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Modelling and Predicting the Growth of *Escherichia coli* and *Staphylococcus aureus* in Co-Culture with *Geotrichum candidum* and Lactic Acid Bacteria in Milk

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Abstract: The growth of two pairs of co-cultures (*Escherichia coli*/*Geotrichum candidum,* and *Staphylococcus aureus*/*Geotrichum candidum*) with a starter culture of lactic acid bacteria was studied in milk at temperatures related to artisanal cheesemaking ripening. For an inoculum of approximately 10^6 CFU/mL, LAB not only induced an early stationary phase of *E. coli* (two strains BR and PS2) and *S. aureus* (strains 2064 and 14733) but affected their death phase. *G. candidum* was found to be the subject of interactions with LAB within a given temperature range only partially. To develop a tertiary model for the growth curves of the populations, a one-step approach was used, combining two types of primary models (Huang and Gimenez and Dalgaard) with secondary square root models for growth rate and lag time. Furthermore, the reparametrized Gompertz-inspired function with the Bigelow secondary model was used to describe the death phase of the *E. coli* and *S. aureus* strains. The prediction ability of the growth of the H-GD tertiary model for co-cultures was cross-validated within the strains and data sets in milk and milk medium with 1 % NaCl.

1. Introduction

A wide range of interactions among microbial populations belongs to actual and challenging subjects of quantitative food microbiology, especially when the research results have applicable predictive potential. Experiments referring to the fate of populations in the background of ongoing fermentation and ripening may provide a substantial view to several questions that usually appear in the practice of artisan cheese. For example, we may ask whether bacterial starters are more suitable and effective against contaminants than naturally present LAB populations with their diversity, actual activity, and acidification ability.

The idea of providing quantitative studies or proofs related to the role of microbial interactions in the microbiological quality and safety of artisanal raw milk products was inspired not only by the history of their production but also by the recent scientific outputs published [1–5]. According to Schoustra et al. [6], traditionally processed foods derived from raw milk may be safe, since adjustment of the doses of the LAB starters can serve as a means of controlling sanitary protection, maintaining bacterial diversity [7,8], and supporting the activity of inherited populations of LAB as well [9]. This is the case of artisanal lump cheese produced in Slovakian mountain areas and sent for processing for Bryndza cheese after a short 8 to 10 days of ripening [10].

Raw milk cheesemaking and ripening affect microbial activity in a variety of ways, resulting in synergistic actions that are bacteriostatic and/or bactericidal against most microbial pathogens [11,12]. Management of acidification by reaching the target values of pH 5.0-5.5 in time is one of the most important factors to ensure the overall quality and safety of the product. The content and activity of natural protection systems such as lactoperoxidase, lysozyme, and lactoferrin, well known for their antibacterial activity in milk, as well as the production of bacteriocins or bacteriocin-like substances, provide additional barriers to proliferation and persistence [13,14]. Furthermore, other

aspects of desired effects include temperature, oxygen accessibility, redox potential, a_w and osmotic pressure, and the concentration of ingredients throughout the entire process [6,7,14].

Except for *L. monocytogenes*, which is not the subject of this research, *E. coli* and *S. aureus* should be included as pathogenic bacteria considered a major safety issue for raw milk cheese. They are most often found in cheeses produced with raw milk and function as an indicator of hygiene deficiencies [14–16]. According to Desmarchelier & Fegan [17], raw milk can become contaminated with *E. coli* directly through animal faeces or indirectly through contaminated farm and dairy environments, equipment, and handling personnel. Although most *E. coli* are harmless commensals, some are known to be pathogenic bacteria, causing severe intestinal and extraintestinal diseases in humans. *The presence of S. aureus* in cheese is associated with post-secretory contamination and it is relevant as it may produce enterotoxins [4,7,13].

Cheese ecosystems may be associated with the presence of unique microbes, leading to unique microbial interactions that can develop remarkable unique sensory characteristics. In general, the microbiota of artisanal cheese consists of complex assemblages consisting of not only prokaryotic but also fungal populations. As an example, the presence of *G. candidum* has been linked to the microbial profile of several artisanal raw milk cheeses from various countries. This yeast has been found in the microbiota of Italian "Robiola di Roccaverano" [18], French "Pélardon" [19], "Saint-Nectaire" and "Reblochon" [20], Brazilian "Minas" [21,22], Spanish “Cabrales” [23] and “Armada” [24] and Slovakian “Bryndza” cheese [25–29]. Besides that, it is used as adjunct culture in the production of soft mould-ripened cheeses (e.g. Brie, Camembert), soft and semi-hard smear-ripened cheeses (e.g. Livarot, Tilsit) [30–32].

Data on the quality of raw milk cheeses and the prevalence of foodborne pathogens are well documented. However, there is less information in the literature on the in-depth knowledge of interactions between microbial populations [33,34]. On the one hand, physiological studies contribute to a better understanding of the behaviour of the microbiota, but studies in predictive microbiology go one step further by modelling and simulating microbial dynamics over time. Therefore, both can provide reproducible complex patterns that give insight into the effect of varying processing environmental conditions on the cheese microsystem [35–37]. As for most artisanal cheeses, ambient temperatures from 15 to 21 °C are applied during fermentation and the early phase of the ripening processes [4,38], we aimed at identifying microbial interactions between a starter culture of lactic acid bacteria, strains of *E. coli*, *S. aureus* and *G. candidum* in detail at a close temperature range, thus contributing to knowledge in artisanal raw milk cheese making. In addition, the other objectives are concerned with the predictive ability to assess and validate the proposed tertiary model for the growth of co-cultures in Slovakian lump cheese that is produced in mountain areas and sent for industrial processing to Bryndza cheese [10].

2. Material and Methods

2.1. Microorganisms and culture conditions

The commercial LAB culture DVS® Fresco® 1000NG (Christian Hansen, Hoersholm, Denmark) and isolate J of *G. candidum* [29] that comes from Slovakian traditional cheese “Bryndza” were used during all co-cultivation experiments. Mesophilic starter culture consisting of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *Streptococcus salivarius* subsp. *thermophilus* was kept frozen at -45 °C. *G. candidum* was refrigerated at 5 °C on plate count skim milk agar slants (SMA; Merck, Darmstadt, Germany) and periodically sub-cultured in diluted SMA agar.

There were four different series of *E. coli* and *S. aureus* co-cultivations using 2 strains of each, BR, PS2 for *E. coli* and 2064, 14733 for *S. aureus*. The BR and PS2 strains of *E. coli* were isolated from a Slovakian traditional “Bryndza” cheese and laboratory-produced pasta-filata cheese from raw cows' milk, respectively. The *S. aureus* strains 2064 and 14733 were isolated from a Slovakian ewes’ lump cheese and milk vending machine biofilm, respectively. All bacterial cultures were maintained in Brain Hearth Infusion (BHI) broth (Sigma-Aldrich, St. Louis, Missouri, USA) at 5 ±0.5 °C before analysis.

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Fungal and bacterial strains belong to the collection of the Institute of Food Science and Nutrition (the Slovak University of Technology in Bratislava, Slovakia). Their identifications were performed or confirmed in the previous works [35,39–42].

2.2. Preparation of inoculum and experiments

Standard suspension of Fresco culture was prepared by inoculation of frozen culture into 100 mL of sterile milk and incubation at 30 ±0.5 °C for 5 h until the stationary phase was reached. Standard suspension of *G. candidum* isolate was prepared from 48 h old culture grown on vertical SMA agar at 30 ±0.5 °C and mixed with 10 mL of sterile saline solution. A standard suspension of *E. coli* and *S. aureus* strains was prepared from a 24 h-old culture grown in BHI broth at 37 °C. The above inoculation procedure was aimed to reach the initial concentration of Fresco at 10^6 CFU/mL, of *G. candidum* at approximately 10^2 CFU/mL and *E. coli* and *S. aureus* strains at approximately 10^3 CFU/mL.

All co-cultivation experiments were performed in 250 mL of pre-tempered ultra-high temperature treated milk with 1.5 g/L fat content (Rajo, Ltd., Bratislava, Slovak Republic) without or with 1 % NaCl (w/v). The incubation was performed in three parallel stages under static conditions at temperatures of 15, 18 and 21 ±0.5 °C, which represent artisanal cheese production [38].

The pH was measured using a WTW Inolab 720 pH-meter (Inolab, Weilheim, Germany) equipped with a SenTix 81 glass electrode (WTW GmbH, Weilheim, Germany) with the same time interval as samples for microbiological quantification. a_w-values were estimated by the LabMaster-aw (Novasina, Lachen, Switzerland).

2.3. Quantification of microorganisms

The counts of LAB, *G. candidum*, *S. aureus* and *E. coli* were determined by the 10-fold dilution method in a saline-peptone solution. To achieve the best possible fit of the model to the curve, time intervals were predefined according to the incubation temperature.

Counts of cocci from Fresco culture were determined on M17 agar (Merck, Darmstadt, Germany) after 48 h incubation at 30 ±0.5 °C according to EN ISO 15214 [43]. *G. candidum* counts were determined on DRBC agar (Biokar Diagnostics, Beauvais, France) after 5 days of incubation at 25 ±0.5 °C according to EN ISO 21527-1 [44]. *E. coli* was counted on Chromocult Coliform agar (Merck, Darmstadt, Germany) after 24 h incubation at 37 ±0.5 °C according to National Standard Method F23 [45]. *S. aureus* was enumerated on Baird-Parker agar (Merck, Darmstadt, Germany) with incubation at 37 ±0.5 °C for 48 h according to EN ISO 6888-1 [46].

2.4. Mathematical models

2.4.1. Modelling the microbial interaction in co-cultures

The primary models of Huang [47] and Giménez and Dalgaard [48] combined with secondary square root models as applied for growth rate and lag time were used to describe competitive growth of the co-cultures series in milk for all isothermal growth curves. The suggested interaction H-GD model with competition coefficients describing the growth of LAB, *G. candidum* and behaviour of *E. coli* and *S. aureus* in inter-species competition were used in this study according to [37]. Thus, the system of the ordinary differential equations with the initial conditions applied for the growth phase and a mixed system of differential equations and nonlinear algebraic equation (G-B model) was used for survivors of *S. aureus* (*Sa*) and *E. coli* (*Ec*) in the death phase, denoted here as index P (P = *Ec* or *Sa*). The equations can be written as follows:

A H-GD model with the competition coefficients

Growth of LAB; tÎá0, tt ñ

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$$\frac{dx_{Lab}}{dt}=\left[\frac{\frac{\mu_{\max}^{Lab}}{\ln 10}}{1+10^{-\alpha\left(t-t_{\lambda}^{Lab}\right)}}\left(1-10^{\left(x_{Lab}-x_{\max,Lab}\right)}\right)\left(1-10^{\left(x_{P}-x_{\max,P}\right)}\right)\right]I_{LP} \quad (1)$$

Growth of P = *Ec* or *Sa*; tÎá0, tt ñ

$$\frac{dx_{P}}{dt}=\left[\frac{\frac{\mu_{\max}^{P}}{\ln 10}}{1+10^{-\alpha\left(t-t_{\lambda}^{P}\right)}}\left(1-10^{\left(x_{P}-x_{\max,P}\right)}\right)\left(1-10^{\left(x_{Lab}-x_{\max,Lab}\right)}\right)\right]I_{PL} \quad (2a)$$

Survival P = *Ec* or *Sa*; tÎá t_t, *t*ñ

$$x=x_{\max,P}+\left(x_{res,P}-x_{\max,P}\right)\exp\left\{-\exp\left[\left(\frac{-\frac{k_{\max,P}}{\ln 10}\cdot e}{\left(x_{res,P}-x_{\max,P}\right)}\right)\left(t_{\lambda,s}-t\right)+1\right]\right\} \quad (2b)$$

$$\frac{dx_{Gc}}{dt}=\frac{k_{Gc}\frac{\mu_{\max}^{Gc}}{\ln 10}}{1+10^{-\alpha\left(t-t_{\lambda}^{Gc}\right)}}\left(1-10^{\left(x_{Gc}-x_{\max,Gc}\right)}\right) \quad (3)$$

$t=0 \quad x_{Lab}=x_{Lab,0} \quad x_{P}=x_{P,0} \quad x_{Gc}=x_{Gc,0}$

where $\mu_{\max}^{i=Lab,P,Gc}$ are the maximum specific growth rates of the LAB, *E. coli* or *S. aureus* and *G. candidum* (*Gc*), respectively, t_l^i are the lag times of microorganisms, α is the lag phase transition coefficient, taking a value of 4 [47]. Concentrations x, which include $x_i = \log N_i$, $x_{0,i} = \log N_{0,i}$, $x_{\max,i} = \log N_{\max,i}$, $x_{res,i} = \log N_{res,i}$ represent the real, initial, maximum and residual (or tail) cell density, N_i, $N_{0,i}$ $A_{\max,i}$ and $N_{res,i}$. I_{LP}, I_{PL} are the competition coefficients representing the effects of LAB (Fresco) on *E. coli* or *S. aureus* and *E. coli* or *S. aureus* on LAB (Fresco), respectively in the H-GD model type R. $k_{\max,P}$ is the maximum death rate of *E. coli* or *S. aureus*, $t_{ls}^{i=Ec,Sa}$ is the survival curve shoulder, t_t is the transitioning breakpoint time from stationary to survival phase for *E. coli*/*S. aureus* that is determined so that the time t_l is equal to zero, k_{Gc} is the reduction coefficient for *G. candidum* growth rate.

As $\mu_{\max}^i$ and t_l^i are a function of temperature, the following secondary square root models were used to incorporate the effect of temperature on growth parameters [49]:

$$\sqrt{\mu_{\max}^{i}}=b_{T,i}\cdot\left(T-T_{\min,i}\right), \quad (4)$$

where regression coefficient b_T ($h^{-1}\times°C^{-1}$) is the slope and depends on additional growth conditions and the microorganism involved, T (°C) is the temperature, and T_{min} is its theoretical minimum for growth.

$$\mu_{\max}^{Ec}=\left[b_{T,Ec}\cdot\left(T-T_{\min,Ec}\right)\right]^2;\ \mu_{\max}^{Sa}=\left[b_{T,Sa}\cdot\left(T-T_{\min,Sa}\right)\right]^2;\ \mu_{\max}^{Lab}=\left[b_{T,Lab}\cdot\left(T-T_{\min,Lab}\right)\right]^2;$$
$$\mu_{\max}^{Gc}=\left[b_{T,Gc}\cdot\left(T-T_{\min,Gc}\right)\right]^2 \quad (5)$$

The square root relation between lag time (t_l) and T was used in the H-GD models according to [50]:

$$t_{\lambda}^{Ec}=\frac{1}{\left[b_{\lambda,Ec}\cdot\left(T-T_{\min,Ec}\right)\right]^2};\ t_{\lambda}^{Sa}=\frac{1}{\left[b_{\lambda,sa}\cdot\left(T-T_{\min,Sa}\right)\right]^2};\ t_{\lambda}^{Lab}=\frac{1}{\left[b_{\lambda,Lab}\cdot\left(T-T_{\min,Lab}\right)\right]^2}.$$
$$t_{\lambda}^{Gc}=\frac{1}{\left[b_{\lambda,Gc}\cdot\left(T-T_{\min,Gc}\right)\right]^2} \quad (6)$$

where $b_{l,I}$ is the regression coefficient.

Next, for the declination phase of *E. coli* and *S. aureus* strains, the reparametrized Gompertz-inspired survival model together with the Bigelow secondary model was used for its versatility of

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fitting linear data and those that have shoulder and/or tailing effects [51]. The z_i parameter was used to help in the theoretical description of the influence of temperature and other factors acting in this phase such as the pH drop and addition of NaCl.

The secondary Bigelow log-linear model was applied to express the dependence of the rate of decrease, k_{max} on temperature as follows:

$$k_{\max}^{Ec} = \frac{k_{ref}^{Ec}}{10^{\frac{T_{ref}-T}{z_{Ec}}}};\quad k_{\max}^{Sa} = \frac{k_{ref}^{Sa}}{10^{\frac{T_{ref}-T}{z_{Sa}}}} \tag{7}$$

where r_{efi}^{t} is k_{max}^{i} at a reference temperature (T_{ref}) and z represents a temperature required for a 10-fold reduction of *E. coli* or *S. aureus* numbers.

2.4.2. Parameter determination and evaluation of model performance

The one-step kinetic data analysis method described by Huang [52] was applied for parameter optimization from the given isothermal growth curves of the co-culture microbial populations. To construct the tertiary H-GD model and minimize the global sum of squared errors (*SSE*), they were analysed concurrently using the H-GD models (Eqs. 1–3) and the secondary square root models (Eqs. 4–7).

In the beginning, the tertiary H-GD model had 19 parameters (Eqs. 1–7): three average maximum values of x_{max}, competition coefficients I_{LP}, I_{PL} and reduction coefficient k_{Gc} in the H-GD model type R and reduction coefficient k_{Gc} (Eqs. 1–3) and parameters b_T, T_{min}, b_l from square-root models (Eqs. 4–6). The parameters N_{res}, k_{ref}, and z are derived from the reparametrized Gompertz-inspired survival model (Eq. 2a) as well as the Bigelow secondary model (7), respectively.

$$p_{H-GD} = \left\{ \begin{array}{l} x_{\max,Lab}; x_{\max,P}; x_{\max,Gc}; I_{LP}; I_{PL}; k_{Gc} \\ b_{T,Lab}; b_{T,P}; b_{T,Gc}; T_{\min,Lab}; T_{\min,P}; T_{\min,Gc}; b_{\lambda,Lab}; b_{\lambda,P}; b_{\lambda,Gc} \\ k_{ref,P}; N_{res,P}; z_P \end{array} \right\} \tag{8}$$

$p_{H\text{-}GD}$ is the vector of parameters of H-GD models for the simultaneous competitive growth of the co-culture series.

The prediction ability of the tertiary H-GD model was tested for a reduced number of parameters (p_E): the average maximum density counts of microorganisms ($x_{max,Lab}$, $x_{max,P}$, $x_{max,G}$), the competition coefficients (I_{LP}, I_{PL}), the reduction coefficient k_{Gc}, the regression coefficient, $b_{l,Gc}$, the maximum declination rates at a reference temperature T_{ref} ($k_{ref,Ec}$, $k_{ref,Sa}$) and z-values (z_{Ec}, z_{Sa}). Regression coefficients $b_{l,Ec}$ for the strain *E. coli* PS2 were also evaluated by using one-step kinetic data analysis. The remaining parameters in Eq. 8, which were previously optimized by nonlinear regression analysis for single cultures [37] or taken from the following scientific articles [41,42], were fixed as constants. This approach has the advantage that some parameters for co-culture growth prediction in milk could be estimated from the growth of individual species.

The goodness of fit of the tertiary H-GD model was evaluated with the global sum of squared errors (*SSE*), the root mean square error (*RMSE*) and the determination coefficient (R^2) to evaluate its suitability to fit the whole set of observation points according to Eqs. 1-7.

$$SSE = \sum_{i=1}^{n} \left(x_i^{\exp} - x_i^{cal} \right)^2 \tag{9}$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i^{\exp} - x_i^{cal} \right)^2}{n - p_E}}, \tag{10}$$

$$R^2 = 1 - \frac{SSE}{SST} \tag{11}$$

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where $x_i^{\exp}$ and x_i^{cal} correspond to the observed and predicted values, respectively, n is the total number of data points, p_E is the number of estimated parameters and SST is the total sum of squared errors.

The prediction capability of the H-GD tertiary model was tested through the Bias (B_f) and Accuracy (A_f) factors [53] on the data sets of different *E. coli* (BR and PS2) and *S. aureus* (2064 and 14733) strains within the temperature range of 15 to 21 °C for the cases without and with 1 % NaCl addition

$$B_f = 10^{\frac{\sum_{i=1}^{n}\left(\log x_i^{cal} - \log x_i^{\exp}\right)}{n}} \quad (12)$$

$$A_f = 10^{\sqrt{\frac{\sum_{i=1}^{n}\left(\log x_i^{cal} - \log x_i^{\exp}\right)^2}{n}}} \quad (13)$$

The accuracy of the H-GD model was checked with the *RMSE* of prediction for each microorganism in the co-culture growth according to [54].

The tertiary model parameters were estimated using the commercial process-engineering software Athena Visual Workbench (Stewart & Associates Engineering Software, Madison, WI; www.athenavisual.com). *SSE*, *RMSE*, as well as bias (B_f) and accuracy (A_f), were calculated using Microsoft Excel (Microsoft, Redmond, Washington, USA).

3. Results and Discussion

3.1. One-Step Analysis of Competitive Growth

By combining co-culture primary growth models with secondary growth and survival models in one step, we were able to describe the growth patterns of three microbial populations. As presented in Figure 1a-1c and 1d-1f for *E. coli* BR in milk and milk with 1 % NaCl, respectively, the following similar characteristics can be recognised. First, LAB played a dominant role in co-cultures; grown at the highest rates that were influenced mostly by the temperature and only partly by 1% NaCl content. Dalcanton, et al. [55] and Medveďová et al. [56] reported a comparable trend regarding the influence of temperature and water activity on the behaviour of LAB. On average, they reached maximum population density (MPD) of 9.32 ±0.07 logs and a stable population increase (the difference between MPD and N_0) of 3.2 ±0,3 log CFU/mL in all co-culture trials in the shortest time. These results aligned with our previous work [37] and those reported for the co-culture growth of *Lactiplantibacillus plantarum* with *S. aureus* by Rodríguez-Sánchez et al. [57].

While grown independently, LAB determined the responses of *E. coli* BR that increased the numbers in 1.23 ±0.32 log CFU/mL for a period in which LAB reached the early stationary phase. *E. coli* reached its stationary phase with a maximum population density (MPD) of 3.91 ±0.18 logs on average and prolonged its duration with increasing temperature in both cases of milk (without and with 1 % NaCl). Consistent with these results, Sreekumar and Hosono [58] reported final counts of *E. coli* 3544 less than 3 logs CFU/mL during co-cultivation with *Lactobacillus acidophilus* SBT2071 in semi-skimmed sheep's milk. Referring to the increase in population, this was not the case for *G. candidum*. However, in milk, as well as at 1 % NaCl, its population reached the highest increase of 4.19 ±0.16 logs, for a longer period than other populations. Naturally, this period was determined by temperature. It can also be seen that the *G. candidum* lag phase was almost identical to the period that covers the LAB lag and exponential phases together.

The growth studies [41,59] demonstrated a similar pattern of growth responses during *G. candidum* and LAB Fresco co-culturing experiments. During the stationary phase, the yeast was able to grow exponentially and reach its stationary phase. This can be explained by both lactate consumption and ammonium production [60], which are related to an increase in pH and tolerance to lactic acid produced by LAB.

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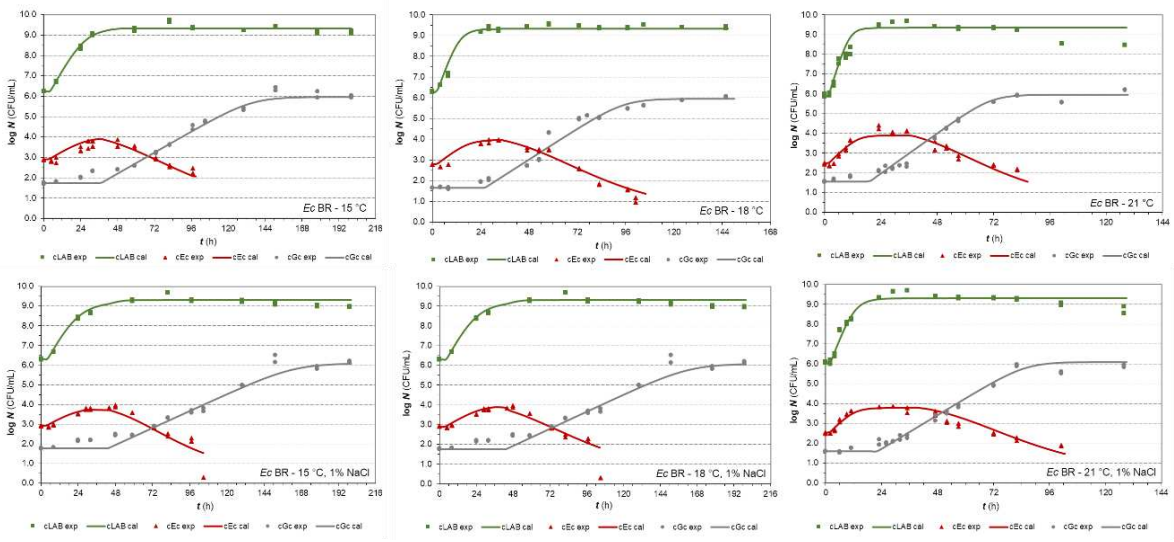

Figure 1. 1a-1c and 1d-1f. Co-culture growth of LAB Fresco, *E. coli* BR and *G. candidum* in milk at 15, 18 and 21 °C (without and with 1 % NaCl, respectively). The continuous lines represent the growth predicted values by the H-GD model and the dots represent the experimental values (® LAB, p Ec, □ Gc).

Similar responses were also found in co-culture trials, in which the previous strain of *E. coli* was replaced by the PS2 strain isolated from Slovakian artisanal steamed and stretched Slovakian cheese. Compared to BR strain, the only visually recognisable differences are referred to as a higher MPD and a population increase of 4.76 ±0.26 log and 1.91 ±0.33 log, respectively, as well as longer durations of stationary phases in general (Suppl. Figure 1 1g-1i and 1j-1l). Thus, naturally, the PS2 strain also grew at a negligible higher rate than the BR strain during the exponential phase.

The *S. aureus* strain 14733 in the same type of co-cultures with LAB and *G. candidum* reached MPD and a population increase of 3.97 ±0.36 and 1.06 ±0.29 log, respectively (Figure 2a–2c and 2d-2f). Both mean values were close to the responses of the *E. coli* BR strain.

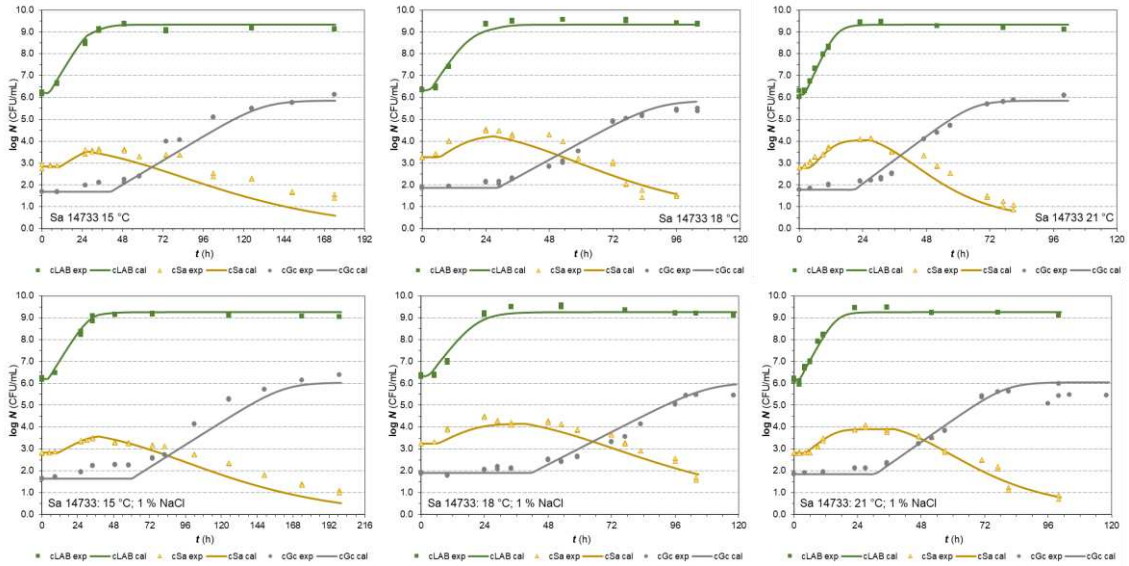

Figure 2. 2a-2c and 2d-2f. Co-culture growth of LAB Fresco, *S. aureus* 14733 and *G. candidum* in milk at 15, 18 and 21 °C (without and with 1 % NaCl, respectively). The continuous lines represent the growth predicted values by the H-GD model and the dots represent the experimental values (® LAB, p Sa, □ Gc).

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The second *S. aureus* strain 2064 [61] was also used in the last series of co-cultures with LAB and *G. candidum*. The results of the tests (Suppl. Figure 2 2g-2j) indicated that this strain appeared to be sensitive to lactic acid or competition in general since it reached MPD in stationary phase less than 4 logs (3.38 ±0.40 log CFU/mL) and the increase in population less than 1 log (0.65 ±0.27 log CFU/mL).

The production of organic acids has a major impact on the quality of the final products during the cheesemaking process. Acidification is usually achieved by the production of lactic acid through the fermentation of lactose by LAB [57,62]. The pH changes in our study followed a sigmoidal behaviour throughout LAB growth but with a delay of approximately 6 to 10 h. This trend is consistent with those reported by Rodríguez-Sánchez et al. [57] when analysing the antimicrobial activity of the LABs against some potentially pathogenic bacteria used as indicators, including *S. aureus*. As LAB growth progressed to the exponential and stationary growth phase, a rapid and significant drop in pH (pH ◎ 5.3) was observed in the second phase.

The parameters estimated for the H-GD model are presented in Table 1 and Table 2 for both *E. coli* and *S. aureus* strains in co-cultures, respectively.

Table 1. Parameters of H-GD model with 95 % highest posterior density interval (Bayesian estimation) for growth of *E. coli* (strains BR and PS2) in co-cultures with *G. candidum* and LAB in milk.

Parameters	*E. coli* (strain BR)		*E. coli* (strain PS2)	
	In milk	1 % NaCl in milk	In milk	1 % NaCl in milk
$x_{max,Lab}$	9.34 ±0.04	9.32 ±0.03	9.36 ±0.04	9.33 ±0.04
$x_{max,Ec}$	4.17 ±0.16	3.95 ±0.10	5.14 ±0.17	5.14 ±0.10
$x_{max,Gc}$	5.96 ±0.08	6.09 ±0.10	5.72 ±0.08	6.04 ±0.17
I_{LE}	1.158 ±0.093	1.254 ±0.100	0.957 ±0.059	0.951 ±0.054
I_{EL}	0.526 ±0.045	0.536 ±0.049	0.588 ±0.042	0.513 ±0.035
k_{Gc}	0.850 ±0.038	0.710 ±0.025	0.931 ±0.048	0.749 ±0.046
k_{ref}	0.101 ±0.006	0.101 ±0.006	0.133 ±0.006	0.081 ±0.006
$x_{res,Ec}$	0.4[a]	0.42 ±0.16	1.20 ±0.29	0.5[d]
z_{Ec}	30.67 ±5.68	32.25[d]	6.38 ±0.70	28.21 ±5.76
$b_{\lambda,Gc}$	0.0109 ±0.0003	0.0101 ±0.0003	0.0096 ±0.0002	0.0085 ±0.0002
$b_{T,Gc}$[b]	0.0228[b]	0.0228[b]	0.0228[a]	0.0228[a]
$T_{min,Gc}$[b]	0.00[b]	0.00[b]	0.00[a]	0.00[a]
$b_{\lambda,Lab}$[c]	0.0343[c]	0.0343[c]	0.0343[b]	0.0343[b]
$b_{T,Lab}$[c]	0.0384[c]	0.0384[c]	0.0384[b]	0.0384[b]
$T_{min,Lab}$	1.11[c]	1.11[c]	1.11[b]	1.11[b]
$b_{\lambda,Ec}$	0.0493[c]	0.0493[c]	0.0365 ±0.0045	0.0366 ±0.0044
$b_{T,Ec}$	0.0421[c]	0.0421[c]	0.052[c]	0.052[c]
$T_{min,Ec}$	4.16[c]	4.16[c]	4.80[c]	4.80[c]

Transition time from stationary to survival phase: t_t (milk) = 36 h; t_t (milk+1 % NaCl) = 44 h. [a] the parameter lower bound. [b] the parameter values are fixed [63]. [c] the parameter values are fixed [37]. [d] the parameter value is not determined.

Except for the facts described above, the data in Table 1 pointed out that the competition coefficients I_{EL} (Eq. 2) in the H-GD model showed a similar growth reduction (< 60 %) for both strains of *E. coli* compared to their original capacity as individual species in milk [37]. On the other hand, the competitive effect of LAB on *S. aureus* strains was stronger and strain-dependent. The coefficients I_{SL}

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in Table 2 were significantly lower and different for strains 2064 and 14733. Thus, they confirmed the higher sensitivity to non-specific inhibition caused by LAB that was found for strain 14733.

Table 2. Parameters of H-GD model with 95 % highest posterior density interval (Bayesian estimation) for growth of *S. aureus* (strains 2064 and 14733) in co-cultures with *G. candidum* and LAB in milk.

Parameters	*S. aureus* (strain 2064)		*S. aureus* (strain 14733)	
	In milk	1 % NaCl in milk	In milk	1 % NaCl in milk
$x_{max,Lab}$	9.43 ±0.03	9.40 ±0.05	9.34 ±0.03	9.25 ±0.03
$x_{max,Sa}$	3.83 ±0.15	4.17 ±0.11	4.43 ±0.12	4.43 ±0.16
$x_{max,Gc}$	5.65 ±0.12	5.82 ±0.17	5.85 ±0.11	6.04 ±0.15
I_{LS}	1.262 ±0.056	1.083 ±0.057	1.064 ±0.044	0.912 ±0.043
I_{SL}	0.308 ±0.144	0.174 ±0.089	0.705 ±0.079	0.526 ±0.054
c_{LS}	-	-	-	-
c_{SL}	-	-	-	-
k_{Gc}	0.995 ±0.067	0.778 ±0.058	0.906 ±0.048	0.850 ±0.055
k_{ref}	0.133 ±0.022	0.102 ±0.007	0.107 ±0.007	0.094 ±0.006
$x_{res,Sa}$	1.47 ±0.13	0.3[c]	0.5[c]	0.5[c]
z_{Sa}	9.46 ±1.21	10.44 ±0.51	11.49 ±1.18	13.79 ±1.67
$b_{\lambda,Gc}$	0.0092 ±0.0002	0.0086 ±0.0003	0.0104 ±0.0003	0.0086 ±0.0002
$b_{T,Gc}$	0.0228 [a]	0.0228 [a]	0.0228 [a]	0.0228 [a]
$T_{min,Gc}$	0.00[a]	0.00[a]	0.00[a]	0.00[a]
$b_{\lambda,Lab}$	0.0343[b]	0.0343[b]	0.0384[b]	0.0384[b]
$b_{T,Lab}$	0.0384[b]	0.0384[b]	1.11[b]	1.11[b]
$T_{min,Lab}$	1.11[b]	1.11[b]	0.0302[b]	0.0302[b]
$b_{\lambda,Sa}$	0.0302[b]	0.0302[b]	0.0409[b]	0.0409[b]
$b_{T,Sa}$	0.0409[b]	0.0409[b]	5.02[b]	5.02[b]
$T_{min,Sa}$	5.02[b]	5.02[b]		

Transition time from stationary to survival phase: t_t (milk) = 23 h; t_t (milk+1 % NaCl) = 23 h. [a] the parameter values are fixed [63]. [b] the parameter values are fixed [37]. [c] the parameter lower bound.

The results in Tables 1–3 showed that the tertiary H-GD model successfully described the competitive growth between species in milk at temperatures related to the ripening conditions of artisanal cheesemaking. The global *RMSE* values for all cases are lower than 0.33 log CFU/mL, which is well within the range of normal experimental error. A dominant level of inoculum (approx. 10^6 CFU/mL) of a starter culture favoured the growth of LAB in milk (the competition coefficient I_{LP} is greater than one in almost all cases and was able not only to induce an early stationary state in *E. coli* (strains BR and PS2) and *S. aureus* (strains 2064 and 14733) for the cases without and with 1 % NaCl addition but also subsequently reduced their population. LAB growth of the LAB slightly suppressed the growth rate of *G. candidum* of its original ability as a single species in milk. The reduction coefficients of the growth rate of *G. candidum* k_{Gc} were within the region 0.710-0.995. Naturally, their values were lower for the cases with NaCl addition (Table 1, Table 2).

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Table 3. Goodness-of-fit indices and models comparison of H-GD model for the *E. coli* and *S. aureus* strains in co-culture with *G. candidum* and LAB Fresco in milk.

Indices	*E. coli* BR		*E. coli* PS2		*S. aureus* 2064		*S. aureus* 14733	
	in milk	1 % NaCl in milk	in milk	1 % NaCl in milk	in milk	1 % NaCl in milk	in milk	1 % NaCl in milk
SSE	14.719	16.080	19.450	25.719	10.625	11.725	15.184	17.592
R^2	0.992	0.991	0.987	0.986	0.991	0.991	0.989	0.988
p_E	10	10	11	11	10	10	10	10
RMSE	0.251	0.254	0.289	0.324	0.280	0.284	0.270	0.282

Number of data points: $n_{EC\ BR}$ (milk) = 244; $n_{EC\ BR}$ (milk+1 % NaCl) = 254; $n_{EC\ PS2}$ (milk) = 244; $n_{EC\ PS2}$ (milk+1 % NaCl) = 256; $n_{Sa\ 2064}$ (milk) = 146; $n_{Sa\ 2064}$ (milk + 1% NaCl) = 155; $n_{Sa\ 14733}$ (milk) = 218; $n_{Sa\ 14733}$ (milk + 1 % NaCl) = 232.

3.2. Model validation

In fermentations, the most important populations consist of the LABs responsible for fermentation and some adjunct culture. In our case, they are represented by Fresco culture and *G. candidum*, respectively. Inviting intensive growth, they create or supply added value to the final characteristics of the fermented product. On the other hand, co-culture studies are usually aimed at the behaviour of microbial contaminants, while the fermentation and adjunct cultures are monitored in the background. Undesirable, pathogenic, or spoilage bacteria play different roles and, in this work, are represented by the strains of *E. coli* or *S. aureus*. To evaluate strain variability or validate the co-culture models, most of their points of view are considered in this section.

3.2.1. *E. coli* strains in co-cultures

First, within the variability evaluation, the *RMSE* values [54] between milk growth and milk with 1 % NaCl were evaluated for each *E. coli* strain. Although the PS2 strain showed the highest difference in milk numbers between 1 and 0 % (*RMSE* = 0.67 log CFU/mL), the *RMSE* for the BR strain was only 0.37 log CFU/mL, indicating that this strain was less sensitive to NaCl addition. In the evaluation of the differences between strains BR and PS2 in the same medium, they were lower in milk without salt addition (*RMSE* = 0.75 log CFU/mL) than in milk with 1 % NaCl (*RMSE* = 1.11 log CFU/mL). Therefore, these values are more about the different behaviour between *E. coli* strains, competitiveness, and sensitivity to NaCl than about variability. The lowest calculated *RMSE* values for LAB in all combinations ranged between 0.22 and 0.30 log CFU/mL. For *G. candidum* sensitive to NaCl, higher *RMSE* values of 0.49-0.69 log CFU/mL were found between co-culture growth in milk without NaCl and milk with the addition of 1 % NaCl.

The prediction capability of the H-GD model was also evaluated through the Bias (B_f) and Accuracy (A_f) factors [53] for each microorganism in the *E. coli* co-cultures. For *E. coli*, the HG-D model data of strain BR were validated with the experimental data of strain PS2. The calculated B_f values for the growth of *E. coli* strains were within 0.993–1.387 and the A_f values ranged from 1.283 to 1.704. With high probability, the prediction of *E. coli* growth was affected by the growth ability and sensitivity of the PS2 strain to the addition of NaCl.

LAB growth was accurately predicted for all co-culture cases and the calculated B_f values were within 0.996 to 1.003, while the A_f values ranged from 1.026 to 1.035 showed that, on average, the predicted value was 2.6 to 3.5 % different (either smaller or greater) from the observed values. Accurate prediction of LAB growth also confirmed the fact that their growth was minimally affected by other co-culture populations such as *G. candidum* or *E. coli* as the values of the interaction coefficient I_{LE} @ 1.0 indicated before (Table 1).

3.2.2. *S. aureus* strains in co-cultures

According to data in Table 2, the fast-growing strain 2064 of *S. aureus* compared with the numbers of strain 14733 showed a large difference in milk and milk with 1 % NaCl (*RMSE* = 0.66 and 0.91 log CFU/mL, respectively). On the other hand, the differences within the same strain but between different media (milk without NaCl vs. 1 % NaCl) were lower in the case of halotolerant *S. aureus*, 0.39 and 0.34 for the strains 2064 and 14733, respectively. Unlike *E. coli*, these *RMSE* values pointed out strain variability. The lowest calculated *RMSE* values for populations in the background were also calculated for LAB in all combinations and ranged between 0.19 and 0.30 log CFU/mL. For *G. candidum* the higher *RMSE* values of 0.51-0.63 log CFU/mL were calculated between their numbers in milk and milk with the addition of 1% NaCl, which also confirmed its sensitivity to NaCl.

Within the validation indices (B_f and A_f), the fast-growing strain 2064 model data and the experimental data of strain 14733 were used. While the calculated B_f values for the growth of *S. aureus* strains were 1.184 and 1.284, the A_f values were 1.371 and 1.500 in milk and 1 % NaCl in milk, respectively.

LAB growth was accurately predicted in all cases of co-culture with *S. aureus* since the calculated B_f values were within 0.980 to 1.016 and the A_f values ranged from 1.023 to 1.035. Furthermore, the values of the interaction coefficient I_{LS} varied between 0.91 and 1.26 (Table 2).

Referring to interpretation of the B_f when used for model performance evaluations involving pathogens, three categories were recommended by [64]. B_f in the range of 0.90 to 1.05 can be considered good; 0.70 to 0.90 or 1.06 to 1.15 can be considered acceptable and less than 0.70 or greater than 1.15 should be considered unacceptable. In almost all cases with two strains of each *E. coli* and *S. aureus* contaminant in our study, the values of the B_f factors were in the range of 0.90-1.05, which means that the H-GD model can be considered as suitable also for growth prediction in co-cultures with three different microbial populations.

4. Conclusions

The behaviour of microbial co-culture populations was successfully described with the H-GD model for the exponential growth phase in combination with secondary Ratkowsky and Bigelow models for declination phase. After the early stationary phase reached by LAB, *S. aureus* responded less competitively and more sensitively to the lactic acid produced by LAB than *E. coli*. However, the different behaviour of the *E. coli* strains found in this work may be associated more with different properties between strains (competitiveness and sensitivity to NaCl) than with only variability, in general. Internal but cross-validation provided acceptable values for the predictions applicable in cheesemaking practice. The LAB culture showed stable growth in all co-culture trials. Growth of *G. candidum* was negligibly reduced compared to the single growth of this yeast in milk, but growth appears not to be inhibited by the presence of LAB nor *E. coli* or *S. aureus* and reached its typical maximum density in a stationary growth phase.

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