

***In Vitro* Testing of Lactic Acid Bacteria from Commercial Yoghurts**

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Abstract. Yoghurt has been known as an excellent source for delivering viable lactic acid bacteria (LAB) in concentrations providing benefits to the human host and microbiome. Different gastrointestinal tract (GIT) simulators have been developed and are successfully used to determine the viability of LAB from fermented dairy products *in vitro* studies. Commercial yoghurts were tested for digestibility using the GIT IT system Labfors 5 (INFORS HT, Switzerland). The length for both the gastric phase and the small intestinal phase was 120 min each. Yoghurt LAB colony-forming units were analysed prior to the GIT simulation test, as well as after the simulation test. The survival rate of LAB was evaluated based on the differences in the viable LAB count at the beginning and at the end of the experiment.

Tested yoghurts' LAB were tolerated in the simulated gastric and small intestinal phases. Samples collected prior to and after the intestinal phase revealed that LAB had adapted and started to grow within 120 min. Our results showed the ability of LAB to recover in the intestinal phase which has been explained by the food matrix, which protects the bacteria from the elimination effect of intestinal secretions.

In addition, differences in survival rates of LAB significantly influenced the overall LAB colony-forming units in the gastrointestinal tract. The chemical composition of the product has an influence on the survival rate of LAB, and it should be studied more thoroughly. *In vitro* studies are quite different from the assay *in vivo* studies; however, this information provides significant data about the viability of LAB from regularly consumed products and helps to modulate the influence of LAB on human microbiota.

Key words: organic milk, yoghurt, lactic acid bacteria, gastrointestinal tract simulator, *in vitro*.

Introduction

Many studies have been performed to establish the evidence for the beneficial effect of LAB used for yoghurt production on the human microbiome. However, the composition and ingredients used for the production of fermented dairy products are important tools for the survival of LAB in the human gastrointestinal tract (Dommels *et al.*, 2009). The study on the survival and functionality of LAB may lead to understanding the role of developed fermented products in the daily diet.

Yoghurt consists of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*, with the addition of other *Lactobacillus spp.* and *Bifidobacterium spp.* species (Aryana & Olson, 2017; Nyanzi, Jooste, & Buys, 2021). Tolerance of *Lactobacillus spp.* to low pH, bile salts, and digestive enzymes (proteolytic and lipolytic) at

biological concentrations has been made to be strain-dependent (Sumeri *et al.*, 2012; Nassinger da Silva *et al.*, 2021). Yoghurt has been known as an excellent source for delivering viable LAB in concentrations providing benefits to the human host and microbiome.

To imitate the human digestive system activity, different gastrointestinal tract (GIT) simulators have been developed and are successfully used to evaluate the viability of LAB applied as starter cultures in commercial products, as well as to understand the potential of commercial products microbiota for health benefits and organism well-being. GIT simulators also help to predict the survival of microbiota in human trials and its advantages.

The study focuses on the survival of yoghurt LAB made from organic and conventional milk in the gastrointestinal tract simulator.

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Materials and Methods

Sample characteristics

Different commercial yoghurts were tested in the GIT simulation system, among them ECO yoghurt, which was partly developed and intensively studied *in vitro* and *in vivo* experiments within the project “Scientifically substantiated fermented milk products development and their clinical studies” (No. 19-00-A01612-000009) activities.

The commercial samples were purchased in retail shops from local producers and kept at 2-6 °C in the fridge until the experiment. The samples used for the study are summarised in Table 1.

Gastrointestinal tract simulation experiments

GIT system Labfors 5 (INFORS HT, Switzerland) was used for the *in vitro* study, and the bioprocess was monitored and controlled by software Iris 6 (INFORS HT). The GIT simulation system consists of a single bioreactor, simulating the conditions in the stomach and in the small intestines (duodenum, jejunum, and ileum), and the bioprocess is automatically controlled with the manual addition of fermentation stocks. The modified method of Minekus *et al.* (2014) was used to determine the survival of viable LAB from analysed yoghurt samples under the influence of stomach media, as well as the bile salts in the small intestines. KCl, KH₂PO₄, NaHCO₃, NaCl, MgCl₂·6H₂O, (NH₄)₂CO₃, NaOH, HCl, CaCl₂, bile

salt, porcine pepsin (EC 3.4.2.3.1), porcine trypsin (EC 3.4.21.4), bovine chymotrypsin (EC 3.4.21.1), porcine pancreatic α-amylase (EC 3.2.1.1), porcine pancreatic lipase (EC 3.1.1.3) were purchased from Sigma-Aldrich (local distributor in Riga, Latvia). The concentration of gastric and intestinal stock solutions and enzymes were calculated in accordance with the procedure reported by Minekus *et al.* (2014). Sample preparation, gastric phase and intestinal phase simulation were performed according to the procedure of Žolnere *et al.* (2018). The samples for LAB survival testing were taken from the stomach media after the gastric phase, and from the intestinal phase media at the end of digestion. The length for the gastric phase was 120 min, and also for the intestinal phase – 120 min. Yoghurt LAB colony-forming units were analysed prior to the GIT simulation test. GIT simulation experiments were performed with duplicate determination.

Enumeration of lactic acid bacteria

Lactobacillus bulgaricus, *Streptococcus thermophilus* and *Lactobacillus acidophilus* can be enumerated on MRS agar (Gebara *et al.*, 2015). *Bifidobacterium spp.* was not analysed during the study. LAB were determined by plating on MRS agar (Scharlau, Spain). Media was prepared according to the standard procedure (ISO 11133-2009) and sample dilutions were performed according to

Table 1

Characteristics of commercial yoghurt samples

Parameter	ECO yoghurt (non-standardised milk)	Greek non-fat yoghurt	Lacto
Energy value, kJ/kcal per 100 g	289-327/69-78	245/58	196/47
Fat, g per 100 g,	3.5-4.5	0.05	2.0
including saturates, g	2.4-3.0	0.0	1.0
Carbohydrates, g per 100 g,	4.5	4.2	3.9
including sugars, g	4.5	4.2	3.9
Protein, g per 100 g	4.8	10.0	3.3
Salt, g per 100 g*	0.1	0.1	0.1
Ingredients	Organic milk, milk proteins, starter	Milk, starter	Milk, starter
Information about starter	<i>Lactobacillus delbrueckii. subsp. bulgaricus</i> , <i>Streptococcus thermophilus</i> ***	<i>Streptococcus thermophilus</i> , <i>Lactobacillus delbrueckii. subsp. bulgaricus</i> ***	<i>Lactobacillus acidophilus</i> , <i>Streptococcus thermophilus</i> , <i>Bifidobacterium spp.</i> **

* only naturally occurring

** information given on the label

*** information received from the producer

ISO 6887-5:2010 using a saline solution. Plates were incubated anaerobically (AnaeroGen™, Gas Pak System, Oxoid, UK) at 37 °C for 72 hours in the incubator Memmert IPP200 (Mettler, Germany). The chosen parameters for the cultivation of LAB corresponded to the recommendations of Coeuret *et al.* (2003).

Individually, ECO yoghurt microbiota was tested. *Lactobacillus subsp. bulgaricus* was tested according to the previously described methodology, but *Streptococcus thermophilus* was determined by plating on M17 lactose agar (Becton Dickinson, USA). Plates were incubated at 45 °C for 72 hours aerobically in the incubator Memmert IPP200 (Gebara *et al.*, 2015). *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* were analysed for understanding the effect of *in vitro* simulation on both species.

Calculation of survival rate

The survival rate of LAB was determined based on the differences in the viable LAB count at the beginning and at the end of the experiment. The survival rate was calculated by the equation described by Sumeri *et al.* (2008):

$$\text{SUR (\%)} = e^{D\tau} \cdot (\text{CFU}_i / \text{CFU}_f) \cdot 100,$$

where: CFU_i – the number of colony-forming units at the end of the intestinal phase;
CFU_f – the number of colony-forming units in the yoghurt prior to the experiment;
 τ – the time of dilution phase (4 h);
D – the dilution rate (0.4 h⁻¹).

Data analysis

Mathematical treatment of data was performed using the one-way variance (ANOVA) and Tukey's test. Differences were considered statistically significant at $p < 0.05$. The mean and standard deviation were determined using the Microsoft Excel 2013 programme.

Results and Discussion

In this study, commercially available yoghurts were analysed. The average lactic acid bacteria (LAB) concentration in analysed commercial yoghurt samples is shown in Table 2.

The commercial product analyses showed very high LAB concentrations above 9 log₁₀ CFU g⁻¹, although the generally accepted therapeutic minimum is 10⁷ CFU g⁻¹ for probiotic strains in yoghurt over the shelf-life (Nyanzi, Jooste, & Buys, 2021). Cabinet Regulation No. 97 (2011) regulates fermented milk products including yoghurt to contain at least 10⁷ CFU g⁻¹ of LAB in a product (Ministru kabineta noteikumi No. 97, 2011), which corresponds to analysed products.

All LAB used for fermented product production were tolerated in the simulated gastric phase and small intestinal phase (Table 2). Tested bacteria of the yoghurts showed different levels of viability for up to 120 min in the simulated stomach media. Results revealed a moderate log cycle reduction (only 2 log cycles) in viability for the ECO yoghurt sample, but a major reduction (up to 4 log cycles) for Lacto and Greek non-fat yoghurt samples. Reductions were statistically significant ($p < 0.05$). The results could be explained by the findings of Veruck *et al.* (2017) that fat globules contribute to the survival of probiotics in the gastrointestinal tract protecting viable cells against low pH in the stomach. ECO yoghurt fat content is twice as high as in Lacto and at least four times higher than in Greek non-fat yoghurt. pH is not the only factor limiting the growth of LAB; the digestion time is also a decisive factor for the viability of cells (Nassinger da Silva *et al.*, 2021). Slower digestion in the stomach phase leads to intensive exposure to LAB in Greek non-fat yoghurt samples due to a very high protein content. This finding could also explain the significantly lower bacteria recovery data in the intestinal phase.

LAB of the tested yoghurts were tolerated in the simulated intestinal phase for 120 min. Samples

Table 2

LAB in yoghurt samples and its survival during GIT

Parameters	ECO yoghurt	Greek non-fat yoghurt	Lacto
Lactic acid bacteria in product, log ₁₀ CFU g ⁻¹	9.34±8.82 ^a	9.30±8.80 ^a	9.41±8.29 ^a
Lactic acid bacteria, log ₁₀ CFU g ⁻¹ :			
✓ after the stomach phase	7.10±6.50 ^a	5.42±4.82 ^b	5.24±4.88 ^b
✓ after the intestinal phase	7.47±7.40 ^a	5.70±4.64 ^b	7.00±6.23 ^a
Survival rate, %	6.62 ^a	0.12 ^b	1.93 ^c

^{a,b,c} means in lines followed by different letters do not differ at $p > 0.05$

Table 3

***Str.thermophilus* and *L.bulgaricus* in ECO yoghurt and their survival during GIT**

Parameters	ECO yoghurt	
	<i>Streptococcus thermophilus</i>	<i>Lactobacillus delbrueckii subsp. bulgaricus</i>
Lactic acid bacteria in product, log ₁₀ CFU g ⁻¹	8.54±7.51 ^a	9.13±8.10 ^a
Lactic acid bacteria, log ₁₀ CFU g ⁻¹ :		
✓ after the stomach phase	5.14±5.07 ^a	7.09±6.40 ^b
✓ after the intestinal phase	6.96±5.93 ^a	7.24±6.36 ^b
Survival rate, %	1.28 ^a	6.38 ^b

^{a,b} means in lines followed by the different letters do not differ at $p > 0.05$

collected prior to and after the intestinal phase revealed that LAB had adapted and started to grow within 120 min. Analysing the viability of probiotic strains, Nassinger da Silva *et al.* (2021) found a decrease between 1 log₁₀ CFU g⁻¹ to approximately 4 log₁₀ CFU g⁻¹ during the passage through the gastrointestinal tract. These data are in line with our study; however, when evaluating the efficiency of probiotics in a simulated gastric environment, Shindea *et al.* (2019) found a decrease in viability at the end of the gastric phase, with the following drop in the probiotic count of 1.03 log₁₀ CFU ml⁻¹ at the end of the intestinal phase.

Our results showed the ability of LAB to recover in the intestinal phase that has been explained by the food matrix, which protects the bacteria from the elimination effect of intestinal secretions.

The average survival rate of LAB was higher for the ECO yoghurt sample when compared with Lacto and Greek non-fat yoghurt samples. This can be explained by several factors. Different starters were used in yoghurt production, and the yoghurt composition, especially protein and fat concentration, as well as the origin of the milk (organic and conventional) differ in samples. However, the differences in initial amounts were not established, the survival rate of LAB differs significantly, and it influences the overall LAB survival in the simulated digestion environment.

Streptococcus thermophilus and *Lactobacillus subsp. delbrueckii bulgaricus*, which are used for ECO yoghurt production, should be capable of surviving passage through the GIT, see Table 3.

According to Nassinger da Silva *et al.* (2021), an amount considered to be adequate for colonisation in the intestines is 7 log₁₀ CFU ml⁻¹, which is in line with our study. This study demonstrated that different survival rates of LAB in ECO yoghurt (Table 2 and Table 3) were linked to organic milk composition. Florence *et al.* (2014) reported that higher survival of probiotics in organic milk is achieved when the

high proportion of unsaturated fatty acids, high trans-vaccenic acid, α -linolenic acid, and low linoleic acid/ α -linolenic acid ratio are established. Our previous studies confirmed (Zagorska & Ciprovica, 2008) that the higher lactoferrin concentration in organic milk and lactoferrin has been reported (Franco *et al.*, 2010) to promote the growth and development of probiotics. With these findings, we could explain the higher survival rate of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* from the analysed ECO yoghurt sample in GIT as well as the results shown in Table 2. Although the species used for ECO yoghurt and Geek non-fat yoghurt production were similar, the origin of milk and the composition of the product explain the differences found in the study.

In vitro studies are quite different from the assay for *in vivo* studies; however, this information provides significant data about the viability of LAB from the regularly consumed products and helps to modulate the influence of LAB on human microbiota.

Conclusions

GIT simulator can be used for comparative survival studies of LAB in yoghurt and for the evaluation of tolerable lactic acid bacteria in yoghurt production.

The LAB displayed a good capability to attach and colonise in the intestine. There were significant differences in the survival rate of analysed commercial yoghurt LAB.

The chemical composition of the product and milk origin have an influence on the survival rate of LAB, and it should be studied more thoroughly.

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