

Study of the influence of *Lacticaseibacillus paracasei* AS-10 on the development of a traditional starter for Bulgarian yoghurt

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Abstract: A study was conducted to investigate the effect of lactic acid bacteria development involved in starter cultures for the production of lactic acid products. On the basis of the data obtained, concerning its influence on a traditional starter for Bulgarian yoghurt, it is possible to interpret its development in a symbiotic culture, its influence on the organoleptic profile of yoghurt and the rate of development of the lactic acid process. To achieve the set objective, cultivation of the selected lactic acid strains was carried out. The data on the rate of development in the different phases, represented by the acid formation curve for a symbiotic traditional culture for Bulgarian yoghurt containing *Lactobacillus delbrueckii ssp. bulgaricus* u *Streptococcus thermophilus*, with *Lacticaseibacillus paracasei* AS-10 added to them, were reported. The conclusions drawn allow the correct and predictable use of the investigated lactic acid bacteria strain in starter cultures for the production of lactic acid products.

1 Introduction

Yoghurt has turned into a popular fermented dairy product. Yoghurt is a dairy product resulting from the fermentation of milk by a group of bacteria known as lactic acid bacteria. They convert milk sugar (lactose) into lactic acid, giving yoghurt its texture and characteristic taste [1]. In addition, yoghurt has long been recognised as a health-promoting product. It is an easily digestible food and can improve the human digestive system by improving gastrointestinal health with the help of beneficial probiotic bacteria [2, 3]. The basic pattern of acid production is controlled by the yoghurt starter [4]. It is known that *Lactobacillus delbrueckii ssp. bulgaricus* u *Streptococcus thermophilus* ca the dominant species in its production [5]. It has been found that all yoghurt products of high quality are characterized by smooth texture, appropriate viscosity, good taste and low level of acidification after fermentation. Among them, the low acidity of post-fermentation is considered to be an important factor in the selection of yoghurt starter [6, 7, 8, 9].

Lacticaseibacillus belongs to the largest group of lactic acid bacteria and includes various facultatively anaerobic, Gram-positive rod-shaped bacterial species. Apart from the genus *Bifidobacterium*, *Lacticaseibacillus* (known by its old name *Lactobacillus*) is the most widely used genus in the production of probiotic foods, especially in fermented dairy products (probiotic milk, yoghurt, bio-sour milk, kefir, cheese). Recently, different strains of *Lacticaseibacillus* have been developed and applied

for fermentation of soy milk, fruits and vegetables, vegetable juices, meat, bread [10, 11].

Lacticaseibacillus paracasei is a member of the potentially probiotic bacteria associated with the *L. casei* group (*L. casei* and *L. rhamnosus*) [12]. *L. paracasei* is a gram-positive bacterium commonly used in probiotics and in the fermentation of dairy products and has been found in the human intestinal tract and mouth [13, 14]. *L. paracasei* is known to have a preventive effect on obesity and cancer development [15, 16, 17]. Scientists have found that representatives of *L. paracasei* are present in the mammalian gut [18, 19] and are identified from many fermented foods [20, 21]. They are widely used in starter cultures for dairy products, often to improve the taste and texture of products, and for their health-related benefits [12, 13]. Scientists have isolated potential probiotic *L. paracasei* cells from kefir grains and applied them in the production of feta cheese [22].

A number of scientists have studied *Lactobacillus bulgaricus* over the years. It has been found to belong to the group of homofermentative lactobacilli that form lactic acid during lactic acid fermentation of lactose [23]. A number of studies have shown that strains of *Lb. bulgaricus* produce the proteolytic enzymes in yoghurt production [24, 25, 26, 27, 28, 29, 30]. The proteolytic properties of *Lb. bulgaricus* are the basis for strain selection to create symbiotic starters for dairy production.

Streptococcus thermophilus is one of the most widely used lactic acid bacteria in dairy products such as yogurt, other fermented products, cheese, and cheese [31]. It has been shown that a huge amount of microorganisms from this strain are ingested by humans on a daily basis [32]. In

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this regard, *Str. thermophilus* is generally recognized as a safe strain by international food safety agencies. The main role of *Str. thermophilus* in the lactic acid process is to provide acidification, fermentation of milk and lactic acid production.

To study fermentation processes in depth, it is necessary to know the kinetics of the process. The growth kinetics of the microbial population, is a key function to determine the rate of development. Its study in lactic acid organisms is performed during or after the fermentation processes of the given bacteria. The main method to determine the kinetics during cultivation is mathematical modelling. Basically, different function systems are used to give a clear picture of the observed dependencies in a system [33]. Structural and non-structural models are mainly used to model the kinetics [34]. Structural models consider the underlying cellular structures, their composition and function and are characterized by an accurate description of fermentation processes, but are also temporarily very complex and require specialized computational software and a lengthy computational process.

In non-structural models, only the total concentration of cells or product is considered. These models do not include cell physiology, but they also accurately describe the lactic acid fermentation process [33, 34].

According to the current views of a mathematical model in determining the kinetics of a process, there are the following requirements for the microorganism under study: 1. To be at population level; 2. To contain a minimum number of experimentally determined constants and parameters, which should be biologically meaningful; 3. To reflect all important processes of a given fermentation and their relationships; 4. Be capable of defining a pathway to improve, regulate, control and scale up the process [33].

Since the main observable is the variation of the biomass concentration of cultured lactic acid bacteria then logistic models have been used to model the kinetics, which contain a clear biological meaning [33, 34].

Despite the research carried out to date, knowledge of *Lacticaseibacillus paracasei* on its development, growth rate and potential products that can be produced using it remains insufficient. In view of the need to know its growth kinetics and its impact on traditional starter cultures for the production of lactic acid products, the present study was conducted. Appropriate mathematical equations and models have been selected for proper determination of the growth rates of microorganisms.

2 Materials and methods

2.1 Microorganisms

In the present work lactic acid bacteria cultured in static medium were used: *Lacticaseibacillus paracasei*

AS-10, cultured as a monoculture and in combination with a traditional starter for Bulgarian yoghurt containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, and the same traditional starter without the presence of *Lacticaseibacillus paracasei* AS-10. The cultivation temperature is 37°C.

2.2 Food environments

2.2.1 MRS (deMan, Rogosa, Sharpe)-agar (Biokar-France)

Ingredients (g/dm³): casein peptone- 10; yeast extract- 4; meat extract- 10; glucose- 20; K HPO₂₄ - 2; sodium acetate- 5; diammonium citrate- 2; MgSO₄ - 0.2; MnSO₄ - 0.04; Tween 80 - 1 cm/dm³; pH adjusted to 5.7. Sterilization - 15 min on 121°C.

2.2.2 M17-agar (Biochar)

Composition- (g/dm³): tryptone – 2.50; meat peptone – 2.50; soybean meal peptone – 5.00; yeast extract – 2.50; meat extract – 5.00; lactose – 5.00; sodium glycerol phosphate – 19.00; MgSO₄ – 0.25; ascorbic acid – 0.50; agar-agar - 15. pH of the medium 7.1 ± 0.2. Sterilization for 15 min at 121°C.

2.2.3 Fermentation medium

10 % reconstituted dry milk sterilised at 121°C for 15 min.

2.3 Determination of the number of active viable cells

The number of viable cells was determined by the 10-fold dilution method according to BDS ISO 7889:2005.

2.4 Determination of titratable acidity

The titratable acidity was determined according to the requirements of BS 1111:1980.

2.5 Cultivation of the strains tested

Culturing of the strains was carried out in a bioreactor with mechanical agitation and a working volume of 1.5 dm³ in 10% sterile reconstituted milk at 42 ± 1 and 37 ± 1°C.

2.6 Modelling growth kinetics

The Gompertz model (1) and a system of Gaussian logistic equations (equation (2) to (6)) were used to model growth and reproduction kinetics [35, 36, 37]. The system of logistic models was solved numerically using the 4th order Runge-Kutta method, and the Solver function in Excel was used to identify the parameters in the models by minimizing the square of the difference between the experimental data and those obtained from the corresponding model [38]. While the parametric identification In the Gompertz model was carried out in

the Curve Expert Professional software using nonlinear regression.

$$\ln N/N_0 = A \cdot \exp \left[-\exp \left(\frac{\mu \cdot \exp(1)}{A} \right) (\lambda - \tau) + 1 \right]$$

$$A = \ln \left(\frac{N_m}{N_0} \right)$$
(1)

$$\frac{dX_{L.b}}{d\tau} = \mu_m \left(1 - \frac{X_{L.b} + \delta X_{L.p}}{X_{mL.b}} \right) X_{L.b}$$
(2)

$$\frac{dX_{S.t}}{d\tau} = \mu_m \left(1 - \frac{X_{S.t} + \alpha X_{L.p}}{X_{mS.t}} \right) X_{S.t}$$
(3)

$$\frac{dX_{L.p}}{d\tau} = \mu_m \left(1 - \frac{X_{L.p} + \varepsilon X_{L.b}}{X_{mL.p}} \right) X_{L.p}$$
(4)

$$\frac{dX_{L.p}}{d\tau} = \mu_m \left(1 - \frac{X_{L.p} + \gamma X_{S.t}}{X_{mL.p}} \right) X_{L.p}$$
(5)

$$\frac{dX}{d\tau} = \mu_m X - \beta X^2 = \mu_m X - \frac{\mu_m}{X_{kp}} X^2$$

$$= \mu_m \left(1 - \frac{X}{X_{kp}} \right) X$$
(6)

where: μ_m is the maximum specific growth rate, h^{-1} ; μ is the specific growth rate, h^{-1} ; N , N and N_0 are the current, initial and maximum concentration of active cells cfu/cm^3 ; λ is the lag phase duration; A is the asymptote of the curve expressing the maximum biomass increase in dimensionless form; $X_{L.b}$, $X_{S.t}$ and $X_{L.p}$ are the current concentration of active cells per *L. bulgaricus*, *S. thermophilus* and *L. paracasei* AS-10 cells cfu/cm^3 ; $X_{mL.b}$, $X_{mS.t}$ and $X_{mL.p}$ are the final concentration of *L. bulgaricus*, *S. thermophilus* and *L. paracasei* AS-10, cfu/cm^3 ; X and X_{kp} are current and final biomass concentration, cfu/cm^3 ; β , α , δ , γ , ε -coefficients of intra-population competition, $cfu/cm^3 \cdot h$; τ -time, h

3. Results and discussion

Knowledge of the relationships among the microorganisms in a symbiotic starter culture is essential in establishing a symbiotic starter culture. For this reason, the mutual influence of *Lacticaseibacillus paracasei* AS-10 and typical microflora (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) starter culture intended for the production of traditional Bulgarian yoghurt was investigated. For this purpose, the growth of viable cells of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the control and the three-strain combination as well as of *Lacticaseibacillus paracasei* AS-10 was monitored. The results of these studies are presented in Fig. 1 and Fig. 2. and the protector starter culture and also the alteration of the active cells to the influence of *In* order to create a symbiotic protector starter, the influence of strain *Lacticaseibacillus paracasei* AS-10 on the developmental temperature for the respective strain was investigated. The dynamics of biomass change during the fermentation process up to the point of milk coagulation was investigated. The results of these studies are presented in Fig. 1 and Fig. 2.

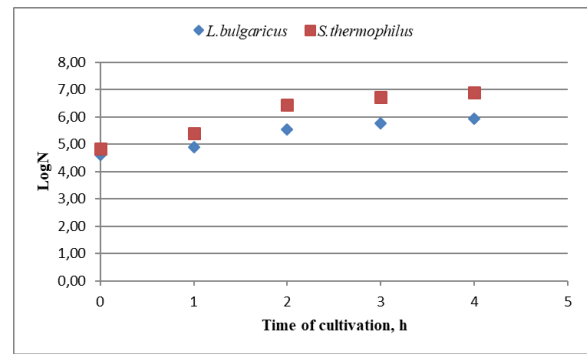


Fig. 1. Dynamics of accumulation of active cells of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the control starter culture

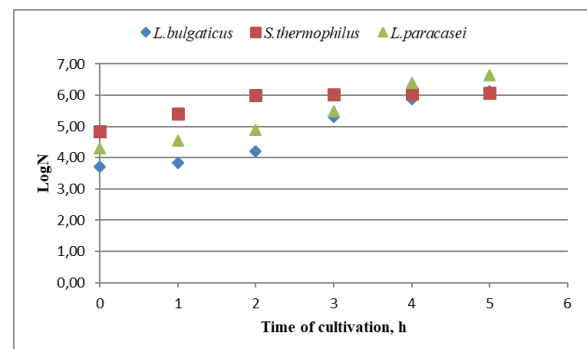


Fig. 2. Dynamics of accumulation of active cells of *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lacticaseibacillus paracasei* AS-10 in the three-strain combination

From the data presented in Fig. 1 and Fig. 2, it can be seen that similar concentrations of active *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cells were

found in the control and the three-strain starter culture at the time of milk coagulation. The concentration of viable cells of *Streptococcus thermophilus* was 6.91 and 6.09 LogN, respectively, in the control and tristate combination, and that of *Lactobacillus bulgaricus* was 5.93 and 6.11 LogN. A commensurate concentration of viable cells was also obtained for *Lacticaseibacillus paracasei* AS-10 in the three-strain starter culture, 6.65 LogN. Mathematical modelling of the growth and reproduction kinetics of the strains in the controls and the three-strain combination was performed with the Gompertz model. The results of these studies were compared with the experimental data and are presented in Fig. 3, Fig. 4 and Fig. 5, and the model parameters are reported in Table 1.

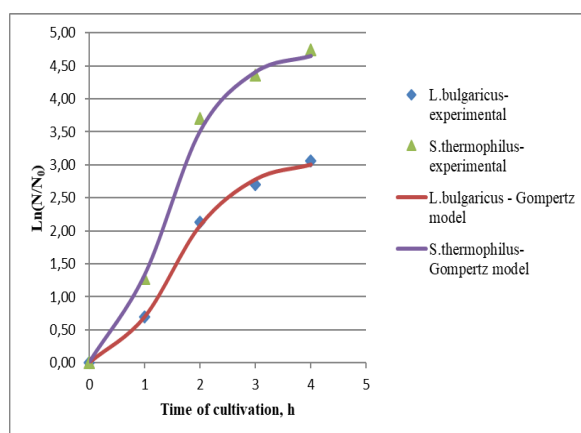


Fig. 3. Comparison of experimental data with those from the Gompertz model for *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the control starter culture

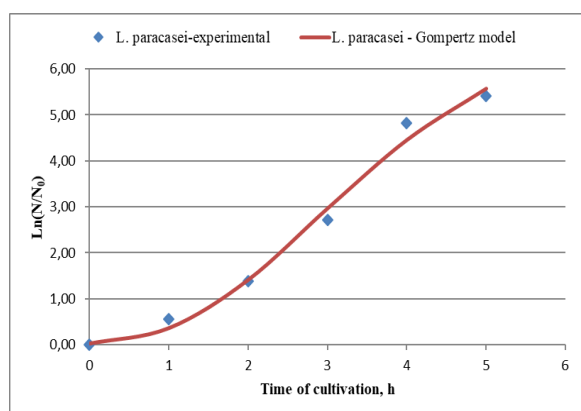


Fig. 4. Comparison of experimental data with those from the Gompertz model for the *Lacticaseibacillus paracasei* AS-10 control

From the data presented in the figures, it is evident that the model agrees very well with the experimental results, which is confirmed by the high correlation coefficients that vary from 0.9893 to 0.9990. This indicates that the model can be used to analyze fermentation processes.

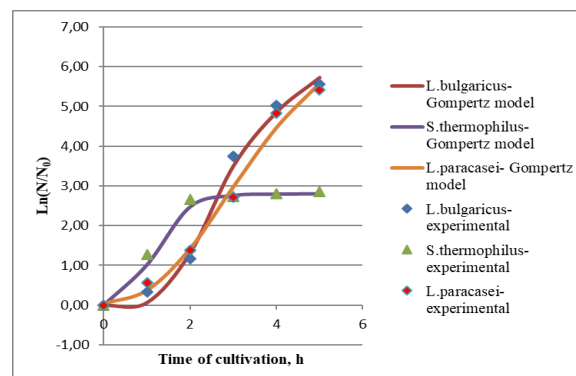


Fig. 5. Comparison of experimental data with those from the Gompertz model for *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lacticaseibacillus paracasei* AS-10 in the three-strain combination

The data presented in Table 1 indicate that *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* in the control starter culture were characterized by low lag phase durations of 0.54 and 0.47 h and high specific growth rates of 1.51 and 2.52 h⁻¹ (Table 1). In the three-strain starter culture, there was a slight decrease in the specific growth rate for *Streptococcus thermophilus*, but retained a high value of 2.17 h⁻¹. The lag phase time for this strain also increased marginally at 0.53 h. However, for *Lactobacillus bulgaricus*, there was an increase in the specific growth rate in the three-strain combination (2.30 h⁻¹) compared to the control starter. This can be explained by the high proteolytic activity of *Lacticaseibacillus paracasei* AS-10, which is associated with a deeper casein degradation and supply of the necessary amino acids and peptides for the growth of *Lactobacillus bulgaricus*. The theoretical lag phase duration for *Lactobacillus bulgaricus* in the three-strain combination was longer (1.43 h) compared to the control starter. However, the free amino acids and peptides formed as a result of the proteolytic activity of *Lacticaseibacillus paracasei* AS-10 led to a growth stimulation of *Lactobacillus bulgaricus* and in the three-strain combination from an initial value of 3.30 LogN was reached to 6.11 LogN for this strain. The increase in the lag phase for both *Streptococcus thermophilus* and *Lactobacillus bulgaricus* was rather due to the smaller amount of these cultures in the combination. From the data presented in Table 1, it is evident that strain *Lacticaseibacillus paracasei* AS-10 was characterized by a long lag phase of 3.10 h when cultured alone (as control), with a specific growth rate of 0.95 h⁻¹ and a milk coagulation time of 14 h, during which time an active cell concentration of 8.89 log N was achieved. When co-cultured with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the three-strain starter culture, the specific growth rate was increased to 1.61 h⁻¹ and the theoretical lag phase duration predicted by the model was shortened to 1.16 h. This indicates that the presence of *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* and their secreted metabolic products have a stimulatory effect on

Table 1. Parameters in the Gompertz model

Strain	Parameters in the Gompertz model						
	Control starter and <i>L. paracasei</i> control			Tristate sourdough starter			R ² control
	μ , h ⁻¹	λ , h	A	μ , h ⁻¹	λ , h	A	
<i>L. bulgaricus</i>	1.51	0.54	3.09	2.30	1.43	5.72	0.9990
<i>S. thermophilus</i>	2.52	0.47	4.73	2.17	0.53	2.80	0.9973
<i>L. paracasei</i>	0.95	3.10	9.34	1.61	1.16	7.32	0.9944

Lacticaseibacillus paracasei AS-10, which is confirmed by the concentration of active cells reached at the time of coagulation (5 h) 6.65 LogN. The parameter A in the Gompertz model, which represents the asymptote of the curve and expresses the maximum biomass concentration in dimensionless form, varies in the range from 2.80 to 9.34 for the respective strains and is commensurate with

experimentally established values ranging from 2.86 to 8.96. From the data obtained from the modelling it can be concluded that a three-strain symbiotic starter culture has been established for the production of fermented yoghurt products. To confirm this, the intra-population competition coefficients were determined using a system of differential equations on a Gaussian logistic curve [37]. The modelling results are shown in Table 2.

Table 2. Kinetic parameters in the logistic models of the Gaussian system

Strain	Parameters in logistics models					
	Control starter and <i>L. paracasei</i> control			Three-strain sourdough starter		
	$\mu_{\max}$, h ⁻¹	X_K , cfu/cm ³	β , cfu/cm ³ .h	$\mu_{\max}$, h ⁻¹	X_K , cfu/cm ³	α , γ , ε , δ , cfu/cm ³ .h
<i>L. bulgaricus</i>	1.03	6.65	0.1548	-	-	-
<i>S. thermophilus</i>	1.13	8.05	0.1318	-	-	-
<i>L. paracasei</i>	0.90	10.47	0.0860	-	-	-
<i>L. bulgaricus</i> + <i>L. paracasei</i>	-	-	-	0.99	5.89	0.0904
<i>S. thermophilus</i> + <i>L. paracasei</i>	-	-	-	1.10	6.72	0.1441
<i>L. paracasei</i> + <i>L. bulgaricus</i>	-	-	-	0.69	7.07	0.0900
<i>L. paracasei</i> + <i>S. thermophilus</i>	-	-	-	0.72	6.83	0.1077

It is evident from the data presented in Table 2 that *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lacticaseibacillus paracasei* AS-10 developed high maximum specific growth rate in both controls and the trichome combination. According to the selected logistic models, the maximum specific growth rate ranged from 0.72 to 1.13 h⁻¹. According to the logistic models, the maximum theoretical biomass concentrations of *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lacticaseibacillus paracasei* AS-10 varied in the range of 5.89 to 10.47 LogN, which were close to the experimental definition, which ranged from 6.09 to 8.89 LogN. Of greater interest are the intra-population competition coefficients, indicating the influence of individual

microorganisms in the tristate combination relative to each other. From the data presented in Table 2, it can be seen that these coefficients are significantly lower than unity for both the controls and the combination and vary from 0.0860 to 0.1548 cfu/cm³.h for the controls and from 0.0900 to 0.1441 cfu/cm³.h for the trichome combination. The low values of the intra-population competition coefficients in the three-strain combination indicate that there is no inter-species competition and associated cell death and growth suppression, further demonstrating the presence of symbiotic relationships among the three strains in the combined starter culture. Furthermore, for *Lactobacillus bulgaricus*, a decrease in the intra-population competition coefficient (0.0904 cfu/cm³.h) was observed in the presence of *Lacticaseibacillus*

paracasei AS-10 compared to the control starter, 0.1548 cfu/cm³.h. This further confirms the conclusions drawn from the modelling with the Gompertz model regarding the stimulatory effect of *Lactocaseibacillus paracasei* AS-10 as a result of its highly expressed proteolytic activity on *Lactobacillus bulgaricus*. In conclusion, it can be said that the prolongation of the coagulation time in the three-strain combination is not due to competition between the individual strains, but to the smaller proportion of the acid-lactating component (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) in the mixed starter culture.

The variation of titratable acidity pH up to the time of coagulation was monitored, and the results of these studies are shown in Fig. 6 and Fig. 7, and the acidification kinetics was also investigated using the following model:

$$\frac{dpH}{pH} = -\nu d\tau \Rightarrow pH = pH_0 e^{-\nu\tau} \quad \text{where}$$

pH and pH₀ are the current and initial value of the active acidity, ν - specific acidification rate h⁻¹, τ - time, h.

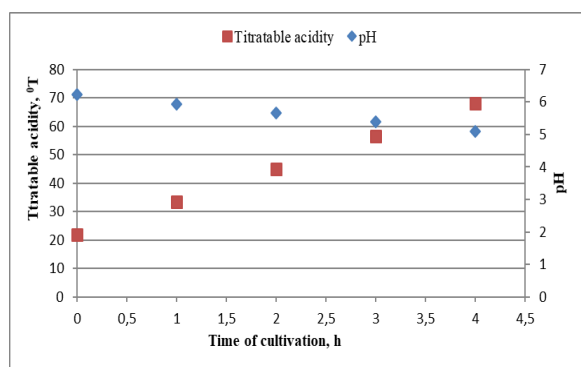


Figure 6. Dynamics of titratable acidity and pH change of the control starter culture

It is evident from the data shown in the figures that for the control starter culture and for the three-strain culture the coagulation of milk occurs at very similar values of titratable acidity - 68 and 65°T. A similar trend is observed for pH - 5.10 and 5.18. The specific acidification rate for the control and the three-strain combination was 0.049 and 0.037 h⁻¹. This lower acidification rate, which is related to the smaller fraction of the acid-lactam component (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) in the tristate combination, is the reason for the prolonged coagulation time compared to the control.

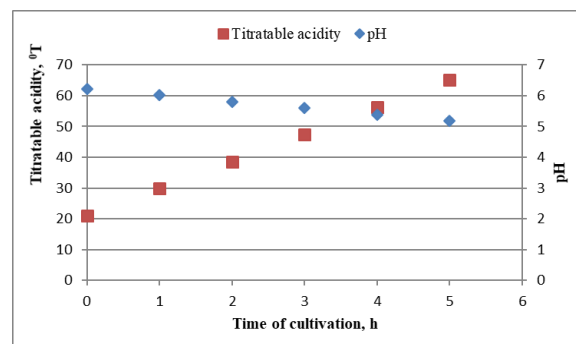


Figure 7. Dynamics of change in titratable acidity and pH of the tristate combination

The milks obtained with the control and the three-strain combination were stored under refrigerated conditions and the dynamics of the change of the active cells of the strains in the starter cultures was monitored. The results of these studies are presented in Fig. 8 and Fig. 9.

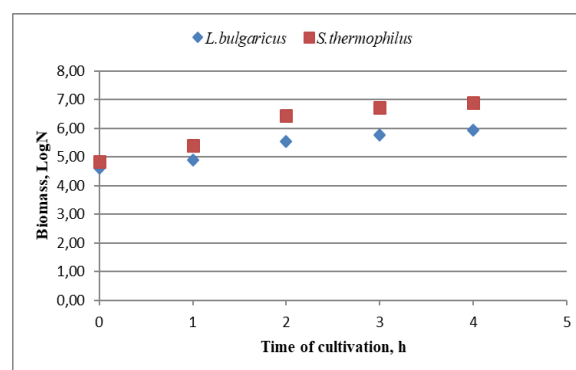


Figure 8. Dynamics of change of active cells of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the control starter culture

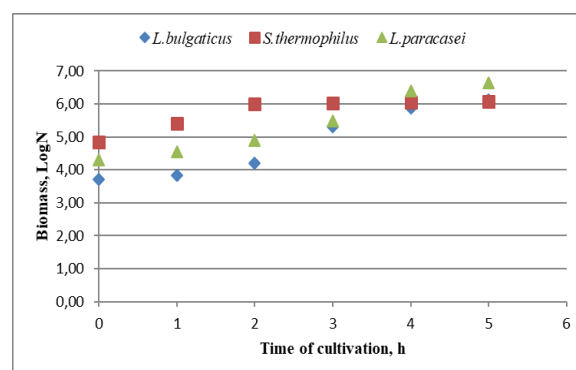


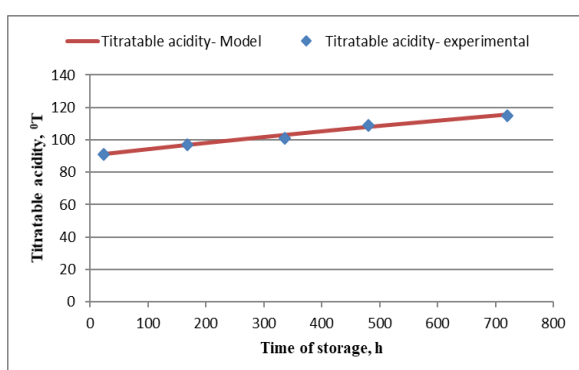
Figure 9. Dynamics of change of active cells of *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Lactocaseibacillus paracasei* AS-10 in the three-strain starter culture

It is evident from the data presented in the figures that no negative influence was observed between the three strains during the storage period. The concentration of active cells of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in the three-strain combination was maintained in the range of 7.00 to 6.32 Log N for *Lactobacillus bulgaricus*, 6.85 to 7.20 Log N for

Streptococcus thermophilus, which values were similar to those of the control starter culture. For *Lactocaseibacillus paracasei* AS-10 in the three-strain combination, a slight reduction in the concentration of active cells was observed in the storage period from 7.60 to 7.30 log N at the end of the storage period. The loss of active cells from lactic acid bacteria during the storage period made the resulting milks functional products.

The post-acid-forming ability of the three-strain starter culture was monitored during storage of the finished milks and compared with that of the control. The following exponential model was used to model the kinetics of post acid formation:

$$K_T = a(b - e^{-q_p \tau})$$



where: k_T -titratable acidity, °T; a and b are empirical coefficients; q_p -specific rate of post-acidification, h^{-1} .

A comparison was made between the experimental titratable acidity data and that predicted by the model, and the results of these studies are presented in Fig. 10 and Fig. 11, and the model parameters in Table 3.

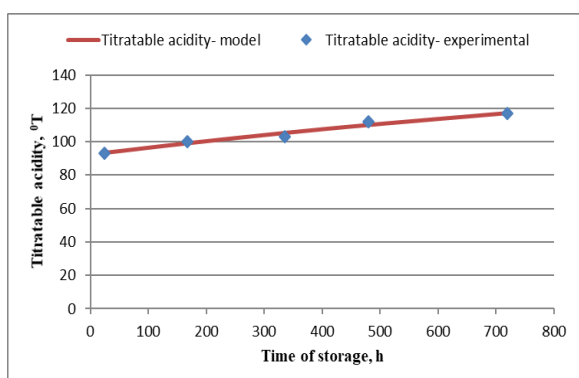


Figure 10. Comparison of experimental titratable acidity data with those from the exponential model for the control starter

Table 3. Parameters in the exponential model

Leaven	a	b	q_p
Control	70.74	2.30	$6.1 \cdot 10^{-4}$
Tree-strain	99.26	1.91	$4.1 \cdot 10^{-4}$

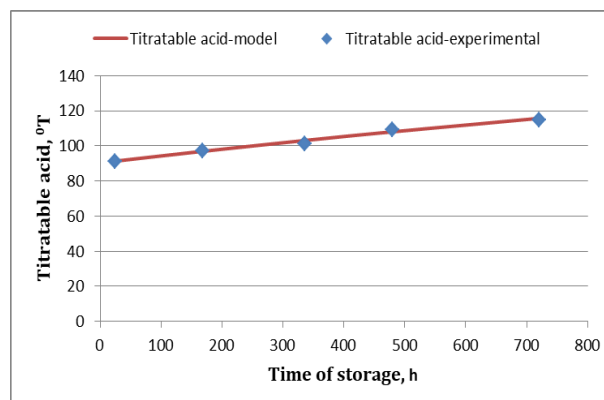


Figure 11. Comparison of experimental titratable acidity data with those from the exponential model for the three-strain starter culture

From the data presented in the figures, it can be seen that the model describes the experimental results with high accuracy. From the data presented in Table 3, it can be seen that the specific rate of post-acid formation and ma commensurate order of magnitude, which is due to the similar values of titratable acidity of the milks obtained with the control and with the three-strain combination.

4. Conclusion

The investigated strain *Lactocaseibacillus paracasei* AS-10 showed satisfactory results in combination with a traditional starter culture for Bulgarian yoghurt. It possesses valuable technological characteristics that make it suitable for use in starter cultures for the production of lactic acid products, improving their overall physicochemical and organoleptic profile. The study of its growth kinetics as a mono and symbiotic culture provides the scientific information necessary to formulate guidelines to producers related to its application in practice.

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