described above. As positive controls, each non-target strain was cultured on MRS at a temperature adapted to its species under anaerobic conditions.

Second, bacteria were isolated directly from raw-milk cheeses. Briefly, 3 Swiss semi-hard raw-milk cheeses (hereafter named A, B, and C) were obtained from the market. Manufacture of the chosen cheese types involved curd cooking at 53 °C (cheese A) and 38 °C (cheese B and C). Ripening took place over 3–5 months (cheese A), 3–4 weeks (cheese B), and 3–4 months (cheese C).

For bacterial isolation, the cheeses were processed as follows: 10 g of cheese sample was homogenized in 90 mL of sterile 0.12 M K_2HPO_4 (Merck, Darmstadt, Germany) solution (pH 7.5) for 3 min in a IUL classic Masticator Homogenizator (IUL Instruments, Barcelona, Spain), after which it underwent serial 10-fold dilution in 10 mL of 0.09% NaCl before being spread in triplicates onto M17, ST, and SPY9.3 agar plates. Plates were incubated according to the protocols described above. Suspected *S. salivarius* subsp. *thermophilus* colonies from each protocol were enumerated based on their typical morphologies: yellow colonies forming a yellow coloration in the ST agar medium for protocol ST (Dave and Shah, 1996), and big, flat, white colonies with no coloration in the SPY9.3 medium for protocol SPY (observation from the present study). As M17 is a selective medium (Ashraf and Shah, 2011), there is no typical colony morphology allowing to distinguishing *S. salivarius* subsp. *thermophilus* from other species and, therefore, all colonies obtained using protocol M17 were counted. When possible, 50 typical colonies per plate were identified as described above to estimate the proportion of false positives. In plates with fewer than 50 typical colonies, all were identified. The total counts, expressed as log(cfu/mL), were adjusted using the proportion of isolates correctly identified as *S. salivarius* subsp. *thermophilus*.

3. Results

3.1. Design of SPY9.3 medium and culture conditions

Colony counts of *S. salivarius* subsp. *thermophilus* strains cultivated at a constant sucrose concentration significantly decreased with increasing pH values, whereas their variance increased (Fig. 1). It should be noted here that the pH of the medium decreased during autoclaving to 9.4, 9.5, 9.6, and 9.8, respectively. On the other hand, increasing sucrose concentrations at a constant pH had no significant impact on the colony counts (Fig. 1). Nevertheless, colonies cultivated at a sucrose concentration of 5 g/L were greyer than colonies cultivated under higher sucrose concentrations. Based on these results, the pH value of the medium before autoclaving was fixed between 9.0 and 9.5 (pH 9.2) to avoid significantly impacting growth. The sucrose concentration was fixed at

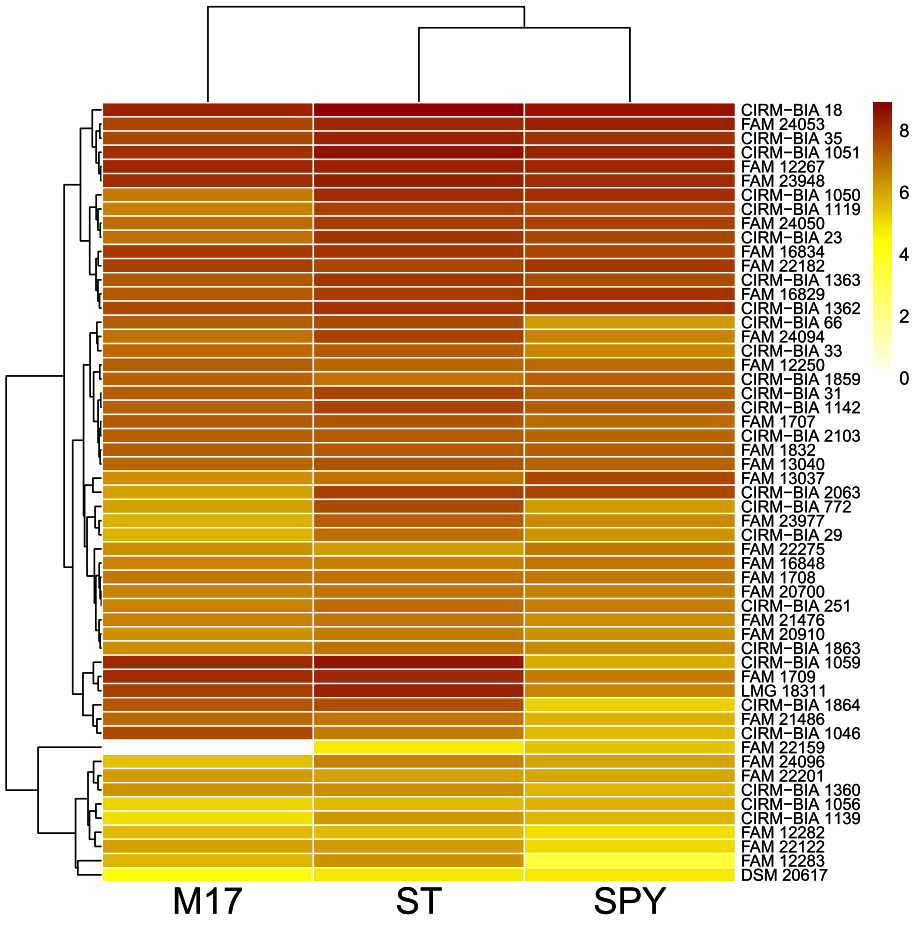

Fig. 2. Heatmap of the mean colony counts, expressed in log(cfu/mL), obtained for each tested strain under the 3 protocols M17, ST, and SPY.

10 g/L to allow the formation of white colonies.

3.2. Sensitivity of culture media

Most of the 55 strains of *S. salivarius* subsp. *thermophilus* were able to grow under all 3 protocols. One exception, FAM 22159, displayed no growth under protocol M17, whereas it reached $5.9 \cdot 10^4$ and $2.8 \cdot 10^5$ cfu/mL with protocols ST and SPY, respectively. No colonies were visible after an extended incubation of 24 h of this strain with protocol M17.

Colony count variations of *S. salivarius* subsp. *thermophilus* among the protocols were strain-specific (Fig. 2). However, the highest counts were usually obtained with protocol ST: 87.0% and 83.6% of the strains had higher colony counts using the ST protocol in comparison with the M17 and SPY protocols, respectively. The SPY protocol led to higher colony counts for 57.4% of the strains in comparison with protocol M17. However, colony counts obtained with protocols M17 and SPY were not significantly different (mean difference 0.07 log(cfu/mL), $p > 0.001$), whereas ST provided significantly different counts as compared with M17 (mean difference 0.42 log(cfu/mL), $p < 0.001$) and SPY (mean difference 0.48 log(cfu/mL), $p < 0.001$).

Most tested strains of *S. salivarius* subsp. *thermophilus* formed big, flat, white colonies under the growth conditions of protocol SPY (Fig. 3) that were already clearly visible after 24 h incubation. The colonies were usually smaller when grown under protocols M17 and ST than under protocol SPY. After 48 h, ST agar plates turned yellow for all *S. salivarius* subsp. *thermophilus* strains under protocol ST, except FAM 1832.

3.3. Specificity of the tested protocols

Irrespective of colony shapes or colors, 20 (33%), 16 (27%), and 9 (15%) of the 60 tested strains displayed unambiguous growth under protocols M17, ST, and SPY, respectively, representing 10 (50%), 8 (40%), and 4 (20%) of the 20 tested species.

All tested strains of *Enterococcus faecalis* and *Enterococcus faecium* were able to grow under all protocols and formed large colonies morphologically similar to those of *S. salivarius* subsp. *thermophilus*. Furthermore, 2 of the 3 tested strains of *Staphylococcus aureus* also formed large colonies under all protocols (Fig. 3). Under protocol ST, *E. faecalis*, *E. faecium*, and *S. aureus* produced yellow colonies and a yellow coloration in the medium. One strain of *Lactococcus lactis* subsp. *lactis* also displayed clear growth under all protocols. No further non-target strain displayed colonies comparable in size to those of *S. salivarius* subsp. *thermophilus* under protocol SPY. However, punctiform colonies of all tested strains of *Pediococcus acidilactici* and *Pediococcus pentosaceus* were visible on SPY9.3, but were usually larger under protocols M17 and ST, albeit without a yellow coloration. One strain of *Lactiplantibacillus plantarum* displayed punctiform colonies under protocols SPY and M17, but also under protocol ST, which were slightly larger and had a yellow coloration. Strains belonging to several non-target species developed colonies under protocols M17 and ST but not under SPY: first, 1 strain of *Lactobacillus delbrueckii* subsp. *lactis* produced tiny colonies under M17 as well as slightly larger colonies under ST, but without a yellow coloration. Two other colonies, from strains FAM 11001 and FAM 12080, clearly grew under M17, but grew very weakly (FAM 11001) or not at all (FAM 12080) under ST. However, all colonies of *L. delbrueckii* subsp. *lactis* under M17 and ST were grey and smaller as compared to those of *S. salivarius* subsp. *thermophilus*. Second,

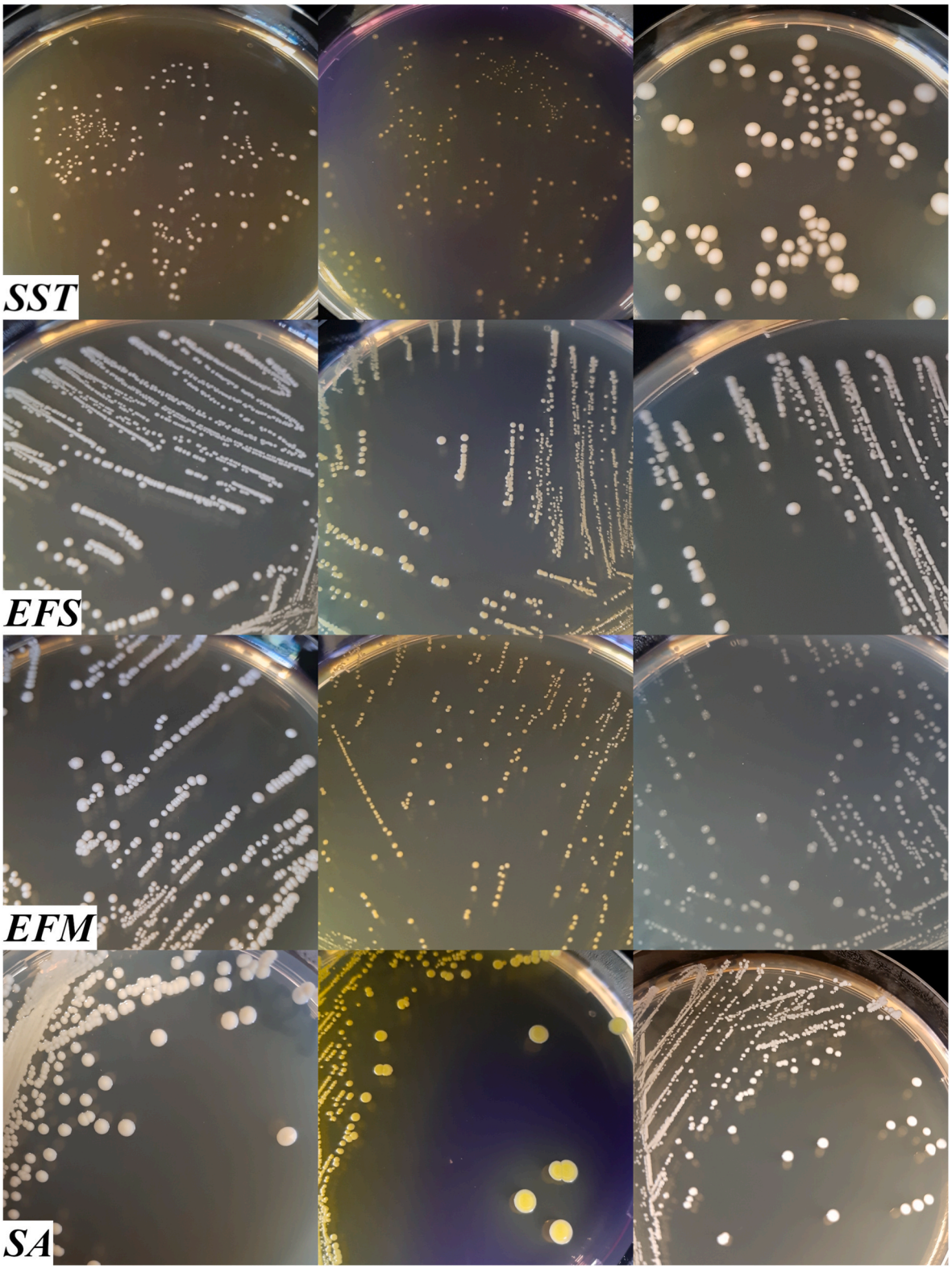

Fig. 3. Typical colony morphologies of *Streptococcus salivarius* subsp. *thermophilus* and of non-target species displaying colony morphologies similar to *S. salivarius* subsp. *thermophilus* grown under protocols M17 (left), ST (middle) and SPY (right). *SST*: *Streptococcus salivarius* subsp. *thermophilus* (FAM 16834); *EFS*: *Enterococcus faecalis* (DSM, 20478); *EFM*: *Enterococcus faecium* (FAM, 19316); *SA*: *Staphylococcus aureus* (FAM 23319).

Lacticaseibacillus rhamnosus clearly grew on M17, with all tested strains forming round, white colonies. The growth of tiny colonies without a yellow coloration was also observed on ST plates. One strain of *Limosilactobacillus fermentum* formed tiny colonies on M17 agar and slightly larger colonies on ST, but without a yellow coloration.

Colony counts from raw-milk cheeses were usually higher with protocol M17 than with SPY or ST, as colonies of *S. salivarius* subsp. *thermophilus* could not be distinguished from other species. Colony counts from cheese C could not be accurately evaluated using protocol M17, as all plates were covered with punctiform colonies. Furthermore,

Table 2
Comparison of the isolation efficiency of *Streptococcus salivarius* subsp. *thermophilus* (*SST*) using the 3 protocols.

Cheese	Total counts (log(cfu/mL))			Proportion (%) of *SST*			Total *SST* (adjusted log(cfu/mL))		
	M17	ST	SPY	M17	ST	SPY	M17	ST	SPY
A	7.39 ± 0.04	5.47 ± 0.02	5.64 ± 0.06	0	98.77 ± 2.14	100 ± 0.00	0	5.40 ± 0.13	5.64 ± 0.06
B	9.13 ± 0.05	9.04 ± 0.01	9.04 ± 0.02	97.33 ± 2.31	100 ± 0.00	100 ± 0.00	8.89 ± 0.25	9.04 ± 0.01	9.04 ± 0.02
C	5.0 ± 0.00	3.99 ± 0.04	3.36 ± 0.09	2.00 ± 2.00	59.54 ± 11.72	50.43 ± 16.03	0.10 ± 0.10	2.38 ± 0.49	1.69 ± 0.49

1 plate was partly covered with a large colony identified as *Bacillus licheniformis*.

The colony counts from cheese were in the same range using protocols SPY and ST (Table 2). After adjusting the colony counts according to the proportion of isolates identified as *S. salivarius* subsp. *thermophilus*, ST and SPY showed the highest recoveries of *S. salivarius* subsp. *thermophilus* isolates from all cheeses. For 2 cheeses, similar recoveries were obtained using ST and SPY, whereas ST performed slightly better for 1 cheese (0.69 log(cfu/mL) higher recovery). In cheese A, non-specific isolates from protocol M17 were *P. acidilactici* and *P. pentosaceus*. In cheese C, most non-specific isolates from all 3 protocols were identified as *E. durans*, *E. faecalis*, and *E. faecium* (data not shown).

4. Discussion

From a quantitative perspective, the newly developed medium SPY9.3 and associated incubation conditions (protocol SPY) proved suitable for the enumeration of *S. salivarius* subsp. *thermophilus*. Indeed, similar colony counts were obtained with this protocol and with protocol M17 (Fig. 2), using medium M17 as currently recommended by the International Dairy Federation for the selective enumeration of this species from yogurt (ISO-7889 and IDF-117, 2003). For the selective cultivation of *S. salivarius* subsp. *thermophilus* from cheese, protocol SPY had the advantage that the colonies of this species displayed a differential morphology in comparison with most other tested species, which was not the case with M17. The larger dimension of *S. salivarius* subsp. *thermophilus* colonies on SPY9.3 may be related to the high pH value and to the production of exopolysaccharides as a protection mechanism (Mende et al., 2016). The observation that colonies of *S. salivarius* subsp. *thermophilus* grown at pH 9.0 were smaller than those grown at higher pH values (data not shown) tends to support this hypothesis.

With regard to selectivity, protocol SPY displayed comparable characteristics to protocol ST (Table 2). However, the latter protocol allowed for the growth of more non-target species and was not able to detect the presence of 1 strain of *S. salivarius* subsp. *thermophilus* (FAM 1832), whose growth neither induced yellow colonies nor resulted in yellow coloration in the medium. In both M17 and ST protocols, strains were incubated in aerobic conditions, favoring the development of *S. salivarius* subsp. *thermophilus* over other species (Tharmaraj and Shah, 2003). Aerobic incubation as a potential means to increase selectivity was, however, not possible with medium SPY, since *S. salivarius* subsp. *thermophilus* displayed no growth on SPY9.3 under these conditions, as reported from preliminary experiments (data not shown).

Incubation temperature and time are other important selectivity factors. *S. salivarius* subsp. *thermophilus* has an optimal growth temperature between 40 °C and 45 °C (Aryana and Olson, 2017). Increasing the incubation temperature from 42 °C to 45 °C or even slightly higher should, therefore, allow increasing selectivity toward this species by hindering the growth of, e.g., *L. lactis* and *L. plantarum*. However, the most misleading non-target species pointed out here, i.e., *E. durans*, *E. faecalis*, *E. faecium*, and *S. aureus* (Fig. 3), all have similar growth temperatures and would thus still be confounded with *S. salivarius* subsp. *thermophilus* (Schleifer and Bell, 2015; Svec and Devriese, 2015). Reducing the incubation time to 18–24 h could further improve the selectivity of the procedure, since all tested *S. salivarius* subsp. *thermophilus* strains had already produced clear colonies after only 24 h (data not shown). This would, nevertheless, not solve the problem of sensitivity toward *E. durans*, *E. faecalis*, *E. faecium*, and *S. aureus*, as these species also formed colonies after 24 h (data not shown). Thus, the identities of the selected isolates should be confirmed in order to detect possible non-target species displaying similar colony morphologies. The MALDI-TOF procedure adopted in this study allowed for rapid and robust identification with very little biomass, making it possible to both directly identify and isolate from single colonies on the plate.

Increasing the pH value above 9.3 (final pH of SPY9.3) may improve the selectivity of the medium, but it may also increase the risk of hindering some strains of *S. salivarius* subsp. *thermophilus* and would additionally not prohibit the elimination of *Enterococcus* spp. Indeed, *S. salivarius* subsp. *thermophilus* does not grow at pH 9.6 (Whiley and Hardie, 2015), whereas many *Enterococcus* spp. still do (Svec and Devriese, 2015). It should be underlined here that in our experiments for the design of SPY9.3, the pH changed during autoclaving and probably continued to do so until the day of inoculation. We suppose that the medium with the highest pH value was at or below 9.6.

It has previously been shown that several lactobacilli, such as *Lactobacillus helveticus* and *L. delbrueckii* subsp. *lactis* and *bulgaricus* are not able to grow above pH 6.7 to 7.1 (Sawatari and Yokota, 2007). Consistently, *L. delbrueckii* subsp. *lactis* was not able to grow under protocol SPY, whereas small colonies of this species were visible with protocol ST. Although ST agar permits differentiating between *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *lactis* by the yellow coloration and the size of the colonies, selective media are usually preferred when a particular species has to be isolated or enriched in the presence of other closely related species (Saccaro et al., 2012).

In conclusion, the protocol SPY described and evaluated in this study improves the selective enumeration and isolation of *S. salivarius* subsp. *thermophilus*. Tested with a complex food matrix, i.e., raw-milk cheese, protocol SPY displayed an increased selectivity compared with the 2 other standard protocols tested.

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Appendix A. Supplementary data

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