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ABSTRACT

Eight *Streptococcus thermophilus* strains of dairy origin isolated in Italy were chosen to investigate autochthonous bacterial diversity in this important technological species. In the present study a comparative analysis of all the 17 *S. thermophilus* genomes publicly available was performed to identify the core and the variable genes, which vary among strains from 196 to 265. Additionally, correlation between the isolation site and the genetic distance was investigated at genomic level. Results highlight that the phylogenetic reconstruction differs from the geographical strain distribution. Moreover, strain M17PTZA496 has a genome of 2.15 Mbp, notably larger than that of the others, determined by lateral gene transfer (including phage-mediated incorporation) and duplication events. Important technological characters, such as growth kinetics, bacteriocin production, acidification kinetics and surface adhesion capability were studied in all the Italian strains. Results indicate a wide range of variability in adhesion properties that significantly clustered strains into four groups. Genomic differences among strains in relation to these characters were identified but a clear correlation between genotype and phenotype was not always found since most of the genomic modifications arise from single nucleotide polymorphisms. This research represents a step forward in the identification of strains-specific functions in *Streptococcus thermophilus* and it

has also the potential to provide valuable information to predict strain specific behaviors in industrial processes.

KEYWORDS

Dairy products, *Streptoccocus thermophilus*, bacterial biodiversity, genome comparison

Highlights

- Genome comparison of all *S. thermophilus* strains publicly available was performed
- Weak correlation between genome evolution and geographical origin was found
- Extracellular polymeric substances and bacteriocin sequences are present but phenotypes were not detected
- Acidification kinetics were highly variable among strains isolated in Italy
- Technological implications of *S. thermophilus* genomic diversity was evaluated

1. Introduction

Streptococcus thermophilus is a thermophilic Lactic Acid Bacterium (LAB) of major importance in the dairy industry. Due to its ability to rapidly ferment lactose, it is widely used as starter to obtain fermented milk products, contributing to milk acidification and organoleptic properties enrichment (Giraffa et al., 2001). The long history of safe use in food production allowed *S. thermophilus* to obtain the status of Generally Recognized as Safe (GRAS) and of Qualified Presumption of Safety (QPS). At present, it is considered the second most important species of industrial LAB after *Lactococcus lactis*. It was estimated that over 10^{21} live cells are ingested annually leading the species to achieve a market value of 40 billion US$, approximately (Iyer et al., 2010). Similarly to other dairy microbes, *S. thermophilus* natural biodiversity decreases with its overuse of industrial starters, hence isolation and characterization of new strains becomes of great importance, since it may lead to the discovery of novel and desirable characteristics, which can fulfil industrial demands (Erkus, Okuklu, Yenidunya, & Harsa, 2014). Strains analyzed in the present study are used as natural starters for Protected Designation Origin (PDO) Italian cheeses, i.e. Fontina, Grana Padano and Mozzarella. These products, obtained from traditional back-slopping procedures, allow the maintenance of the microbiota present in the environment where they are produced. Considering the number of Italian cheese factories involved in dairy productions (ISTAT, 2014), the Italian dairy microbiota can be considered a potential important source of new *S. thermophilus* strains. Nonetheless, until now such biodiversity has been explored only partially both from the genetic and phenotypic point of view (Andrighetto et al., 2002, Morandi and Brasca, 2012). Thanks to the next generation sequencing technology, whole *S. thermophilus* genome sequences are publicly available. Such information allowed to study more in depth the genetic structure of many metabolic activities of the species, such as amino acid metabolism (Hols et al., 2005), arrangement of the proteolytic system (Hols et al., 2005), resistance to bacteriophage (Li et al., 2016), biosynthesis of folate (Iyer et al., 2010), metabolism of urea (Mora et al., 2004) and biofilm formation (Couvigny et al., 2015).

More generally, *S. thermophilus* genomes have been so far analyzed and compared with other related species, specifically with pathogenic streptococci (Hols et al., 2005). Within this framework, Rasmussen et al. (2008) used microarrays to perform a comparative genomic analysis of different *S. thermophilus* strains to demonstrate the presence of variable subsets of genes responsible for ecological and technological differences. One of the most interesting technological properties reported in comparative studies is related to the ability to synthesize extracellular polymeric substances (EPS) (Flemming and Wingender, 2010, Mora et al., 2002). Recently, the beneficial effects of EPS in fermented milk have been recognized and linked to their role as thickeners and

stabilizers of the product, together with healthy effects, such as their activity on human blood pressure and gastrointestinal tract health (Caggianiello et al., 2016). To date, 28 different EPS gene clusters are known in *S. thermophilus* (Iyer et al., 2010, Vuyst et al., 2011, Wu et al., 2014).

This species has been tested as bio-preservative to control growth of pathogenic and spoilage bacteria in dairy products by production of bacteriocins (Kongo, 2013). These molecules, produced by food grade lactic acid bacteria, are classified into two classes based on their modification status. Known *Streptococcus* bacteriocins belong to class I and class IIb (Egan et al., 2016) and the identification of genes encoding for bacteriocins is a quite difficult task due to their small size and high variability in sequence composition (Willey and van der Donk, 2007).

The present study used the results obtained thanks to the most innovative Next Generation Sequencing approach (Treu et al., 2014a, Treu et al., 2014b, Treu et al. 2014c) to provide more details on the genetic organization of *S.thermophilus* at whole genome level. The overall biodiversity of Italian *S. thermophilus* was studied by comparing eight isolates, coming from PDO cheese productions. It is known that subsets of features specifically characterizing different strains are extremely important when they are forced to face environmental changes (Hols et al., 2005). The geographical effect on biodiversity was examined by comparing the genome sequences of all strains available in the literature. Furthermore, technological characters related to food production were investigated in the Italian strains and linked to genomic data.

2. Material and methods

2.1. Strain used

Considering all surveys regarding *S. thermophilus* species, seventeen genomes publicly available in the NCBI database in August 2015 (Table 1) were used. Other species used as outgroups or references for specific analysis are described in the correspondent paragraphs. In the present study gene finding and annotation for all the strains were newly performed using RAST (Rapid Annotation using Subsystem Technology) service which orders annotated genes into subsystems, subcategories and categories, following the SEED structure (Aziz et al., 2008). Gene function is assigned by sequence attribution to protein families (FIGfams). Eight strains isolated form Italian dairy products were used for phenotypic tests (Table 1). Unless otherwise indicated, *S. thermophilus* strains were grown overnight at 37°C in M17 broth (Oxoid, UK). For long-term maintenance, grown cultures were stored at −80 °C in in 40% (v/v) glycerol and 5% skim milk (Sigma-Aldrich, Italy).

2.2. Phylogenetic and genomic analyses

Genomic data of 17 *S. thermophilus* genomes along with *Streptococcus macedonicus* 33MO, *Streptococcus pneumoniae* NT_110_58 and *Streptococcus salivarius* JIM8777 were used to estimate phylogenetic relationships by combining two methods. The first phylogenetic tree was obtained using PHYLIP package (Tuimala, 2005) with neighbour–joining method. This method utilized single nucleotide polymorphisms (SNPs) of the whole genome alignment computed using Mauve software (Darling et al., 2004) with a procedure previously described (Treu et al., 2014). In the second case, the phylogenetic tree was built using PhyloPhlAn software (Segata et al., 2013) which determines microbial phylogeny on the basis of 400 conserved proteins alignment. Phage proteins were recognized by RAST gene functional attribution and their organization was manually explored localizing sequences coding for phage proteins. Laterally transferred regions were identified using Alien Hunter software (Vernikos and Parkhill 2006). From the output, sequences putative functions were identified by blastp alignment. Gene duplication analysis was performed according to Campanaro et al. (2014) by clustering total strains proteins using CD-HIT software (Li and Godzik, 2006). Two different analyses were performed using 90% and 99% identity of sequence and minimal length similarity of “0.5”.

2.3. Gene content evaluation

For each *S. thermophilus* strains, annotated gene were attributed to subsystem and a features list was created based on subsystem gene abundance. Feature lists were used to elaborate hierarchical

clustering (HCL) using MeV (MultiExperiment Viewer) software (Saeed et al., 2003). Strains functional relationship was computed using the "linkage method" process for determining cluster-to-cluster distances and the "Euclidean distance" for distance calculation. A comparison on subsystem gene abundance was conducted analyzing the resulting heatmap and the most interesting subsystems were manually investigated in detail. For the specific subsystem 'Iron acquisition and metabolism', the following strains of *Streptococcus pyogenes* were used*:* M1GAS, MGAS10270, MGAS10394, MGAS1075, MGAS2096, MGAS315, MGAS5005, MGAS6180, MGAS8232, MGAS9429, SSI-1 and str. Manfredo. *Lactobacillus fabifermentans* T30PCM01 genome was also included in the analysis (Treu et al., 2014d). On the basis of features lists, non-redundant common and non-common strain features were identified using R software, custom script (R Development Core Team, 2008). Strain specific features were assigned to the SEED categories using RAST.

2.4. Identification of sequences related to technological properties

S. thermophilus proteolytic activity was studied by verifying sequence presence of species specific main components, namely the cell-envelope protease, Ptrs, and the protein responsible for its anchoring to bacterial membrane, Sortase A (SrtA). Sequences of *S. thermophilus* MN-ZLW-002 (YP_006340201.1 and YP_006340309 for Ptrs and Srt A respectively) were used to perform blastp search using strains genome as reference sequence.

Exopolysaccharides-related genes were analyzed considering subsystems completeness and sequence similarities. Genes assigned to "EPS" and "CPS" were identified for each strain, their number of copies and the organization into operons were recorded. Sequences were clustered using CD-HIT at 50%, 80%, 90% and 100% of identity in order to understand their degree of similarity. Promoter regions of the main EPS operon of each strain were compared against a reference strain, ND03, previously studied for its abilities in EPS production (Sun et al., 2011). Bacteriocins are important strain specific compounds and BAGEL3 software (van Heel et al., 2013) was chosen to determine microorganism potentiality to produce novel compounds. Putative biosynthetic gene clusters were identified in the genome sequence (Egan et al., 2016).

2.5. Strain growth curve

For growth curve determination, a loopful of cells from frozen stocks was inoculated in 10 ml of M17 broth pre-warmed at 37°C and incubated at 37°C overnight. The cultures were used to inoculate 200 ml of fresh M17 broth to a concentration of 10^5 cells/ml and bacterial growth was monitored by plate counts. To estimate growth parameters, 3 different mathematical models were used, namely Gompertz (Zwietering et al, 1990), Baranyi (Baranyi and Roberts, 1994) and Huang (Huang, 2008).

Models goodness-of-fit were evaluated using four indexes, namely bias factor (BF), accuracy factor (AC), sum of squares error (ESS) and AIC index according to Huang 2010 in order to establish the most suitable model for growth parameters prediction. Data were analyzed using XLSTAT (version 2011, Addinsoft, USA).

2.6. Acidification kinetics and protease activity determination

A loopful of bacteria from freshly grown M17 plates was used to inoculate 10 ml of 10% (w/v) skim milk (Oxoid, IT), previously sterilized by autoclaving 10 min at 110°C. Inoculated samples were kept in a water bath at 42°C until milk coagulation. Only strains able to coagulate milk within 16 hours were further tested, as follows.

Flasks containing 250ml of sterilized 10% (w/v) skim milk (Oxoid, IT) were inoculated with 2% (v/v) of cultures obtained as describe above. Flasks were incubated in a water bath at 37°C and the pH recorded continuously for 24h by a pH electrode (Micros, Siap+Micros, Treviso, Italy) immersed in the medium and connected to a software system for data acquisition. According to Dandoy and collegues (2011) results were expressed as maximum acidification rate (V_m), defined as the maximum slope of the pH curve (dpH/dt) and time required to reach two pH values, pH 5.2 and 4.6.

The PrtS proteinase phenotype was phenotypically determined on bacterial colonies grown on semi skimmed milk agar plate according to Morris et al. (2012). For each strain, 5 µl of a routinely grown culture were dropped on lactose-free skim milk plates and incubated at 37°C for 24h and 48h. Strains were considered to express protease activity when a transparent halo appears around the culture drops. Each experiment was repeated at least 3 times and statistical analyses were performed using R software.

2.7. Exopolysaccharides production and antimicrobial activity

Strains were tested for EPS production using a colorimetric assay. Strains were routinely grown in liquid M17 broth and used to inoculate a fresh M17 broth (1% v/v) dispensed into microtiter plate wells (200µl per well). Cultures were incubated at 37°C and the increase in absorbance (OD_{600}) was monitored every 30 min, after gently shaking for 30 sec. After 24h, biofilm formation was quantified as described by Maragkoudakis et al. (2013). OD value recorded for empty wells plus three standard deviations were used as control. Results were evaluated by ANOVA tests performed by R. For scanning electron microscope (SEM) analysis, routinely grown cultures were refreshed by adding 10% (v/v) of fresh medium into sterile Petri dish containing a glass coverslip and statically incubated at 37 °C for 24 h. Coverslips provide the adhesion surface for bacterial cells, therefore they were recovered after M17 broth gently removal and PBS buffer washing (NaCl 137 mM, KCl 2.7 mM,

198 Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4). Sample fixation, dehydration and assembly were
199 performed as described by Campanaro et al., 2014. Samples were observed and photographed by a
200 Quanta 200 SEM (FEI, Hillsboro, OR, USA).
201 Antimicrobial activity was determined by agar-spot test (Rossi et al., 2013). Details on strains and
202 protocols are reported in the Supplementary information.
203

3. Results and discussion

3.1. Phylogenetic reconstruction

Eight *S. thermophilus* strains isolated from Italian dairy products were sequenced to investigate autochthonous bacterial diversity of this important technological starter species (Giraffa et al., 2001, Treu et al., 2014a, Treu et al., 2014b, Treu et al. 2014c). In the present study a comparative analysis of all *S. thermophilus* genomes presently available in public databases (Table 1) was performed.

Although all strains were collected from dairy environments, they derived from different food-making processes and from milk of different mammals (Table 1). Two independent phylogenetic analyses were performed, one based on the conserved proteins and another based on single nucleotide polymorphisms (SNPs) detected comparing the whole genome sequences. . Since M17PTZA496 was found to possess a large strain-specific region which was excluded from the whole genome computation, the second tree was aimed to avoid this bias. The two resulting phylogenetic trees are concordant in defining the relationships among strains (Fig. 1). It is clear that the phylogenetic reconstruction does not follow geographic distribution since strains isolated in the same continent are rarely clustered together. Indeed, strains isolated in Europe are widespread on the phylogenetic tree while American and Asian strains show a higher proximity. Within European strains, some Italian strains are phylogenetically close, namely TH982/TH985 isolated in Campania region and TH1435/TH1436 isolated in Friuli Venezia Giulia region. These two strain couples were collected from the same food matrices, buffalo and goat milk respectively (Table 1). Therefore it cannot be excluded that both factors contributed in determining genome similarity.

Finally, both phylogenetic approaches resulted in a concordant topology indicating higher distance of strain M17PTZA496 from the others (Fig. 1). I can be hypothesized that this strain is undergoing an evolutionary process that could eventually resolve into its diversification.

3.2. Genetic diversity of strain M17PTZA496

Genetic diversity ascribed to strain M17PTZA496 depends from the highest number of SNPs and from the presence of a large strain-specific genomic region. In fact, the 17 genomes considered in the present study have a comparable size (from 1.93 to 1.74 Mbp) with the notable exception of strain M17PTZA496 (2.15 Mbp), which carries almost 0.3 Mbp more genetic information than the average (Table 1). To clarify the origin of M17PTZA496 differentiation, lateral gene transfer (including phage-mediated incorporation) and gene duplication event were investigated.

Phages are widespread in dairy environment and therefore phage resistance systems are considered a technological character of major interest (Goh et al., 2011). It is known that several genes belonging

to CRISPR/cas system increase their expression during response to bacteriophage attack (Wu et al., 2014). Interestingly, when compared with the other strains, M17PTZA496 shows the lower number of CRISPR/cas genes (Table 1). Moreover, M17PTZA496 contains a higher number of unknown proteins, or other related to "phages/prophages" category proteins compared to the other strains: 81 proteins in M17PTZA496 and from 5 to 15 in the others. Most of these proteins are positioned in a phages-rich protein region (PH) spanning approximately 42 Kbp in *contig23* (from 1266 to 43435 bp; Fig. 2), related to phage functioning (i.e. tail proteins, replication, packaging machinery, tail fiber proteins, capsid proteins and lysis). From these results it's possible to hypothesize that the reduction in CRISPR/cas genes content of M17PTZA496 strain has increased its susceptibility to phage infection resulting into acquisition of new genetic material.

Analysis of laterally transferred regions (LTR) performed by Alien Hunter software led to the identification of four LTRs, two of which consisting in large genomic islands (Fig. 2), named ISL1 and ISL2, with a size of 37.5 and 77.5 Kbp respectively (in *contig11* from 192500 to 230000 bp and in *contig61* from 1462450 to 1540000 bp). Results of sequences similarity search demonstrated that the acquired genes are not closely related but span a wide range of different functions, including transport and stress response (Supplementary Table S1). While ISL1 encodes features clearly recognized as part of the normal genetic pool of the *Streptococcus* genus, on the contrary ISL2 includes genes having similarity with different species. Moreover, similarity values obtained are lower in ISL2, which could indicate a higher decay rate or a stronger selective pressure on the second region (i.e. on average 99% of identity with E-values of 7.7E-19 and 70% of identity with E-values 4.8E-6 respectively). Based on these results it is possible to hypothesize that ISL2 was included in M17PTZA496 genome prior to ISL1. The two smaller regions, "Island3" and "Island4", have a size of 15 Kbp and 7.5 Kbp respectively and large part of the proteins present in these regions have unknown functions.

Together with LTR and gene loss, gene duplication and regulatory divergence of paralogues are fundamental in determining bacterial functional and technological properties (Snel et al., 2002). A specific analysis was performed on duplicated genes identified on the Italian isolates, in order to investigate M17PTZA496 paralogues content. Present findings revealed 60 clusters of genes with similarity >90% (6 with similarity >99%), mainly assigned to mobile elements and related proteins (Supplementary Table S2). Considering the paralogues clusters with >90% similarity, three groups were arbitrarily defined according to the number of strains having the same paralogues. In class **(I)** several strains possess duplicated genes in cluster, in class **(II)** only strains from Fontina have duplicated genes in cluster while the other have only one copy of the sequence, and in class **(III)** duplicated genes are present in strain M17PTZA496while in the other strains there is only a single

copy of the gene. This classification revealed that 30% of paralogues were found in at least three strains. Interestingly, a small amount of unknown genes (5%) is present in both strains isolated from the same environment (M17PTZA496 and MTH17CL396). More than half of the paralogues clusters (63%) are present only in M17PTZA496 and most of them are ribosomal proteins while others have specific functions (e.g. UDP-glucose 4-epimerase) (Supplementary Table S2). The remaining 8% of paralogues is not included in the above classification because they are duplicated mobile elements exclusively present in strain M17PTZA496 and absent in the other strains.

Finally, 18 out of 60 paralogues clusters belonging to class **(III)** are composed by genes located in a specific genomic region (DR) in *contig69* (from 82019 to 93062 bp), which probably underwent a single duplication event producing the second copy located in *contig*71 (from 7519 to 18640 bp; Fig. 2).

3.3. Functional variability

Phenotypic differences among strains could be determined by variation in the number of genes assigned to specific SEED functional categories.. Hierarchical clustering was performed on the profiles reporting the number of genes for each functional category in order to evaluate similarities among 17 strains. This analysis was also useful to identify correlations between gene content and strain geographical isolation sites (Fig. 3). Variations in gene abundance of each functional category were used to build a dendrogram reporting the level of divergence between strains (Fig. 3).

Results obtained from hierarchical clustering are highly different from the phylogenetic reconstruction. In particular, in the "functional clustering" Italian strains formed a compact cluster, while they were found to be distantly related from a phylogenetic point of view. Only strains TH982 and TH985 clustered together with strains distantly isolated, forming a branch separate from the others.

Besides SEED categories describing basic metabolism, such as protein, RNA and DNA metabolism, a subset of functional categories are particularly important to define distinctive characters among *S. thermophilus* strains . These highly variable categories were organized for discussion purposes into four groups: **(a)** "Cofactors, vitamins, prosthetic groups and pigments" **(b)** "Cell wall and capsule" and "Membrane transport", **(c)** "Stress response" and **(d)** "Nitrogen metabolism" and "Amino acids and derivatives" (Fig. 3).

The first group **(a)** includes the "Cofactors, vitamins, prosthetic groups pigments" category and it is mainly due to variation in "Riboflavin, FMN, FAD" and "Folate and pterines" subcategories, which are involved in the production of the most important cofactors, key targets for the development of new vitamin-enriched products (Russo et al., 2014; Divya and Nampoothiri, 2015).

Three SEED subcategories, “capsular and extracellular polysaccharides”, “cell wall and capsule” and “membrane transporter” are the most relevant in the second group **(b)**. Their importance derives to the role of extracellular polysaccharides (EPS) in determining technological characteristics (see par. 3.6.), such as organoleptic and healthy properties of the fermented end-products (Awad et al., 2005). Interestingly, different strains evidenced a high variability in the number of genes of the third group **(c)**, represented mainly by three subcategories: “osmotic stress”, “oxidative stress” and “detoxification”. In detail, concerning “osmotic stress”, genes related to choline and betaine uptake and biosynthesis were specifically found in 8 strains (Fig. 3). Under standard conditions, Gram-positive bacteria possess turgor pressure higher than Gram-negative and respond to hyper-osmotic condition accumulating protective compounds such as glycine betaine (Sleator and Hill, 2001).

The fourth group **(d)** is characterized by genes involved in nitrogen and amino acids metabolism. The main differences are related to “lysine, threonine, methionine and cysteine”, “nitrogen metabolism” and “histidine metabolism”, which ranged from 0 to 14 genes depending on strain. This finding is in contrast with a previous study revealing a strong conservation in the amino acids metabolism among strains (Hols et al., 2005).

3.4. Strain-specific features

A global comparison of the whole gene content among strains of the *S. thermophilus* species allowed the identification of strain specific features, varying in number from 196 to 265, representing 17-21% of the total genes with known function (Fig. 4; Supplementary Table S3). Four functional categories accounted for a large part of strain diversity, namely “amino acids and derivatives”, “carbohydrates”, “DNA metabolism” and “membrane transport” covering almost 50% of the specific genes (11, 12, 13 and 10% respectively). The contribution of these categories to strain variability was in accordance with previous findings obtained by comparative genome hybridization experiments (Rasmussen et al., 2008). Moreover, one of the most interesting subcategories found is involved in stress response (Supplementary Table S3). In detail, only CNRZ1066 and LMG 18311 possess the “acid resistance” subcategory, composed by four genes encoding the glutamate transporter and known to be involved in acid tolerance. These genes were specifically detected in species belonging to the *Streptococcus* genus (Krastel et al., 2010). Other important subcategories describing strain specialization are “oxidative stress”, which includes proteins involved in the protection from reactive oxygen species (ROS) and glutathione homeostasis.

High variability is also affecting the “iron acquisition and metabolism” category, which is known to be connected with oxidative stress response in *Streptococcus* pathogenic species (Tsou et al., 2010). A comparison was performed among *S. thermophilus* strains, 12 *Streptococcus pyogenes* strains

available in the RAST database, selected for the presence of several iron related genes (Ge, Sun, & He, 2009), and *Lactobacillus fabifermentans* T30PCM01 that is the *Lactobacillus* strains with the largest genome so far identified (Campanaro et al., 2014). It was reported that adaptation to the dairy environment probably influenced iron requirement of lactic acid bacteria, in fact bacterial growth is unaffected by iron deprivation (Pandey et al., 1994). Indeed, the analyses of *S. pyogenes* strains underlines that this species possesses from 15 to 18 genes ascribed to iron metabolism while *L. fabifermentans* has only 5. Strain comparison performed among *S. thermophilus* strains revealed a strong heterogeneity in the number of genes related to iron metabolism which varies between 3 and 15. In particular, eight strains showed a strong reduction in iron related gene content, possessing only 3 copies of the ferrous iron transporters (*EfeUOB*) and having completely lost the "Heme, hemin uptake and utilization systems in Gram positives" and "Iron acquisition in *Streptococcus*" subcategories.

3.5. Acidification kinetics

Growth dynamics strictly affect the technological behavior of *S. thermophilus* strains along with their capability to rapidly acidify the milk. Three mathematical models were taken into account to describe bacterial growth kinetics and Gompertz model was selected for all the strains with the exception of MTH17CL396. A model fit assessment criteria, based on the MTH17CL396 growth curve, led to the selection of the Huang model for this strain (Huang, 2010). Growth curves results showed interesting differences among strains (Table 2), considering the three main parameters: lag phase (λ), maximum growth rate (μ_{max}) and stationary phase concentration (N_{max}). Strain 1F8CT showed the longest lag phase, the higher μ_{max} and the lowest stationary phase cell concentration, thus appearing the least performing strain from a technological point of view. On the contrary, strain M17PTZA496 showed the shortest lag phase and the highest stationary phase cell concentration, while TH1477 had the highest μ_{max}.

Acidification capability is important to assure a good outcome of dairy processes and to enhance food safety by hampering the development of undesirable bacteria (Gaden et al., 1992). To evaluate strain performances, fermentation kinetics and acidification capability were monitored in skim milk medium. A preliminary test was performed to assess whether strains were capable to induce coagulation of caseins within 16 h incubation. Only strain 1F8CT failed and was excluded from the following analysis, also considering its bad results in terms of growth kinetics.

Results obtained from 24 h acidification kinetics show that all strains reached pH 5.2 (Table 2) and 5 out of 7 went below 4.6, which is the value required for caseins coagulation and for the inhibition of pathogen growth. Maximum acidification rate (Vmax) analysis showed no significant differences in

strains acidification profile, nevertheless ANOVA analyses reveal statistical differences in the time required to reach the two pH point considered. Dandoy et al. (2011) reported a significant correlation between acidification kinetics and activity of PtrS, a cell-envelope proteinase (CEP) of *S. thermophilus*. In the *Streptococcus* genus, CEPs are generally anchored to the cell wall by sortase A (SrtA) and recently it was reported that *S. thermophilus* proteinase could be released in the medium (Chang et al., 2012). To better understand whether this proteolytic system is present and active in the strains under evaluation, sequence similarity analyses were performed. Results obtained by comparing the reference amino acid sequences of strains MN-ZLW-002 with strains' genotype indicate the presence of SrtA in all strains, with average identity of 97%.On the contrary, PrtS gene sequence was detected only in TH1435, with some minor differences when compared to the reference strain.

3.6. Exopolysaccharides production

The genomic organization of the genes involved in exopolysaccharides (EPS) production could be summarized as follows: **(1)** regulatory genes *(epsA, epsB),* **(2)** genes involved in determining the number of repeated units and their export *(epsC, epsD),* **(3)** genes related to the biosynthesis of the repeated units for exopolysaccharide synthesis *(epsE, epsF, epsG, epsH,* and *epsI),* and **(4)** genes active in the polymerization and export *(epsK, epsL, epsM)* (Iyer et al., 2010)

Gene content analysis performed on the EPS-subsystems on the eight *S. thermophilus* strains isolated in Italy showed several differences (Table 3). It is known that the presence of gene sequences coding for the exopolysaccharide biosynthesis transcriptional activator (EpsA), manganese-dependent protein-tyrosine phosphatase (EpsB) and tyrosine-protein kinase transmembrane modulator (EpsC) is not sufficient to determine the "ropy" phenotype (Stingele, Neeser, & Mollet, 1996). Results highlight the presence in all the strains of a "core" subset constituted by 5 genes, namely the previous cited genes together with tyrosine-protein kinase (EpsD) and undecaprenyl-phosphate galactose phosphotransferase (rfbP), indicating their putative role in cell adhesion and aggregation (Sun et al., 2011). Nevertheless, significant variations in gene copy number and sequence similarity were observed in the "core" genes (e.g. TH982 *vs* TH1435).

Additionally, there are other EPS-related genes, generally called glycosyltransferase (*gtf)* (Stingele et al., 1996) and *cps* gene cluster, known to have sequence similarity with *eps* (Bolotin et al., 2004). Results indicated that most of the studied strains possess *gft* gene sequences (Table 3). Moreover, even though genes belonging to **(3)** and **(4)** were not detected, their homologous *cps* sequences are present in 1F8CT, M17PTZA496 and TH982. As a concluding remark, genetic results evidenced these strains as promising EPS producers, in particular strains TH982 possessing 13 EPS-related

genes. An additional investigation performed on the arrangement of these genes revealed that they appear to be organized in three operons. The main important operon, composed of the "core" genes, was found in all the analyzed strains, while two additional operons encoding the *cps* genes were present only in 1F8CT and in M17PTZA496. A more detailed analysis performed on the promoter region of the "core" operon revealed a 34 bp insertion located between the -35 and -10 regions in strain TH982 (Fig. 5a).

The insertion in the promoter region of strain TH982, absent in the other strains and in the well-known EPS producer strain ND03 (Sun et al., 2011), suggests a possible response for its low production notwithstanding its richness in EPS related genes.

An in-vitro surface adhesion test was performed and highlighted significant differences among strains ($P < 0.001$; Fig. 5b). Statistical analysis organized strains into four groups according to their adhesion capacity: *a)* absent, *b)* low, *c)* medium and *d)* high. Only in MTH17CL396, the best performing strain, results were statistically significant.

Finally, SEM images of MTH17CL396 and TH985 (representatives of high and low adhesion capability, respectively) provided additional information on EPS and allowed to correlate adhesion results with the amount of EPS produced. No clear biofilm formation was visible in SEM images but a variation in cell-to-cell binding capability is clearly evident. In particular, MTH17CL396 displayed the highest robustness in cell anchorage, reflected in a more complex structure and cell chains interaction (Fig. 5c and 5d). Even in the absence of complex extracellular matrix production, a strong cell-to-cell binding could be relevant in producing compact clusters that could help protecting cells during gastrointestinal transit. In this direction, further analyses could lead to discover interesting gastrointestinal survival properties of strain MTH17CL396 due to its highest ability in cell aggregation.

3.7. Bacteriocin production

S. thermophilus strains are normally well represented in the spontaneous microbial consortia of artisanal cheeses and fermented milks. This is in part favored by the production of organic acids and bacteriocins that hamper proliferation of competitor microorganisms (Morandi & Brasca, 2012).

Phenotypical test performed against a selection of marker bacteria (similar to pathogens or food spoilers) and against three lactic acid bacteria did not reveal any inhibitory activity. Conversely, whole genome analysis based on bioinformatics mining by BAGEL3 software (van Heel et al., 2013) identified several lantibiotic-related (LR) genes in all the strains (Table 4). The main findings of this analysis are the presence of 8 LR genes in strains 1F8CT and the occurrence of self-immunity elements in four strains which confer resistance to their own bacteriocin (Table 4). On the contrary,

MTH17CL396 is the only strain without genes directly correlated to lantibiotics. Moreover, genes encoding putative pore-forming proteins were found in five out of eight strains (Table 4). These evidences support the idea that the identified genes may be involved in different functions that could also be possibly related to quorum-sensing mechanisms. It is known that bacteriocin synthesis is strictly linked to quorum-sensing regulation and their role as pheromone was previously demonstrated (Renye and Somkuti, 2013). Alternatively, it could also be possible that the antibacterial substances produced by the identified genes could be active against microorganisms different from those tested in this study (Rossi et al., 2013).

4. Conclusion

In the present study a comparative analysis of 17 *S. thermophilus* genomes was performed and the influence of geographical origin on genetic variability was assessed. Results indicated that strains isolated in the same continent infrequently cluster together. A notable finding is the high divergence detected for M17PTZA496 strain, which has an enhanced genome size due to several recombination events. Several genetic traits related to technological phenotypes were found in all strains, with a considerable degree of genome variability determined by the presence of several SNPs. These findings reflected important differences among phenotypes that were detected among strains. Strains-specific functions of *Streptococcus thermophilus* were investigated and valuable information regarding characters of technological relevance were obtained.

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Figure legends

Fig 1. Phylogenetic trees of *S.thermophilus* strains. a) Analysis performed considering a panel of 400 conserved proteins. Strain IDs are numbered according to their geographic origin: Europe (1), Asia (2), Australia (3) and North America (4). *S. salivarus*, *S. pneumoniae* and *S. macedonicus* were used as outgroups. b) Analysis performed using total SNPs extracted by whole genome alignment.

Fig 2. Circular genome map of M17PTZA496 strain. Forward gene COG annotations, reverse gene COG annotations, %GC plot and GC skew are reported in circles from outside inwards. Laterally transferred islands are highlighted as green arrows (ISL1 and ISL2), "phages, prophages" protein rich region (PH) as red arrow and duplicated region (DR) as yellow arrows. In the magnification a schematic representation of the duplicated region is reported. Gene abbreviations indicate: DNA-directed RNA polymerase alpha subunit (DNA poly; EC 2.7.7.6), translation initiation factor 1 (TIF), adenylate kinase (AK, EC 2.7.4.3), preprotein translocase secY subunit (PT, TC 3.A.5.1.1); all the other features refer to ribosomal proteins.

Fig 3. Hierarchical cluster of **s**trains annotations. Hierarchical clustering was performed on genes identified for each functional class of the SEED subsystem. Color scale on the top of the heatmap allows the identification of the gene numbers for the strains reported. The clustering on top of the figure shows similarities between strains. Italian strains are colored in dark grey, other strains in light grey; strains possessing genes related to osmotic stress are indicated with an asterisk.

Fig. 4. Strain specific features. For each strain reported in the x axis, genes were assigned to 24 functional categories of the SEED subsystem. The number of genes in the y axis for each functional category is proportional to its parcel height.

Fig. 5. Strains adhesion properties. EPS operon promoter analysis (a). Results from colorimetric assay (b); dotted line indicates the detection threshold. Letters above the bars correspond to the statistically identified groups. Images of cells aggregation in MTH17CL396 (c) and TH985 (d) by Scanning Electronic Microscope.

492 **Tables**

493

494 **Tab. 1.** Geographical origin and genomic information of the *S. thermophilus* strains present in
495 GenBank database (August 2015) used in the present work.

496

Strain	Isolation matrix	Geographic origin	Genome size (Mbp)	No. of ORF	No. of CRISPRs	NCBI accession number	Reference
1F8CT	curd from Raw cow milk	Veneto, Italy	1.74	1864	25	AZTK00000000	(Treu, Vendramin, Bovo, Campanaro, & Corich, 2014a)
M17PTZA496	Fontina cheese	Valle d'Aosta, Italy	2.13	2221	4	AZJT00000000	(Treu, Vendramin, Bovo, Campanaro, Corich, et al., 2014b)
MTH17CL396	Fontina cheese	Valle d'Aosta, Italy	1.82	1935	25	AZJS00000000	(Treu, Vendramin, Bovo, Campanaro, Corich, et al., 2014b)
TH982	Buffalo mozzarella curd	Campania, Italy	1.79	1924	24	AZTL00000000	(Treu, Vendramin, Bovo, Campanaro, & Corich, 2014a)
TH985	Buffalo mozzarella whey	Campania, Italy	1.83	1952	26	AZTM00000000	(Treu, Vendramin, Bovo, Campanaro, & Corich, 2014a)
TH1435	Raw goat milk	Friuli Venezia Giulia, Italy	1.79	1925	23	AYSG00000000	(Treu, Vendramin, Bovo, Campanaro, Corich, et al., 2014c)
TH1436	Raw goat milk	Friuli Venezia Giulia, Italy	1.79	1899	24	AYTT00000000	(Treu, Vendramin, Bovo, Campanaro, Corich, et al., 2014c)

TH1477	Raw cow milk	Veneto, Italy	1.9	1986	20	AZTJ00000000	(Treu, Vendramin, Bovo, Campanaro, & Corich, 2014a)
ASCC 1275	Dairy starter	Australia	1.85	1948	30	ASM69888v1	(Wu et al., 2014)
CNRZ1066	Yogurt	France	1.8	1918	14	ASM1184v1	(Bolotin et al., 2004)
JIM 8232	Milk	France	1.93	2076	21	ASM25339v1	(Delorme et al., 2011)
LMD-9	Industrial starter	USA	1.86	1930	21	ASM1448v1	(Hols et al., 2005)
LMG 18311	Yogurt	UK	1.8	1892	27	ASM1182v1	(Bolotin et al., 2004)
MN-BM-A02	Traditional dairy products	China	1.85	1953	29	ASM100801v1	(Shi et al., 2015)
MN-ZLW-002	Yogurt	China	1.85	1941	23	ASM26267v1	(Kang et al., 2012)
ND03	Traditional dairy products	China	1.83	1935	23	ASM18287v1	(Sun et al., 2011)
SMQ-301	Mozzarella whey	Canada	1.86	1952	24	ASM97166v1	(Labrie et al., 2015)

497

Tab. 2. Growth and fermentation parameters of *S.thermophilus* strains. Maximum acidification rate (V_{max}) is expressed as ΔpH unit per 10^{-4} per minute, T_{pHx} refers to the time required to achieve pH 5.2 and 4.6 respectively, **: p-value<0.001.

Strain ID	Growth curve parameters			Acidification performance			
	λ (h)	μ (h^{-1})	log Nmax (cell/ml)	ΔpH	V_{max}	$T_{pH\ 5.2}$** (h)	$T_{pH\ 4.6}$** (h)
1F8CT	2 .93	0 .53	8 .5	-	-	-	-
M17PTZA496	1 .12	0 .42	10 .3	0 .99	29	10	-
MTH17CL396	1 .16	0 .36	9 .1	0 .98	61	16	-
TH982	2 .05	0 .41	9 .5	1 .74	33	7	13
TH985	1 .46	0 .35	9 .9	1 .26	24	12	23
TH1435	1 .46	0 .35	9 .6	1 .50	57	4	6
TH1436	1 .53	0 .45	9 .0	1 .62	52	4	7
TH1477	2 .21	0 .26	9 .2	1 .41	43	6	14

Tab. 3. Extracellular polysaccharides genes in *S. thermophilus* strains genome. GTF: glycosyltransferase family group 1 or 2, EpsA: exopolysaccharide biosynthesis transcriptional activator, EpsB: manganese-dependent protein-tyrosine phosphatase, EpsC: tyrosine-protein kinase transmembrane modulator, EpsD: tyrosine-protein kinase, CpsF: polysaccharide biosynthesis protein, CpsG: glycosyl transferase, CpsM: polysaccharide biosynthesis protein. If not differently specified, the percentage of identity for gene clustering by amino acidic sequences similarity was 100%.

Strain ID			Operon "core"					Second operon		
	GTF1	GTF2	EpsA	EpsB	EpsC	EpsD	EpsE	CpsF	CpsG	CpsM
1F8CT		1	1	1	1	1	1	1	1	1
M17PTZA496		1	1	1	1	1	1;1(80%)	1	1	
MTH17CL396	1		1	1	1	1	1			
TH982	1	1	1	1;1(80%)	1;1(90%)	1;1(90%);1(80%)	1			1
TH985			1	1;1(80%)	1(90%);1(80%)	1(90%);1(80%)	1			
TH1435			1	1	1	1	1			
TH1436			1	1	1	1	1			
TH1477	1		1	1	1	1	1			

513 **Tab. 4.** Genes encoding for bacteriocins in *S. thermophilus* strains. Total numbers of genes belonging
514 to lantibiotics, ABC transporter and non-lantibiotic compounds are reported.
515

Strain ID	Lantibiotic related genes	ABC transporter	Lactococcin LcnD-like	Pore-forming peptide	Bacteriocin self-immunity protein
1F8CT	8	3	1	0	0
M17PTZA496	1	2	1	0	1
MTH17CL396	0	5	1	1	0
TH982	4	2	0	0	0
TH985	2	1	1	2	0
TH1435	2	4	1	2	1
TH1436	3	2	1	1	1
TH1477	1	2	1	1	1

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