Food Microbiology 53 (2016) 18–29

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Use of the dynamic gastro-intestinal model TIM to explore the survival of the yogurt bacterium *Streptococcus thermophilus* and the metabolic activities induced in the simulated human gut

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ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form
28 February 2015
Accepted 21 May 2015
Available online 28 May 2015

Keywords:
Streptococcus thermophilus
TIM (TNO gastrointestinal model)
Gastric acid resistance
Survival
RIVET (Recombinase *In Vivo* Expression Technology)

ABSTRACT

Streptococcus thermophilus, a lactic acid bacterium used to produce yogurts and cheeses is more and more considered for its potential probiotic properties. This implies that additional information should be obtained regarding its survival and metabolic activity in the human Gastro-Intestinal Tract (GIT). In this study, we screened 30 *S. thermophilus* strains for urease, small heat shock protein, and amino-acid decarboxylase functions which may play a role in survival in the upper part of the GIT. The survival kinetics of 4 strains was investigated using the TIM, a physiologically relevant *in vitro* dynamic gastric and small intestinal model. The three strains LMD9, PB18O and EBLST20 showed significantly higher survival than CNRZ21 in all digestive compartments of the TIM, which may be related to the presence of urease and heat shock protein functions. When LMD9 bacterial cells were delivered in a fermented milk formula, a significant improvement of survival in the TIM was observed compared to non-fermented milk. With the RIVET (Recombinase *In Vivo* Expression Technology) method applied to the LMD9 strain, a promoter located upstream of *hisS*, responsible for the histidyl-transfer RNA synthesis, was found to be specifically activated in the artificial stomach. The data generated on *S. thermophilus* survival and its adaptation capacities to the digestive tract are essential to establish a list of biomarkers useful for the selection of probiotic strains.

1. Introduction

Streptococcus thermophilus is a bacterium used in the food industry as a starter for the production of cheeses and yogurts. The main role of *S. thermophilus* in dairy fermentation is a rapid milk acidification by the production of large quantities of lactic acid but also participates in the production of secondary fermentation products which contribute to the aromatic and textural properties of the fermented products (Iyer et al., 2010). The very long history of *S. thermophilus* use in the dairy industry led to the GRAS (Generally Recognized As Safe) status assignment by the American Food and Drug Administration and the QPS (Qualified Presumption of Safety) status by the European Food Safety Authority (EFSA). Yogurt, which is the product of milk fermentation by the two lactic acid bacteria *S. thermophilus* and *Lactobacillus delbrueckii* sp. *bulgaricus* has obtained in 2010 the EFSA scientific substantiation of health claim for lactose digestion improvement. The target population is individuals with maldigestion and the yogurt should contain at least 10^8 CFU live starter microorganisms per gram of

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http://dx.doi.org/10.1016/j.fm.2015.05.007
0740-0020/

fermented product (EFSA, 2010). The physiological effect is attributed to the microbial β-galactosidase which degrades milk lactose during the fermentation process but also during transit through the gastro-intestinal tract (GIT) after consumption (Drouault et al., 2002; Mater et al., 2006). *In vitro* and *in vivo* studies testing strains of *S. thermophilus* suggest that they would also possess other health probiotic properties in immuno-modulation, antioxidant and colonization resistance functions (Guarner et al., 2005; Ito et al., 2008; Ogita et al., 2011).

While the internationally endorsed definition of probiotics (FAO/WHO, 2002) involves that microorganisms are alive at administration time, there are still questions on the fact that they should arrive in a viable state when they reach the target tissues. When a probiotic property involves a metabolic activity like production of lactic acid for example, bacterial cells need to stay alive until they reach the target tissue and at the site of probiotic action. However when components of dead or lysed cells are responsible for the health effect, then bacterial cell survival would not be required or desired. It is therefore essential to know the survival ability and the physiological status of the probiotic cells in the site where they are programmed to exert their functions. When probiotics are taken by oral route, bacteria have to deal with a series of adverse physical and biochemical conditions including low stomach pH, emulsifying and antimicrobial effect of bile, digestive enzymes and peristaltic elimination, to cite only a few. Unlike Bifidobacteria and some *Lactobacillus* also used in dairy production, starter yogurt species *Lactobacillus bulgaricus* and *S. thermophilus* which have been selected for industrial applications are usually reported to show low survival capacities in the human upper GIT (Conway et al., 1987; Marteau et al., 1997). However, faecal recovery of viable *S. thermophilus* after yogurt consumption has demonstrated that a substantial number of bacterial cells could survive the human GIT transit. For example, in a study where volunteers consumed daily dose of $10^{11.4}$ of *S. thermophilus* provided in yogurt, viable cells could be recovered from most faecal samples with total viable count ranging from $10^{2.6}$ to $10^{6.5}$ CFU per gram (Mater et al., 2005). Moreover, a metagenomic study aiming at cataloging microbial communities in human faecal samples revealed the presence of *S. thermophilus* DNA in more than 90% of the European individual tested (Qin et al., 2010). Taken together, these data results indicate that *S. thermophilus* has a high human population occurrence and a demonstrated ability to survive the human GIT.

Because stomach is the first harsh environment encountered after *S. thermophilus* oral consumption, it is important to screen strains for functions favoring survival in the acidic gastric environment. Previous studies assessing survival rates of *S. thermophilus* exposed to simulated gastric juices already showed that viable cell counts commonly dropped by more than 2–4 $\log_{10}$ units especially when pH value was 2.5 or below (Conway et al., 1987; Ziarno, 2010). Other studies indicated that *S. thermophilus* could not survive at the stress of one hour incubation at pH 2.0 (Fang et al., 2013; Kim et al., 2006) while incubation at higher values of pH 2.5, 2.8 or 3.0 did not considerably affect cell viability (Fang et al., 2013; Grimoud et al., 2010). Experiments assessing gastric acid tolerance usually incubate bacterial cells in HCl based solutions in static conditions. Such experimental assays do not take into account the sequential stresses that bacteria undergo through GIT and the impact of food matrices containing ingredients that may improve bacteria viability in the digestive environment (Huang and Adams, 2004; Iyer et al., 2010; Bove et al., 2013). A more realistic prediction of resistance to upper GIT conditions can be achieved with the dynamic TIM model (TNO gastrointestinal model) which closely simulates the physico-chemical conditions found in the human stomach and small intestine (Blanquet-Diot et al., 2012; Minekus et al., 1995). The main parameters of human digestion such as body temperature, changing in pH, transit time, sequential supply of digestive enzymes and bile salts as well as passive absorption of nutrients and water are modeled, according to *in vivo* data obtained from studies on human volunteers. The TIM device can be used to monitor and compare the survival rates of probiotic strains with the possibility to test different food matrices like milk, fermented milk or ordinary meals.

For an optimal use of probiotic strains, we also need to better understand the physiological status of the bacteria during their passage through the human GIT. Among available techniques exploring the physiological state of bacterial cells, the RIVET (Recombinase *In Vivo* Expression Technology) enables the identification of genes which are specifically expressed in any complex environment (Bron et al., 2004; Junjua et al., 2014). When tested in the stomach, the RIVET analysis is expected to provide essential information about how cells specifically respond to the acidic challenge in comparison to normal growth conditions observed in laboratory medium. Mechanisms of acid-tolerance response (ATR) developed by Gram positive bacteria include strategies of (1) export $H+$ ions with proton pumps (GAD and F1F0 ATPase), (2) alkalinization of the extracellular environment by production of amines by amino-acid decarboxylase or urease systems, (3) production of general shock proteins or enzymes protecting or repairing proton-damaged proteins, (4) changes in the composition of the cell envelope (Cotter and Hill, 2003).

The first objective of this work was to screen a collection of 30 *S. thermophilus* strains for functions presumably important in gastric acid resistance i.e. urease, heat shock protein, and decarboxylase for the amino-acid tyrosine, lysine, glutamate and arginine substrates. From these results, four strains were selected and their capacities to survive in the different compartments of the digestive system were determined in the simulated human TIM system. Finally a RIVET analysis was performed with the acidic resistant LMD9 strain to identify functions specifically activated when *S. thermophilus* cells reside in the gastric compartment of the TIM model.

2. Materials and methods

2.1. Bacterial strains, growth, transformation and PCR conditions

The *S. thermophilus* strains used in this work are listed in Table 1. They were isolated in our laboratory from either yoghurt or cheese (P. Bracquart, Laboratory URAFPA, Nancy, France), or issued from the CNRZ (Centre National de Recherches Zootechniques, INRA, Jouy-en-Josas, France) or ATCC (American Type Culture Collection, Manassas, VA, USA) collections. Strains were stored at −80 °C in reconstituted skim milk (10%, w/v). STUL5001, the LMD9 derivative strain containing the *loxP-specR-loxP* cassette in the STER_0891 locus (Junjua et al., 2014) was used for the RIVET library construction. *S. thermophilus* strains were grown in anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) at 42 °C in M17 medium (Terzaghi and Sandine, 1975) supplemented with 2% (w/v) lactose (LM17) or in 10% (w/v) milk (powdered semi-skimmed milk, fast dissolution, Régilait). When needed, antibiotics purchased from Sigma (Saint Quentin Fallavier, France) were added at the following concentrations: 300 μg/mL for spectinomycin, 20 μg/mL for streptomycin or 5 μg/mL for erythromycin. *S. thermophilus* competent cells were prepared in Chemically Defined Medium in the optimal conditions as described by Gardan et al. (2009). For long term storage at −80 °C, cells were pelleted by centrifugation, suspended in 1/10 volume of the initial culture supplemented with glycerol (14%) and frozen in liquid nitrogen.

Escherichia coli TOP10 (Invitrogen, Breda, the Netherlands) was used as an intermediate cloning host for the construction of RIVET library. TOP10 cells were grown in aerobic conditions at 37 °C in LB