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# IMPACT OF ENGINEERED *STREPTOCOCCUS THERMOPHILUS* TRAINS OVEREXPRESSING *GLY*A GENE ON FOLIC ACID AND ACETALDEHYDE PRODUCTION IN FERMENTED MILK

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## ABSTRACT

The typical yogurt flavor is caused by acetaldehyde produced through many different pathways by the yogurt starter bacteria *L. bulgaricus* and *S. thermophilus.* The attention was focused on one specific reaction for acetaldehyde and folic acid formation catalyzed by serine hydroxymethyltransferase (SHMT), encoded by the *glyA* gene. In *S. thermophilus*, this enzyme SHMT also plays the typical role of the enzyme threonine aldolase (TA) that is the interconvertion of threonine into glycine and acetaldehyde. The behavior of engineered *S. thermophilus* strains in milk fermentation is described, folic acid and acetaldehyde production were measured and pH and counts were followed. The engineered *S. thermophilus* strains StA2305 and StB2305, have the *gly*A gene (encoding the enzyme serine hydroxymethyltransferase) overexpressed. These engineered strains showed normal growth in milk when it was supplemented with Casitione. When they were used in milk fermentation it was observed an increase in folic acid and in acetaldehyde production by StA2305 and for StB2305 it was noticed a significative increase in folic acid formation.

**Key words:** acetaldehyde; *Streptococcus thermophilus; glyA* gene; serine hydroxymethyltransferase and folic acid.

## INTRODUCTION

Yoghurt is a product obtained through milk fermentation by a specific yoghurt starter culture consisting of a mixture of two species of lactic acid bacteria (LAB), *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*). It is interesting to observe that in fermented milk there is a higher concentration of folic acid (up to 200µg/L) in than in milk (from 20 to 60µg/L). As well some others LAB, *S. thermophilus* is known to produce folic acid during growth in milk, the extent is strain dependent and the final concentration also depend on the *L. bulgaricus* strain used, once they consume this vitamin (10).

The typical yoghurt flavour is caused by lactic acid, which imparts an acidic and refreshing taste, and a mixture of various carbonyl compounds like acetone, diacetyl, and acetaldehyde of which the latter is considered the major flavour component (6). In the yogurt bacterium *S. thermophilus,* the only enzyme with threonine aldolase activity (interconvertion of threonine into acetaldehyde and glycine) seems to be the serine hydroxymethyltransferase (SHMT, EC.2.1.2.1.) (1). This is an important enzyme involved in the metabolism of not only glycine

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and serine, but of folate in all organisms (5). Overexpression of the *glyA* gene (encoding for SHMT), showed an increase in TA activity and in acetaldehyde and folic acid formation when the engineered strains were grown on M17 medium (1).

The aim of this work was to investigate the role and the importance of SHMT in folic acid and acetaldehyde formation by using the engineered *S. thermophilus* strains for milk fermentation. These mutants could be interesting for the dairy Industry application due to its fuctional propriety (Higher folic acid content).

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

The *S. thermophilus* strains used in this study are listed in Table 1. The strains were grown over-day at 42ºC in M17 medium (Oxoid) supplemented with 1% lactose at 42ºC and used to inoculate (1%) sterile skim milk and incubated at 42ºC overnight. Growth media (milk and M17) for the mutants were supplied with 5mgmL$^{-1}$ of chloramphenicol.

**Table 1**. The *Streptococcus thermophilus* strains used in this study.

| Strain | Characteristics | Reference | Medium supply |
|---|---|---|---|
| **A054** | Wild type | Mercenier *et al*., 1988 | - |
| **StA2305** | A054 harbouring pNZ2305* | Chaves *et al*., 2002 | 5 μg mL$^{-1}$ of Cm |
| **NIZOB130** | Wild type | Yoghurt starter | - |
| **StB2305** | B130 harbouring pNZ2305* | Chaves *et al*., 2002 | 5 μg mL$^{-1}$ of Cm |

*pNZ2305 plasmid: Cm$^r$ 5.5kb pNZ276 derived, carrying a 1250-bp *gly*A gene from NIZOB130 strain.

### Milk fermentation

Fermentation for each strain was carried out in duplicate in two independent replicated experiments; samples were taken at intervals of 2h (0h to 12h) and after 24h. The decrease of pH during this incubation period was continuously monitored (every 2 min) using two systems: Microprocessor pH/ION and Microbe System 6.05 (NIZO food research). Samples were taken for microbial counts (serial dilutions and deep-plated on M17-agar supplemented with 1% lactose). Plates were incubated at 42ºC/48h and results were expressed as log of colony-forming units. The acetaldehyde (kit according to the instructions of the suppliers Boehringer) and folic acid content were measured in triplicate after 24h. The folic acid produced during fermentation was measured using a microbiological assay (2,4,7,8). The samples were submitted to a conjugase treatment that allows the folic acid produced inside of the cells to go outside due to the beak down of its tail.

## RESULTS AND DISCUSSION

Milk was inoculated with all four strains (Table 1) and incubated at 42ºC. Due to the poor growth of the mutants in milk observed (1A and 1C), milk was supplemented with 0.2% casitone (Difco) in order to increase the available source of nitrogen and therefore improve growth (1B and 1D). It is possible to observe in Figs. 1A and 1C, that StA2305 and StB2305 grew less (one log difference) than their respective parental strains and they also produced less lactic acid. When these four strains were grown on M17 medium, the acidification and growth rate of all of them were nearly identical (1). This result showed that the engineered strains were not able to grow and acidify milk in the same level than the parental strains but they can grow easily in a rich medium like M17, Probably, because in milk they had problems to break down the proteins in order to get the aminoacids they need to grow.

**Table 2**. Folic acid production after 24h of milk fermentation by four strains of *S. thermophilus* A054 and NIZOB130 (wild types), StA2305 and StB2305 (engineered strains), in presence and absence of casitone (CN), and with and without a conjugase enzyme treatment.

| | | Mean ± SD | | | |
|---|---|---|---|---|---|
| | | TOTAL | | SUPERNATANT | |
| | Strain | No-conjugated | Conjugated | No-conjugated | Conjugated |
| **Milk** | **A504** | 22.72±1.57 | 22.74±2.34 | 2.30±0.12 | 2.60±0.40 |
| | **StA2305** | 49.42±6.08 | 45.36±4.13 | 5.07±0.63 | 5.27±0.25 |
| **Milk** | **NIZO B130** | 37.56±3.51 | 42.11±2.62 | 2.27±0.67 | 2.64±1.37 |
| | **StB2305** | 43.74±11.64 | 50.38±6.43 | 7.10±1.43 | 6.73±2.74 |
| **Milk +2% CN** | **A504** | 18.23±1.38 | 26.49±2.06 | 1.53±0.31 | 1.80±0.47 |
| | **StA2305** | 28.72±1.83 | 41.84±2.79 | 2.06±0.65 | 2.99±1.11 |
| **Milk +2% CN** | **NIZO B130** | 20.39±1.99 | 27.36±2.81 | 0.85±0.13 | 0.76±0.05 |
| | **StB2305** | 28.79±5.09 | 46.39±3.07 | 2.66±0.72 | 3.64±1.01 |

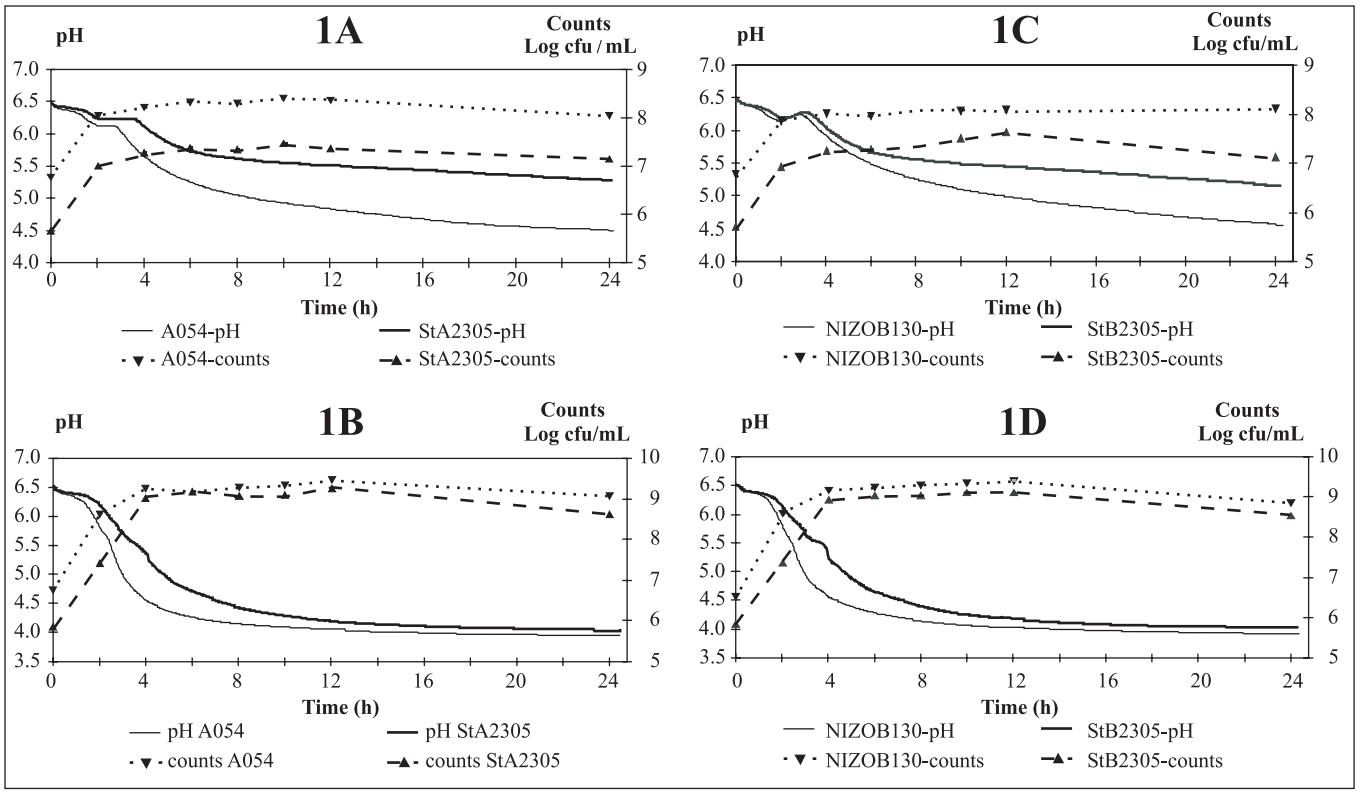


**Figure 1.** Mean values of pH and counts (log cfu ml$^{-1}$) of the *S. thermophilus* strains: A054 and NIZO B130 (wild types) and engineered StA2305 and StB2305 (engineered strains) during 24h at 42ºC in milk fermentation. In 1A and 1Cit is without supplementation of 0.2% casitone (CN) and in 1B and 1D milk was supplemented with 0.2% casitone.

Milk fermented with StA2305 produced 22% more acetaldehyde them the wild type and for StB2305 and NIZOB130 there was no difference observed (Fig. 2). When they grow on M17, is observed an increase in acetaldehyde production on both *glyA*-overexpressing strains (1). The behaviour of StB2305 could be due to the poor growth observed. When the milk was supplemented by casitone there was a significative increase (around tree time) in acetaldehyde formation for all four strains, probably because of the improvement in the growth rate.

All the engineered strains showed a higher folic acid (FA) formation compared to their parental strains in all conditions (Table 2). In milk FA production was higher than in milk supplemented with casitone (except for AO54). In the supernatant there was a higher increase in FA content than in total folic acid in all samples.

Taken all results together it is possible to conclude that in the engineered *S. thermophilus* strains grew at the same rate and reached the same final pH as the wild type as long as the milk was supplemented with casitone. And StA2305 produced more acetaldehyde and folic acid when compared to its parental strain.

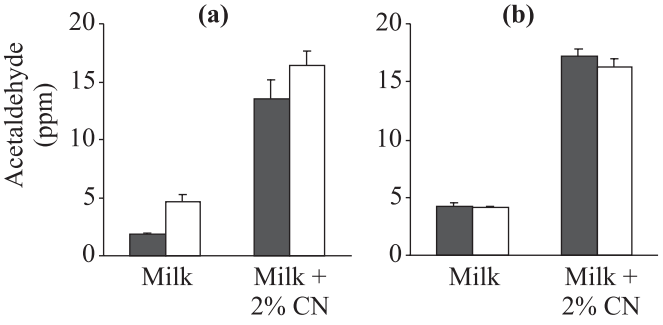


**Figure 2**. Acetaldehyde production after 24 h of milk fermentation by four strains of *S. thermophilus* in presence and absence of casitone: (a) A054/ StA2305 and (b) NIZOB130/ StB2305 wild-type strains (grey filled bar)/ engineered strains (open bar) strains, respectively.

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## RESUMO


### Impacto de linhagens de *Streptococcus thermophilus* com aumento da expressão do gene *glyA* na produção de ácido folico e acetaldeído em leite fermentado

O acetaldeído, responsável pelo sabor e aroma característicos de iogurte, é produzido por diferentes vias metabólicas pelas bactérias lácticas: *Streptococcus thermophilus* (*S. thermophilus*) e *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*). Neste trabalho, a atenção foi focada especificamente na reação para a formação de acetaldeído e de ácido fólico, catalisada pela enzima serina hidroximetil transferase (SHMT), codificada pelo gene *glyA*. A enzima SHMT catalisa diversas reações e, no caso da bactéria *S. thermophilus*, ela exerce também a atividade característica da enzima treonina aldolase (TA), definida como a interconversão do aminoácido treonina em glicina e acetaldeído. Foram construídas linhagens de *S. thermophilus* (StA2305 e StB2305) com super expressão do gene *gly*A. Estas linhagens modificadas apresentaram crescimento normal quando o leite foi suplementado com hidrolisado de caseína (Casitione). Quando foram usadas para fermentação de leite, observou-se: aumento na produção de ácido fólico e acetaldeído por StA2305 e aumento significativo na formação de ácido fólico por StB2305.


**Palavras-chave:** Acetaldeído; *Streptococcus thermophilus;* serina hidroximetil transferase (SHMT); gene *glyA* e ácido fólico.

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