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Proteolytic activity and production of γ-aminobutyric acid by *Streptococcus thermophilus* cultivated in microfiltered pasteurized milk

Running Title: Proteolysis and GABA production by *S. thermophilus*

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ABSTRACT

A set of 191 strains of *Streptococcus thermophilus* were preliminarily screened for the presence of the genes codifying for cell envelope-associated proteinase (*prtS*), and for glutamate decarboxylase (*gadB*) responsible for γ-aminobutyric acid (GABA) production. Growth and proteolytic activity of the *gadB* positive strains (nine presenting the prtS gene and 11 lacking it) were studied in microfiltered pasteurized milk. Degradation of both caseins (capillary electrophoresis) and soluble nitrogen fractions (HPLC), and changes in the profile of free amino acids (FAA, ion-exchange chromatography) were evaluated at inoculation and after 6 and 24 hours incubation at 41°C. None of the strains was capable of hydrolyzing caseins and β-lactoglobulin and only two hydrolyzed part of α-lactalbumin, these proteins being present in their native states in pasteurized milk. Contrariwise, most strains were able to hydrolyze peptones and peptides. For initial growth, most strains relied on the FAA present in milk, whereas, after 6 hours, *prtS*$^+$ strains released variable amounts of FAA. One *prtS*$^+$ strain expressed a PrtS- phenotype and two *prtS*$^-$ strains showed a rather intense proteolytic activity. Only five strains (all *prtS*$^+$) produced GABA, in variable quantities (up to 100 mg/L) and at different rates, depending on the acidification strength. Addition of glutamate did not induce production of GABA in non-producing strains that, however, unexpectedly showed to adopt the degradation of arginine into citrulline and ornithine as an alternative acid resistance system and likely as a source of ATP.

KEYWORDS: *Streptococcus thermophilus*, microfiltered milk, proteolysis, free amino acids, GABA, arginine, citrulline, ornithine.

INTRODUCTION

Among lactic acid bacteria, *Streptococcus thermophilus* represents the second most important species of lactic acid bacteria (LAB) of industrial interest, after *Lactococcus lactis* and it is the only species of this genus recognized as "Generally Regarded As Safe" by the FDA[1]. *S. thermophilus* is widely present in raw milk and is a major dairy starter used in the manufacturing of both artisanal and protected designation of origin (PDO) cheeses[2-3], in traditional yoghurt preparation, in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus*, and more generally in fermented milk products.

It was highlighted that *S. thermophilus* in milk satisfies its amino acid requirement by efficient biosynthetic capacities and by cooperation with other species of the dairy environment, being the most studied the proto-cooperation with *Lb. delbrueckii* subsp. *bulgaricus*. The proteolytic system of *S. thermophilus* consists of (i) an extracellular cell envelope protease, called PrtS and belonging to the subtilisin-like serine protease family [4-6], (ii) an efficient transport system for import of amino acids and oligopeptides, and (iii) a pool of intracellular peptidases for further degradation. PrtS is reported to be present only in a minority of the strains studied to date. Non-expression of *prtS* gene is reported as well[1]. Letort et al.[7] evidenced that growth of *S. thermophilus* in liquid milk shows two different exponential phases and that expression of PrtS

takes place in the second one. In general, the proteolytic activity allows the $PrtS^+$ strains in pure culture to grow and produce acid in milk more rapidly than the $PrtS^-$ strains.[8] More recently, the existence of a range of cell-associated extracellular peptidase activities was reported for a strain with $Prts^-$ phenotype, including aminopeptidase, carboxypeptidase, peptidyl dipeptidase and X-prolyl dipeptidyl peptidase.[9] The ability of *S. thermophilus* to hydrolyze undenatured (native) β-lactoglobulin (b-Lg) and α-lactalbumin (a-La) whey proteins in milk has not been elucidated yet, as most of the studies have been conducted on heat denatured whey proteins. A single strain of *S. thermophilus* inoculated in heat-sterilized reconstituted whey powder promoted the hydrolysis of up to 10% b-Lg and 2-3 fold more a-La.[10]

Amino acid biosynthesis pathways were identified by in silico studies[6] and the number and nature of amino acids essential for growth were found to be strain dependent with some strains exhibiting no absolute amino acid requirement.[4,11,12] It is worthy to note that most of these studies were conducted on a limited number of strains and this fact might explain some contradictory results. Very little information is available on the changes in free amino acid content of milk during growth of *S. thermophilus*. At this regard, it has to be considered that milk contains few free amino acids and peptides. However, milk native proteases, such as plasmin and cathepsins, contribute to initiate casein degradation[13,14] and thus provide more easy access to the microbial proteases.

Presence of bioactive substances in foods is receiving increasing attention within the scientific community. Among these, γ-aminobutyric acid (GABA), a non-protein amino acid that is widely distributed in nature[15], possesses a variety of beneficial effects and physiological

functions, such as neurotransmission, induction of hypotension and secretion of insulin and plasma growth hormone [16,17] as well as stimulation of immune cells.[18,19] Ability to produce GABA was evidenced in *S. thermophilus* and related to the presence of the *gadB* gene. [20]

LAB strains producing GABA were observed among *Lactococcus lactis* (22), *Lactobacillus brevis* (23), *Lb. buchneri* (24), *Lb. helveticus* (25), *Lb. paracasei* (26), *Lb. plantarum* (27) and *Lb. sakei* (28) species.

Other LAB species able to produce GABA are *Lactococcus lactis*[21], *Lactobacillus brevis*[22], *Lb. buchneri*[23], *Lb. helveticus*[24], *Lb. paracasei* [25], *Lb. plantarum* [26], and *Lb. sakei.*[27] In microbes, production of GABA has been reported to increase acid tolerance.[28] During cell growth, the intracellular pH decreases as a consequence of the accumulation of organic acids. The glutamic acid decarboxylase (GAD) system converts one molecule of glutamate into one molecule of GABA consuming an intracellular proton, thus shifting the cytosole pH towards neutrality. GABA is then released into the extracellular environment, thereby contributing to alkalinisation.[29]

The capability of producing GABA is expected to be dependent on both the degree of GAD activation and availability of free glutamate[28]. High levels of glutamate may be released by proteolytic enzymes in dairy products, since milk proteins are rich in this amino acid.[30] To our knowledge, few studies have considered fermented milks and cheeses as potential vehicles for GABA.[31-34] In this context, the biodiversity of the microbiota in raw milk cheeses represents an unmatched source of strains to investigate for GABA production ability.

The aim of this work was to investigate the proteolytic activity of *S. thermophilus* strains in mildly heated (pasteurized) milk and to shed light on their behaviour with respect to available

free amino acids and production of GABA. To conduct this study, 165 wild strains isolated from both artisanal and PDO raw milk cheeses as well as 26 strains from commercial starter cultures were preliminarily screened for the presence of the *prtS* and *gadB* genes; on this basis, 20 strains were selected for this study.

MATERIALS AND METHODS

Bacterial strains. A set of 191 *S. thermophilus* strains, including 165 wild strains from the bacterial collection of the Institute of Sciences of Food Production of the National Research Council of Italy (CNR-ISPA, Milan, Italy), previously isolated from Italian raw milk cheeses, and 26 strains from the Sacco S.r.l. (Cadorago, Italy) culture collection, was used in this study (**Table 1**). The strains were previously identified by partial 16S ribosomal DNA sequence analysis as described by Morandi et al.[35] and analysed by RAPD-PCR analysis with primers M13, D11344 and D8635 to exclude clonal relatedness.[2] Twenty strains harbouring the ORF*gadB* gene were selected for the study of proteolytic activity and GABA production in milk.

Detection of *prtS* and ORF*gadB* genes. DNA was extracted according to Cremonesi et al.[36] from 1 mL of overnight grown bacterial culture, incubated in M17 broth (Biolife, Milan, Italy) at 37 °C by the DNA Isolation System Kit (M-Medical, Genova, Italy), according to the manufacturer's recommendations. Genomic DNA was used in the PCR reactions to detect the presence of the *prtS* (531 bp) according to Galia et al.[37] using the primer Prt/For (5'-TAC GGT GAA TGG TTT AACG-3') and Prt/Rev (5'-AAT TAC TTT ACT ACC AAC CG-3'). In

addition, the presence of ORF*gadB* (1.380 bp) gene was tested with primers P3/For (5'-ATG AAT GAG AAG CTA TTC AGA GAG AT-3') and P4/Rev (5'-TTA ATG ATG GAA GCC ACT GCG GATG-3')[20]. Amplification conditions were: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Final extension was carried out at 72 °C for 5 min. All the PCR reactions were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). Each DNA amplification was performed in 200-µL microtubes using 25 µL of reaction mixture containing 50-100 ng DNA template, PCR Master Mix 2X (Thermo Fisher Scientific Inc., Waltham, MA, USA), 10 µM of the primer pair and double-distilled water to achieve the final volume. Amplification products were separated on a 1.5% agarose gel GellyPhor (Euroclone, Pero, MI, Italy) stained with GelRed™ (Biotium, Hayward, CA, USA). Molecular size markers (TrackIt™ 1 Kb Plus DNA Ladder, Life Technologies, Carlsbad, CA, USA) were included in each agarose gel.

Milk incubation trials. Before incubation trials, strains were inoculated twice in succession in reconstituted skim milk (10% w/v) and incubated at 37 °C overnight. To avoid presence of contaminating bacteria, somatic cells and spores**,** microfiltered pasteurized (75° C for 15 s) milk (MPM) was used for incubation trials. Freshly produced MPM was taken at an industrial plant (Tetrapack, Arhus, Denmark), immediately frozen and kept at -18 °C until inoculation. Stationary-phase-grown cells from overnight cultures of each *S. thermophilus* strain were inoculated in six sterile tubes containing 50 mL MPM at a final cell density of 7.01±0.37 log CFU mL^{-1} and incubated at 41 °C for 24 h. The optimal conditions (41 °C) for *S. thermophilus*

147 growth in milk were also applicable to the production of GABA, since Yang et al.[45]
148 demonstrated that 40 °C, pH 4.5 were the optimal conditions for GAD activity.
149 A negative control (non-inoculated MPM) was included in each incubation trial. In selected
150 experiments, MPM was supplemented with glutamic acid (Sigma-Aldrich, Milan, Italy) to
151 increase the concentration by 1 mMol L^{-1}. At each sampling time, the content of three tubes
152 was mixed and pH (Metrohm, Origgio, CO, Italy) and viable cell count enumeration of *S.*
153 *thermophilus* were determined and aliquots taken for proteolysis analyses.
154
155 ***S. thermophilus*** **viable cell count.** During the incubation trials, 1 mL of the cultured MPM
156 samples was taken after 0, 6 and 24 h, serially diluted on the decimal scale in one-quarter-
157 strength Ringer's solution and plated on M17 agar (Biolife) in duplicate for viable counts. The
158 plates were incubated under aerobic conditions at 37 °C for 48 h.
159
160 **Estimation of proteolysis and GABA production in incubated milk samples.** Individual
161 casein fractions were evaluated by capillary zone electrophoresis (CZE). One mL of incubated
162 MPM sample was clarified by centrifugation (3000*g* for 10 min), added with 10 mL of 10 M
163 urea sample buffer and, after 4 hours, filtered on 0.22 µm disposable filter and analysed by
164 CZE. Buffers and separation conditions for CZE were as described by Masotti et al.[39] Analyses
165 were carried out using a P/ACE System MDQ unit (Beckman Instruments Inc., Fullerton, CA,
166 USA), equipped with a diode array detector operating at 214 nm and a coated capillary column
167 (DB-WAX 126-7012; Agilent Technologies Inc., Santa Clara, CA, USA), 500 mm x 50 µm i.d.,
168 with slit opening of 100 x 800 µm.

The soluble nitrogen components were analysed by high-performance liquid chromatography (HPLC). Ten mL of incubated MPM samples were adjusted to pH 4.6 using 1 M HCl, centrifuged (Du Pont Instruments Sorvall RC-5B, 12000*g* for 20 minutes at 10 °C) and filtered through a sterile 0.22-µm filter. The filtrate was analysed by HPLC according to the conditions described by De Noni et al.. [40] An Alliance workstation (Waters, Milford, MA, USA) was used, coupled with a 996 DAD detector (Waters) operating at 205 nm. The concentration of α-lactalbumin (a-La), β-lactoglobulin (b-Lg), proteose-peptone (PP) and small peptides (SP) were calculated using the external standard calibration curves as described by Pellegrino et al. [41]

Free amino acids were analysed by ion-exchange chromatography as described by D'Incecco et al.. [42] The same filtrate as for HPLC was adjusted to pH 2.2 using 1 M HCl and filtered on 0.2 µm RC filter prior to injection into the chromatograph. A Biochrom 30+ (Biochrom Ltd, Cambridge, UK) automatic amino acid analyzer was used operating under the conditions provided by the manufacturer. Briefly, a 10-step elution program with six lithium citrate buffers of increasing pH and ionic strength was adopted, post-column derivatization with ninhydrin and detection at 440 and 570 nm. Injection volume was 100 µL, and quantification was performed using 4-level calibration lines for 21 amino acids in the range 0.75-22.5 mg L^{-1}.

Statistical analysis. Statistical treatment of data was performed by means of the SPSS Win 12.0 program (SPSS Inc., Chicago, IL, USA). Data were submitted to Student's t-test. $P<0.05$ was assumed as significance limit.

RESULTS AND DISCUSSION

Presence of* prtS *and* ORFgadB *genes and growth behaviour of strains. The presence of *prtS* and ORF*gadB* genes were firstly investigated in 191 *S. thermophilus* strains of different origins. The screening of a spectrum of strains was necessary since cell envelope-associated proteinase is rather exceptional in *S. thermophilus* and GABA-production varies widely.[1, 22] In fact, a total of 72 strains (38%) harboured the PrtS gene but only 20 (10%) were ORF*gadB* positive (Table 1, Fig. 1). These last were considered as the putative producers of GAD and chosen for this study.

In view of characterizing their proteolytic activity, strains were grown in MPM where proteins are present in their native status, i.e. caseins are aggregated into large micelles and whey proteins are soluble globular monomers. On the contrary, in reconstituted skim milk, usually adopted as growth medium, proteins are extensively glycosylated by the Maillard reaction[43] and the additional sterilization treatment (e.g. 110 °C for 10 min) induces whey protein denaturation and binding to the casein micelle surface[44], so impairing access to proteases. Of relevance here, the temperature (41 °C) for *S. thermophilus* growth in milk was shown to be suitable for the production of GABA, and the acidic pH due to lactic acid fermentation proved to maximize the GAD activity and specificity.[45]

Although the initial concentration of viable cells was comparable among strains (7.01 ± 0.37 log CFU mL^{-1}), important differences in growth and acidifying activity were evidenced (Table 2). The commercial starters showed the highest acidification rate and a rapid growth after 6 to 24 h, reaching final loads nearly one log higher than wild strains. Vice versa, the TR strains isolated from Trentingrana were characterized by the lowest growth and acidification rates,

213 with final pH values often remaining above 6 (Table 2). *S. thermophilus* strains isolated from
214 raw milk cheeses showed an intermediate behaviour. Only nine out of the 20 studied strains
215 harboured the *prtS* gene (Table 2). Overall, *prtS*$^+$ strains showed highest counts and lowest pH
216 values after 24 hours. Previous studies evidenced that high milk-acidifying capacity in *S.*
217 *thermophilus* is associated with high cell wall proteinase activities.[6, 46]
218
219 ***Proteolytic activity.*** The proteolytic activity of the 20 strains was firstly assessed through the
220 evaluation of changes in the free amino acid (FAA) content (Fig. 2). The initial content of FAA
221 in the uncultured MPM (negative control) (n=4) ranged from 83 to 93 mg L^{-1}, in agreement
222 with the figures recently reported by Pellegrino et al. [41] for raw milk, and remained constant
223 throughout the 24-hour observation period (not shown). On the contrary, a decrease of FAA
224 content was observed for most of the strains during the initial 6 h of incubation, with a
225 subsequent increase (Fig. 2). This finding is consistent with the fact that *S. thermophilus*
226 growth in milk is diauxic and that the first growth phase relies on the utilization of free amino
227 acids and peptides while PrtS proteinase synthesis in pure culture only begins in the
228 intermediate non-exponential growth phase.[7] There were, however, a few exceptions. Three
229 *prtS*$^+$ strains, namely SC1, VS429 and SE95, proved to release FAA also during the first 6 h,
230 roughly corresponding to the first exponential growth phase, whilst *prtS*$^+$ BT232 kept uptaking
231 FAA from MPM over the whole incubation period (Fig. 2). Between 6 and 24 h, the FAA
232 content remained steady or slightly decreased for most of the *prtS*$^-$ strains (Fig. 2), whereas
233 VAL40 and VS436 released FAA at a rate comparable to that of *prtS*$^+$ strains.

With the aim of identifying the preferred protein substrates for the proteolytic activity of different strains, the casein and the pH 4.6-soluble nitrogen fractions in the MPM samples taken after 6 and 24 h were analysed by CZE and HPLC respectively. Three *prtS*$^+$ (SE95, SC2 and BT232) and three *prtS*$^-$ (VS436, VAL40 and TR12) strains were selected for this assessment on the basis of their characteristic behaviour in releasing and uptaking FAA as previously observed (Fig. 2). The small decrease of αs1-casein (2%) and β-casein (5-10%) observed in all of the samples after 24 h of incubation, being comparable to that of the control sample, was attributed to the residual activity of plasmin (data not shown). In fact, although microfiltration allowed to lower plasmin activity in MPM by removing the somatic cells, which carry part of the enzyme activation system,[13] our incubation temperature was close to the optimum temperature for plasmin activity in milk, i.e. 37 °C.[47] Our results are not in contrast with those of Miclo et al.[48] who demonstrated that *prtS*$^+$ strains of *S. thermophilus* were able to hydrolyze purified casein fractions individually dissolved in sodium phosphate buffer at pH 7.5. In fact, it is known that, until strong acidification occurs, casein fractions in milk are aggregated into large micelles and thus less susceptible to proteolysis. [49]

The main soluble whey proteins, namely a-La and b-Lg, as well as proteose-peptones (PP) and small peptides (SP) were all evaluated from HPLC chromatograms.[41] Values and relative changes of these fractions in MPM at the different sampling times are shown in Table 3. Identifying single peptides and studying their production and subsequent degradation was of limited interest in the context of this study. The negative control proved to be stable throughout the incubation time except for the PP which increased as an expected result of plasmin activity on β-casein. Among the tested strains, none was able to hydrolyze b-Lg and only two (SE95

and VAL40) operated a limited hydrolysis of a-La (Table 3). This finding confirmed the strong resistance of native whey proteins to proteolysis, [41, 50] due to their globular structure stabilized by disulphide bonds and, more relevantly, explained the unavailability of free cysteine. This amino acid is only present in whey proteins and is reported to be essential for growth of some strains of *S. thermophilus* and stimulating for others[11]. Strain SE95 confirmed to have a strong proteolytic activity which begins quite early, as already observed. In fact, besides the reduction of a-La, the net content of PP was lower than in the control at 6 h and then levelled off, indicating that additional PP formed by plasmin were progressively hydrolyzed to SP and these last, in turn, to FAA. Compared to SE95, strain SC2 showed a lower proteolytic activity in the first step of growth (Table 3), when the consumption of FAA was fast (Fig. 2), and thereafter a more extensive degradation of SP to which corresponded a high rate in FAA accumulation. In contrast, the lack of degradation of SP and the progressive consumption of FAA observed for *prtS*$^+$ BT232 strain concurred to evidence that this strain expressed a PrtS$^-$ phenotype, highlighting the non-expression of *prtS* gene. Interestingly, strains VS436 and VAL40, although *prtS*$^-$, were able to hydrolyze PP (Table 3). These two strains initially consumed part of the content of FAA available in milk but successively both were able to increase it, albeit to a limited extent (Fig. 2). This finding might be explained by the presence of a broad range of extracellular peptidases on the cell wall of *prtS*$^-$ strains, as recently reported by Hafeez et al.[51] The inability of TR12 to hydrolyze whey proteins and peptides were evident from data in Table 3, confirming that growth of this strain in MPM mostly relied on available FAA as a source of nitrogen. The low proteolytic capacity of TR strains of this study, all isolated from natural whey starters, i.e. mixed cultures containing *Lactobacillus* spp., is consistent with the results

of Courtin et al.[52] showing that PrtB of *L. bulgaricus* largely contributes to the optimal growth of *S. thermophilus.*

Free amino acid profile. Changes in average molar concentration of individual FAA are shown in Table 4 whereas the whole data set is presented in S1. Only in few cases, differences between *prtS*$^+$ and *prtS*$^-$ strains were statistically significant after 6 h of incubation, since standard deviation was very high. Literature on amino acid requirements of *S. thermophilus* is scarce and sometimes contradictory because of the different conditions of the assessment. [4, 6, 11] Nevertheless, there is wide agreement on the fact that type and number of amino acids essential for growth of *S. thermophilus* are strain-dependent.[6] Although PrtS allows *S. thermophilus* better to grow alone in milk, the efficiency of transport systems as well as the intracellular peptidases and *de novo* synthesis of amino acids are also responsible for such a high variability.[7] This fact can be argued from the evidence that, while several FAA (glutamate, aspartate, methionine, proline) evolved similarly between *prtS*$^+$ and *prtS*$^-$ strains, although with different rates, others (lysine, leucine, tyrosine, glycine) showed an opposite behaviour. Interestingly, proline content increased 6 times in MPM incubated with *prtS*$^+$ strains and represented up to 20-28% of total FAA after 24 h incubation. This relevant increase may be related to the presence of an extracellular X-prolyl dipeptidyl aminopeptidase (PepXP) observed in *S. thermophilus.*[9, 10, 48] Interestingly, Derzelle et al.[53] reported that PepXP is specifically upregulated in *S. thermophilus* when grown in milk with respect to a synthetic medium (M17). Glycine was the only amino acid to reach a significantly higher concentration in *prtS*$^-$ than in *prtS*$^+$ strains (Table 4). Concerning the behaviour of the branched chain amino

300 acids, it is noteworthy that isoleucine did not accumulate and showed the same final
301 concentration in both groups, whilst for leucine and valine the final concentrations were
302 remarkably higher in *prtS*$^+$ strains. This different behaviour is consistent with the presence of
303 two different biosynthetic pathways in *S. thermophilus*, one leading to formation of isoleucine
304 and the other to leucine and valine.[54] It should be mentioned that, consistently with their lowest
305 proteolytic activity among *prtS*$^-$ strains, the six TR strains were unable to release methionine
306 (not shown). For most FAA, the behaviour here described for *prtS*$^+$ strains agrees with that
307 found by Stulova et al.,[55] who however investigated a single strain grown in reconstituted skim
308 milk. Major differences with our data regarded the content of aspartate and glutamate that,
309 according to these authors, continuously increased.
310 ***GABA production.*** Production of GABA was examined in relation to free glutamate and
311 glutamine behaviour because both these amino acids may be involved in the metabolic
312 pathway.[8] As mentioned above, only five out of the 20 strains harboring the *gadB* gene were
313 capable of producing GABA in MPM (Fig. 3). This observation suggests that a silent GAD
314 gene might be present in the non-producing strains as a consequence of a frameshift mutation,
315 resulting in inactive forms of GAD, as also evidenced by Somkuti et al..[20] All of the GABA-
316 producing strains were *prtS*$^+$, and the highest levels were found for the strong proteolytic
317 VS429, SE95 and SC1 strains. Interestingly, while comparable residual quantities of glutamate
318 were found in MPM samples incubated with GABA-producing strains, the levels of glutamine
319 varied greatly. In particular, free glutamine increased during incubation in the high proteolytic
320 strains, as expected, while the trend was opposite in the less proteolytic ones (SC2 and BT122)
321 and was comparable to that of non-producing strains, regardless whether *prtS*$^+$ or *prtS*$^-$ (not

shown). Remarkably, no free glutamine was detected in TR strains, although synthesis of this amino acid was reported by Monnet et al.[12] to be essential for growth of *S. thermophilus* in milk. Variable amounts of glutamate were found in MPM incubated with no-GABA producing strains (Fig. 3). These strains likely consumed glutamate to a different extent for synthesizing other amino acids they need for growth through the glutamate dehydrogenase/α-ketoglutarate pathway[6]. According to Stulova et al.,[55] glutamate could supply ammonia for up to 25% of the amino acids in the biomass. Among *prtS*$^-$ strains, VAL40 and VS436 actually produced a very limited amount of GABA. Both these strains evidenced a peculiar proteolytic activity towards PP (Table 3) which are rich in glutamate[30]. An early production of GABA was observed for strains BT122 and VS429, while for strains SC1, SC2 and SE95 the production initiated at a later stage (Fig. 4). This different behaviour might be related to the different acidification rate of the strains, as can be argued from the pH values recorded at 6 h (Table 2). In fact, BT122 and VS429 reached the lowest pH among wild strains.

In parallel incubation trials, MPM was supplemented with 1 mmol L^{-1} glutamate. All *prtS*$^+$ and a selection of *prtS*$^-$ strains were tested. As shown in Fig. 5, almost all the available glutamate was converted into GABA by the five producing strains, although the relative differences in the yield changed for some of them. In particular, for SC1 the yield was close to that of VS429, whereas in not supplemented MPM it was a half (Fig. 3). For both these strains the amount of glutamine increased with the addition of glutamate. Previous studies[45, 56] evidenced the amount of produced GABA to be strictly dependent on glutamate availability, although experimental conditions were not always comparable to ours. In order to evaluate GABA production by *Lactococcus lactis* subsp. *lactis* in milk, Gardner-Fortier et al.[21] added 10 mmol L^{-1} of

344 glutamate (10 times more than in this study) and, after 5 days of incubation, found amounts of
345 GABA (50-80 mg L^{-1}) close to those here obtained (83-108 mg L^{-1}) (Figure 5). Total
346 conversion of GLU into GABA occurred reaching levels found to promote significant decrease
347 of blood pressure,[31, 57] and it can rationally be expected that higher GABA conversion yield
348 could be obtained in presence of higher GLU content. Wu et al. [58] recently observed that
349 selected strains of *L. brevis* increased production of GABA when co-cultured with *S.*
350 *thermophilus*. GABA production was not induced in the non-producing strains, with the
351 exception of BT232 that, however, converted into GABA less than 5% of the added glutamate.
352 Consumption of glutamate by strains unable to perform decarboxylation was confirmed to be
353 strain-dependent (Fig. 5). VAL40 used approximately 35% of the whole amount of available
354 glutamate without increasing the production of GABA.
355 As already mentioned, decarboxylation of glutamate to GABA represents an acid resistance
356 mechanism for several bacterial species to survive in the acidic environment. Similarly, an
357 arginine-mediated pathway, involving the arginine deiminase (ADI) system, is adopted by a
358 variety of lactic acid bacteria[42,59,60] as an alternative acid resistance system. Arginine is
359 stoichiometrically converted to citrulline and this last to ornithine producing two mol of
360 ammonia per mol of degraded arginine. Although, to our knowledge, this mechanism has not
361 been described for *S. thermophilus* in milk, we evidenced that strains unable to produce GABA
362 generally operated a more extensive degradation of arginine (Fig. 6). In fact, in MPM samples
363 incubated with these strains, with the exception of SC3 and SC5, at least half of free arginine
364 was converted into citrulline and ornithine, irrespective of the absolute amounts. Furthermore,

this pathway gave a bioenergetic benefit to these strains because of the ATP generated by degradation of arginine.

This study provides new evidence on proteolytic pathways of *S. thermophilus* in milk. A range of *S. thermophilus* strains, all sharing the *gadB* positive character, was examined by evaluating the ability to hydrolyze individual protein fractions and to accumulate or use single FAA in a minimally heat-treated milk. Interesting systematic differences were observed between $PrtS^+$ and $PrtS^-$ strains, although several strains displayed an intermediate behavior. A strain-dependent adoption of specific proteolytic pathways has been found in *S. thermophilus* grown in minimally treated milk, where proteins are present in the native form and overall availability of FAA is restricted. Some wild strains from raw milk cheeses were almost unable to release FAA, indicating their habit of growing in mixed population. Our data showed the capacity of synthesizing GABA to be infrequent in *S. thermophilus* and the yield to be dependent on the available amount of glutamate. Since *S. thermophilus* is largely used in a variety of fermented foods, strain screening based on this trait may be of interest in the manufacturing of functional foods. The five strains able to fully convert the glutamate into GABA could represent microbial factories for industrial GABA production. Notably, new evidence was given that strains unable to produce GABA may adopt the degradation of arginine into citrulline and ornithine as an alternative pathway helping to raise intracellular pH and as a source of energy. Our findings demonstrated that the characterization of single strains with respect to their actual proteolytic activity and ability of using peptides and specific FAA in milk provides strategic information to identify those having the most suitable behaviour for manufacturing of targeted fermented dairy products.

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Figure captions

Fig. 1 – PCR products obtained with primers for the *prtS* (a) and ORF*gadB* (b) in different *S. thermophilus* strains (lanes 2-7); MW marker, kb (lane 1)

Fig. 2 - Evolution of total content of free amino acids (FAA) (mg L^{-1}) in milk samples during incubation with different strains of *prtS*$^+$ and *prtS*$^-$ *S. thermophilus*

Fig. 3 - Free glutamine (GLN), glutamate (GLU), and γ-amino butyric acid (GABA) (µmol L^{-1}) in milk samples after 24 hours incubation with different strains of *S. thermophilus* and in the non-inoculated control (blank) sample

Fig. 4 - Evolution of free γ-amino butyric acid (GABA) content (µmol L^{-1}) in milk samples during incubation with different *prtS*$^+$ strains of *S. thermophilus*

Fig. 5 – Free glutamine (GLN), glutamate (GLU), and γ-amino butyric acid (GABA) (µmol L^{-1}) in milk samples added with glutamate (1 mmol L^{-1}), after 24 hours incubation with different strains of *S. thermophilus*, and in the non-inoculated control (blank) sample.

Fig. 6 - Free arginine (ARG), citrulline (CIT) and ornithine (ORN) (µmol L^{-1}) in milk samples after 24 hours incubation with different strains of *S. thermophilus* producing or non-producing γ-amino butyric acid (GABA), and in the non-inoculated control (blank) sample

Table 1 – Origin of the *S. thermophilus* strains investigated in this study and presence of *prtS* (extracellular cell envelope protease) and *gadB* (glutamate decarboxylase) genes

Product of origin	**Source**	**strains**	***prt*S$^+$**	**ORF*gadB*$^+$**
Commercial starter cultures		26	26	5
Asiago PDO[a]	Cheese	12	11	-
Bitto PDO[a]	Fresh curd	28	3	1
	Cheese	4	3	1
	Whey culture	11	-	-
Fontina PDO[a]	Cheese	2	-	-
Formaggella Luinese PDO[a]	Fresh curd	3	2	-
Formaggella Valle di Scalve[a]	Cheese	14	2	-
Formagèla Valseriana[a]	Fresh curd	6	-	-
	Cheese	9	-	-
	Milk culture	14	8	2
Semuda[a]	Cheese	6	5	1
Silter[a]	Cheese	15	3	1
Soft cheese	Cheese	9	6	-
Trentingrana PDO[a]	Whey culture	10	-	9
Valtellina Casera PDO[a]	Fresh curd	19	3	-
	Cheese	3	-	-
Total		**191**	**72**	**20**

[a] Raw milk cheese

Table 2 – Growth and acidification of *S. thermophilus* strains used in this study during incubation in MPM for different times

Product of origin	Strain	*prtS*	6 hours		24 hours	
			Log CFU mL^{-1}	**pH**	**Log CFU mL^{-1}**	**pH**
Commercial starter	SC1	+	7.48±0.09	4.91	9.70±0.12	4.12
	SC2	+	7.66±0.21	5.19	9.40±0.26	4.79
	SC3	+	7.04±0.19	4.68	9.20±0.15	4.16
	SC4	+	7.58±0.22	4.85	9.04±0.22	4.18
	SC5	+	7.43±0.17	4.64	9.34±0.19	4.14
Bitto PDO	BT122	+	7.62±0.25	5.09	8.59±024	4.33
	BT232	+	7.54±0.10	6.60	8.58±0.29	4.84
Formagèla Valseriana	VS429	+	7.56±0.30	5.19	8.79±0.08	4.13
	VS436	-	7.62±0.29	5.51	8.95±0.16	4.57
Semuda	SE95	+	7.00±0.14	6.56	9.00±0.23	4.52
Silter PDO	VAL40	-	7.28±0.11	5.56	8.66±0.17	4.82
Trentingrana PDO	TR12	-	7.63±0.32	6.70	8.49±0.28	6.11
	TR13	-	7.32±0.19	6.86	8.49±0.20	5.04
	TR14	-	7.54±0.31	---	8.43±0.17	6.17
	TR15	-	7.51±0.24	---	8.45±0.12	6.15
	TR16	-	7.63±0.18	---	8.57±0.23	5.99
	TR17	-	7.36±0.23	---	8.51±0.14	5.95
	TR18	-	7.49±0.22	---	8.46±0.27	6.01
	TR27	-	7.71±0.17	6.71	8.56±0.16	4.80
	TR37	-	7.78±0.33	6.80	8.58±0.13	5.03

Table 3 – Absolute and relative changes of soluble nitrogen fraction content in MPM inoculated with various strains of *S. thermophilus* and incubated for different times

Strain	Time hours	pH	b-Lg[a] mg L^{-1}	*Change %*	a-La[b] mg L^{-1}	*Change %*	PP[c] mg L^{-1}	*Change %*	SP[d] mg L^{-1}	*Change %*
Control	0	**6.71**	3347		1105		864		760	
	6	**6.67**	3335	*0*	1124	*0*	1276	*48*	785	*3*
	24	**6.54**	3360	*0*	1114	*0*	1531	*20*	769	*0*
SE95 *prtS*+	0	**6.71**	3339		1186		873		786	
	6	**6.56**	3349	*0*	1149	*-3*	1049	*21*	996	*27*
	24	**4.52**	3325	*0*	962	*-16*	1033	*0*	877	*-12*
SC2 *prtS*+	0	**6.80**	3351		1112		855		793	
	6	**5.19**	3353	*0*	1123	*0*	1247	*45*	851	*7*
	24	**4.79**	3344	*0*	1145	*0*	1388	*11*	719	*-15*
BT232 *prtS*+	0	**6.78**	3325		1136		870		766	
	6	**6.45**	3342	*0*	1107	*0*	1014	*17*	830	*8*
	24	**4.63**	3374	*0*	1084	*0*	1148	*13*	991	*19*
VS436 *prtS*-	0	**6.80**	3342		1116		896		792	
	6	**5.51**	3369	*0*	1101	*0*	862	*-4*	804	*0*
	24	**4.57**	3382	*0*	1104	*0*	899	*4*	970	*8*
VAL40 *prtS*-	0	**6.70**	3347		1124		847		786	
	6	**6.09**	3370	*0*	1127	*0*	1000	*18*	788	*0*
	24	**4.64**	3312	*0*	1075	*-5*	724	*-28*	922	*17*
TR12 *prtS*-	0	**6.70**	3373		1124		847		786	
	6	**6.70**	3391	*0*	1109	*0*	1207	*42*	882	*12*
	24	**6.11**	3366	*0*	1102	*0*	1452	*20*	1013	*29*

[a] b-Lg: β-lactoglobulin. [b] a-La: α-lactalbumin. [c] PP: proteose peptones.[d] SP: small peptides

Table 4 – Concentration (µmol L^{-1}) (mean ± standard deviation) of FAA in MPM inoculated with single strains of *S. thermophilus* (9 *prtS*$^+$ and 11 *prtS*$^-$ strains) and incubated for different times

	0 hours	**6 hours**		**24 hours**	
		prtS+	***prtS-***	***prtS+***	***prtS-***
Asp	21.2 ± 2.5	10.3 ± 5.3	10.8 ± 4.0	8.8 ± 5.3	8.8 ± 6.1
Thr	11.8 ± 1.5	11.1 ± 6.1	6.6 ± 2.7	18.8 ± 6.3*	10.7 ± 8.0*
Ser	10.6 ± 0.7	6.4 ± 5.0	2.6 ± 1.5	9.6 ± 4.1*	4.8 ± 3.1*
Asn	4.7 ± 1.6	13.6 ± 16.1	0.4 ± 0.8	32.4 ± 29.0*	2.2 ± 3.8*
Glu	327.6 ± 27.8	148.4 ± 103.5	211.1 ± 77.1	90.9 ± 65.8	113.4 ± 43.7
Gln	43.2 ± 21.4	29.1 ± 25.0*	2.5 ± 0.7*	37.1 ± 35.7*	2.4 ± 3.1*
Gly	97.5 ± 9.4	17.1 ± 29.8	40.1 ± 21.6	6.4 ± 5.2*	50.7 ± 22.6*
Ala	48.4 ± 2.2	36.51 ± 21.0	30.2 ± 8.6	82.5 ± 43.2	70.5 ± 15.3
Cit	7.3 ± 2.6	4.1 ± 1.3	5.4 ± 1.2	5.2 ± 3.1*	8.6 ± 1.7*
Val	10.5 ± 2.2	8.8 ± 8.4	4.3 ± 1.3	14.5 ± 10.0*	7.2 ± 4.7*
Met	0.0 ± 0.0	3.2 ± 2.9	3.4 ± 3.9	12.9 ± 10.6	13.2 ± 16.2
Ile	9.9 ± 0.8	6.4 ± 8.0	3.6 ± 1.4	9.3 ± 4.6	9.8 ± 5.9
Leu	9.2 ± 0.5	16.5 ± 12.8	3.8 ± 0.8	22.7 ± 15.8*	5.1 ± 1.5*
Tyr	6.6 ± 0.4	11.9 ± 9.3	2.7 ± 0.6	16.4 ± 11.5*	3.7 ± 1.1*
Phe	0.0 ± 0.0	24.1 ± 11.1*	4.6 ± 1.2*	25.7 ± 16.2*	8.5 ± 3.8v*
GABA	0.0 ± 0.0	33.0 ± 63.6	1.1 ± 1.6	109.3 ± 118.0*	1.6 ± 2.8*
Orn	4.9 ± 0.9	5.4 ± 0.9	6.0 ± 1.0	6.2 ± 2.1*	9.3 ± 1.2*
Lys	16.4 ± 2.4	30.0 ± 16.7*	7.9 ± 4.2*	50.1 ± 23.8*	5.3 ± 2.0*
His	2.8 ± 0.4	10.1 ± 6.6*	1.8 ± 1.6*	9.2 ± 8.6*	0.6 ± 1.0*
Arg	15.3 ± 2.5	17.1 ± 11.2	11.0 ± 7.6	21.0 ± 12.6*	9.8 ± 4.2*
Pro	32.9 ± 7.3	128.4 ± 60.0*	49.5 ± 22.1*	182.3± 25.8*	79.6± 43.7*

* Difference between *prtS*$^+$ and *prtS*$^-$ strains significant at $p < 0.05$

Fig. 1

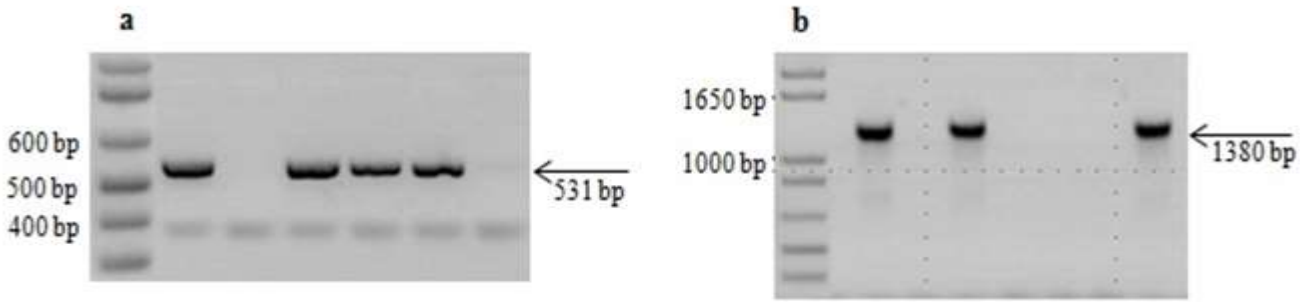
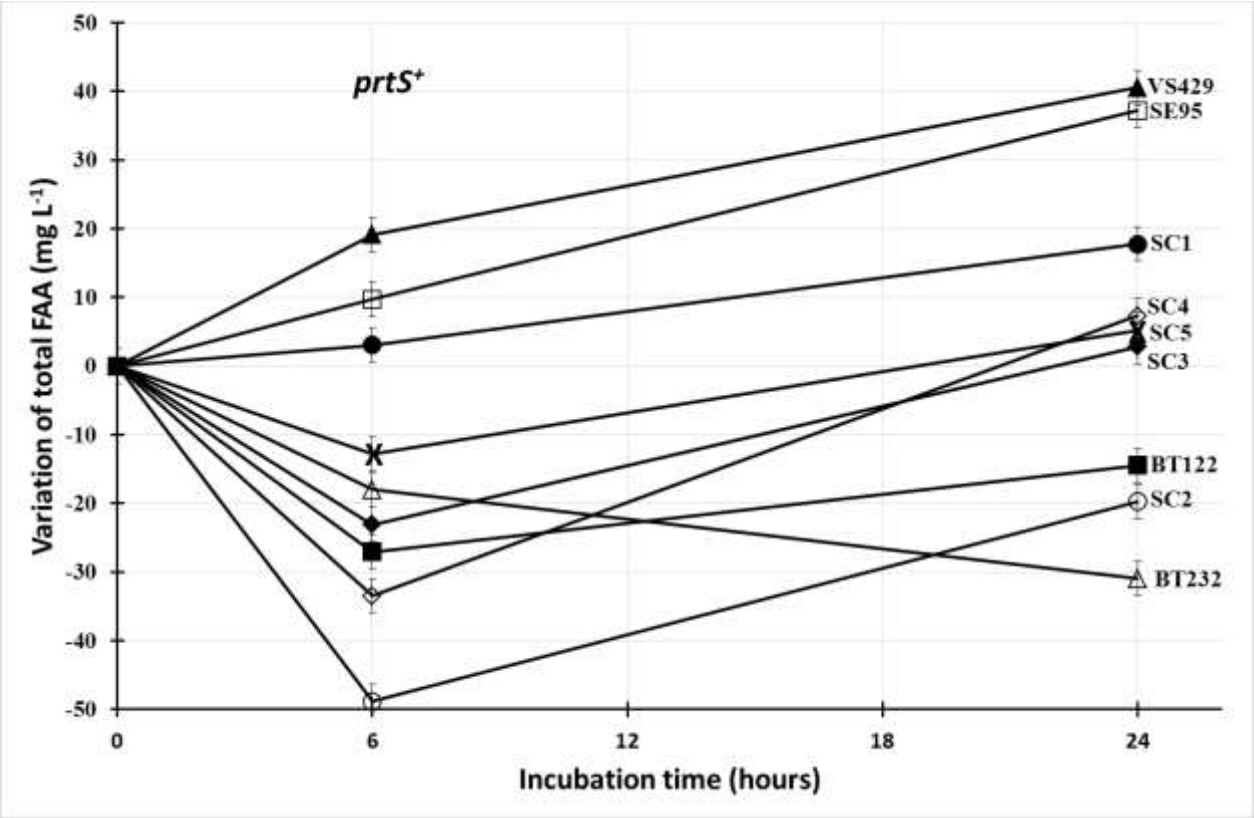


Fig. 2


prtS+
Variation of total FAA (mg L-1)
Incubation time (hours)
VS429
SE95
SC1
SC4
SC5
SC3
BT122
SC2
BT232

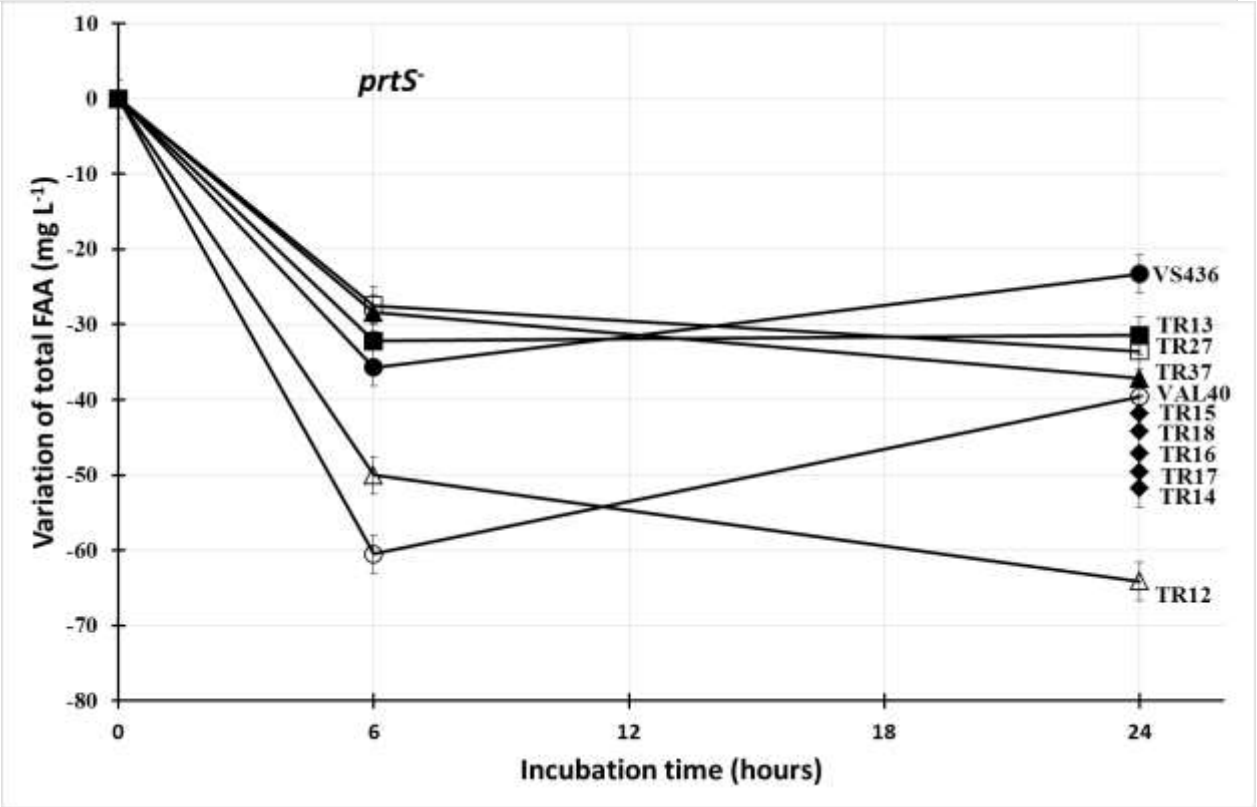

prtS-
Variation of total FAA (mg L-1)
Incubation time (hours)
VS436
TR13
TR27
TR37
VAL40
TR15
TR18
TR16
TR17
TR14
TR12

Fig. 3

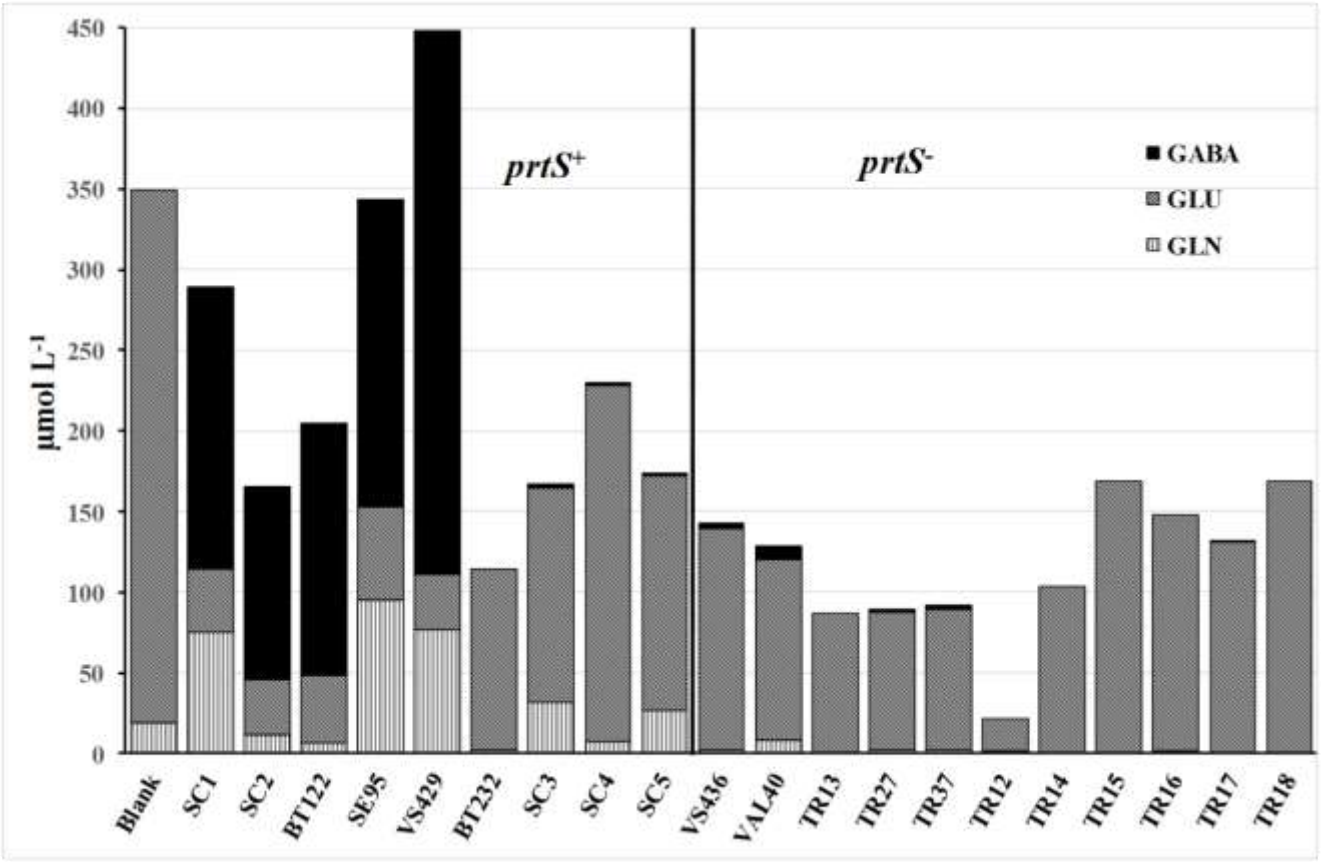

450
400
350
300
250
200
150
100
50
0
µmol L-1
prtS+
prtS-
GABA
GLU
GLN
Blank
SC1
SC2
BT122
SE95
VS429
BT232
SC3
SC4
SC5
VS436
VAL40
TR13
TR27
TR37
TR12
TR14
TR15
TR16
TR17
TR18

Fig. 4

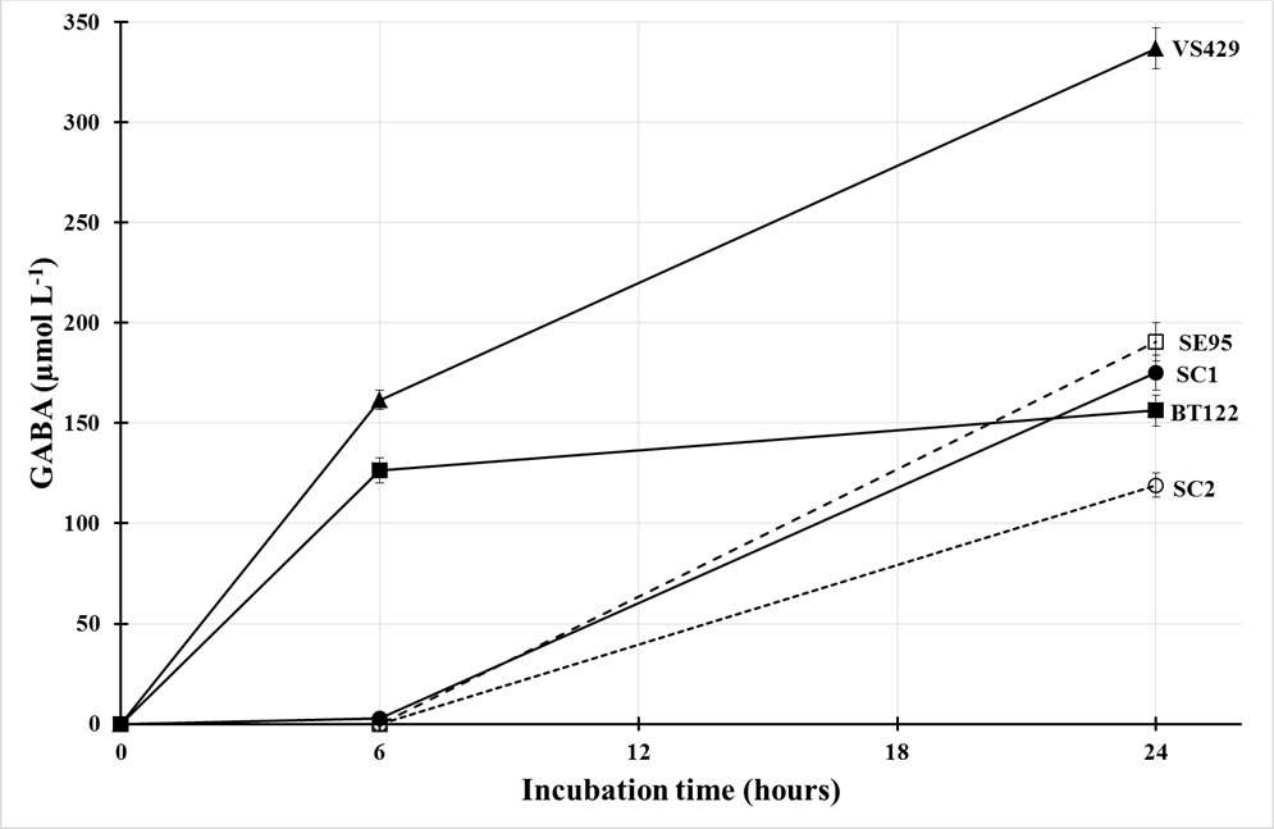

350
300
250
200
150
100
50
0
GABA (µmol L-1)
0
6
12
18
24
Incubation time (hours)
VS429
SE95
SC1
BT122
SC2

Fig. 5

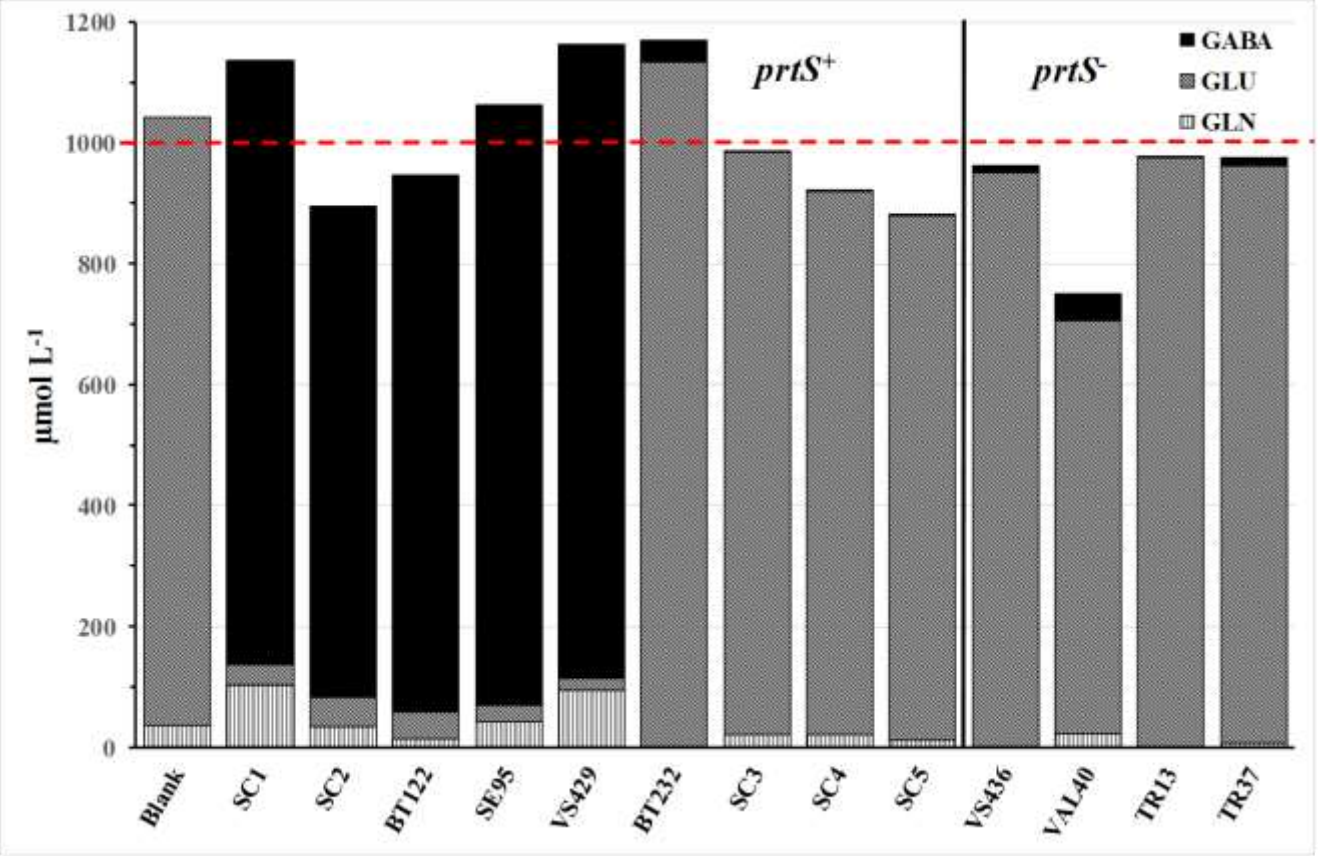
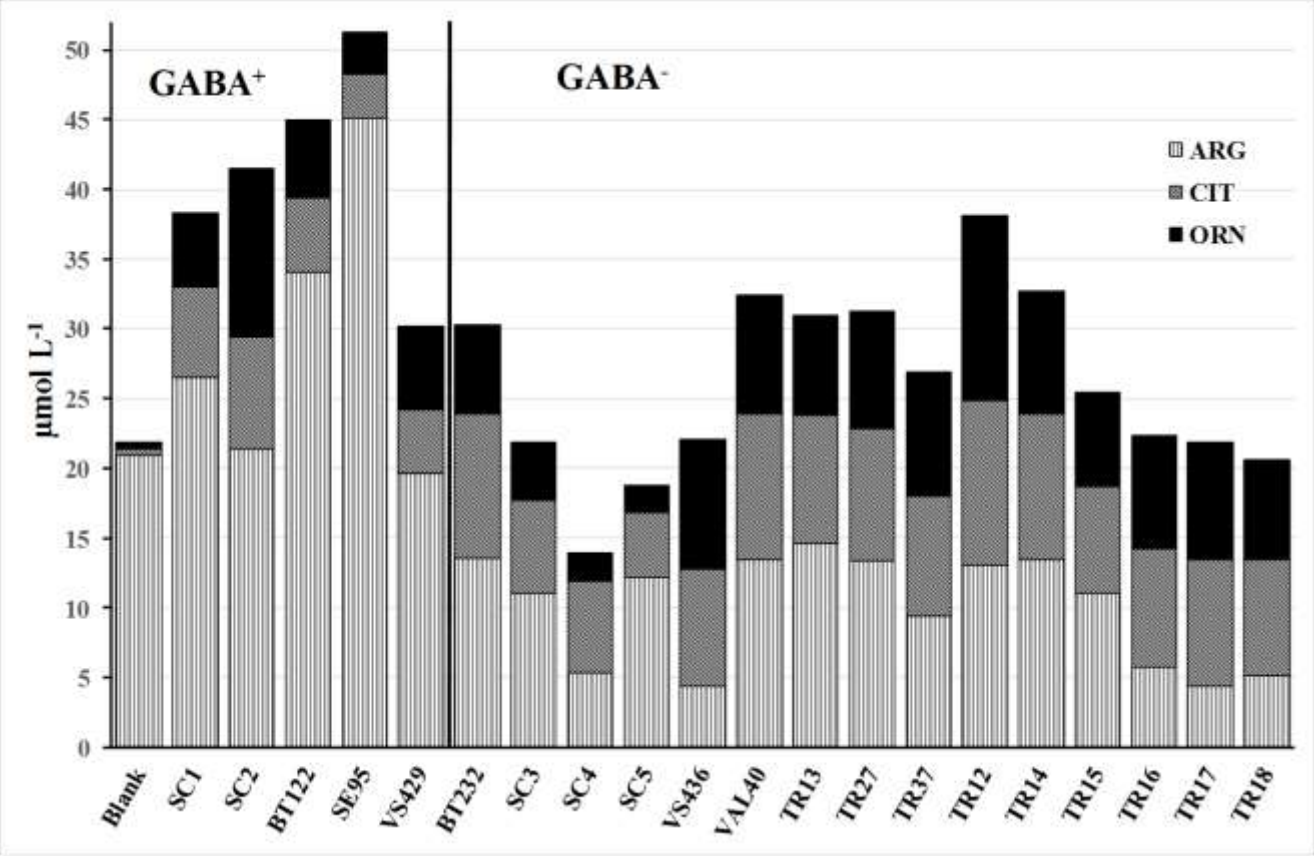
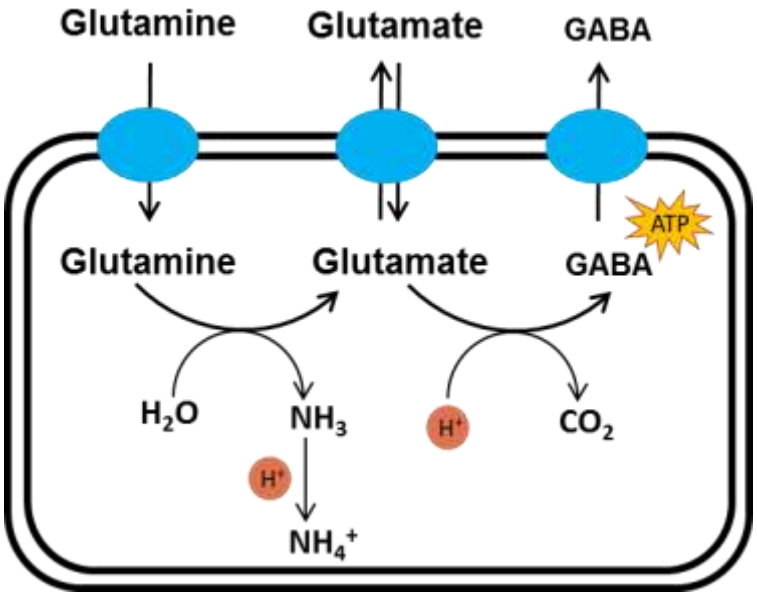
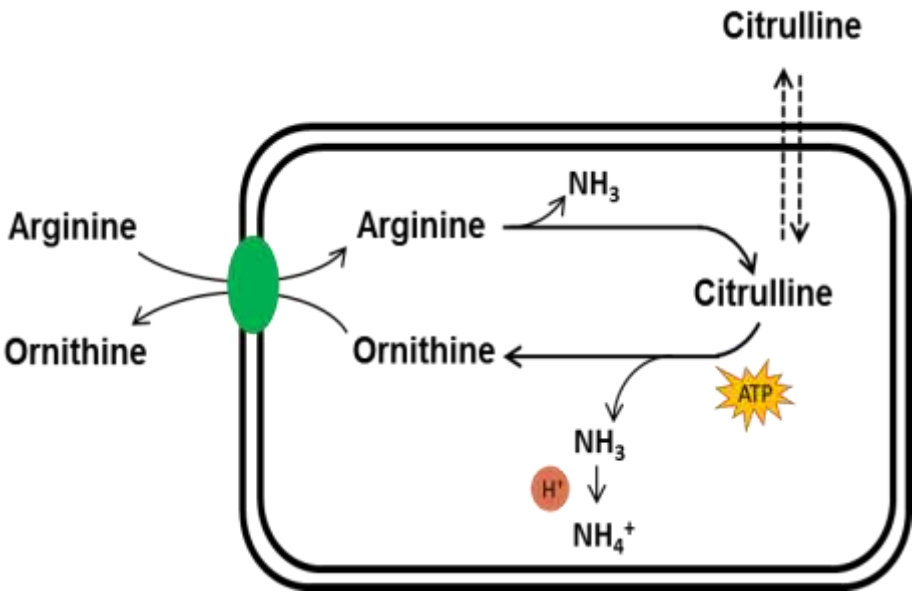

1200
1000
800
600
400
200
0
µmol L-1
prtS+
prtS-
GABA
GLU
GLN
Blank
SC1
SC2
BT122
SE95
VS429
BT232
SC3
SC4
SC5
VS436
VAL40
TR13
TR37

Fig. 6


GABA+
GABA-
ARG
CIT
ORN
µmol L-1
0
5
10
15
20
25
30
35
40
45
50
Blank
SC1
SC2
BT122
SE95
VS429
BT232
SC3
SC4
SC5
VS436
VAL40
TR13
TR27
TR37
TR12
TR14
TR15
TR16
TR17
TR18

TOC


Glutamine
Glutamate
GABA
ATP
Glutamine
Glutamate
GABA
H_2O
NH_3
H^+
CO_2
H^+
NH_4^+


Citrulline
Arginine
Arginine
NH_3
Citrulline
Ornithine
Ornithine
ATP
NH_3
H^+
NH_4^+