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Modelling the biphasic growth of non-starter lactic acid bacteria on starter-lysate as a substrate

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Abstract

Since cheese is poor in energy for bacterial growth, it is believed that non-starter lactic acid bacteria growth and flavour development are supported by the nutrients from lysis of the starter culture. This study was performed to investigate the dynamics of interaction between starter and non-starter strains from cheese. A starter culture lysate was prepared by enzymatic digestion and tested as a growth substrate for *Lactobacillus* sp. strains. The two starter culture strains of *Lactococcus lactis* were also tested on the starter-lysate. All seventeen strains were individually inoculated at the level of $5.0 \log_{10} \text{ cfu mL}^{-1}$ in M17 broth, with or without 10 % starter-lysate, and incubated at 30 °C for 140 h. The optical density_{600 nm} was modelled with the primary log-transformed Logistic model with delay and lag phase duration, maximum specific growth rate as well as maximum population density obtained. Biphasic growth was mainly observed when the strains were able to utilize the starter-lysate as an energy source. To deal with the lack-of-fit related to the biphasic growth, the observed data points of the curve were divided after graphic evaluation and according to deviation of the residuals from the range ± 0.05 . Modelling was then

performed in two phases by applying the same primary Logistic model in each of the two parts of the growth curve. Values of root-mean-square error and graphic evaluation indicated the good fitting of the data with the suggested approach. The growth of the two *Lactococcus lactis* strains was not affected by the starter-lysate. However, thirteen of the non-starter strains had their growth rates increased. The increase was greatest for *Lactobacillus rhamnosus* KU-LbR1, which reached maximum optical densities of 0.23 and 0.58 in the absence and the presence of starter-lysate, respectively. No effect of the starter-lysate was shown for the growth of *Lactobacillus curvatus* strains. The extend of the growth of non-starter strains on the starter-lysate was shown to be species and strain dependent.

Keywords: *Lactobacillus*; *Lactococcus*; bacterial interaction; enzymatic digestion; cheese isolates; biphasic model

Introduction

The microbial ecology of cheese involves a rich and complex interaction between the starter lactic acid bacteria (SLAB) and the non-starter lactic acid bacteria (NSLAB). The SLAB in the cheese decline throughout ripening while growth of NSLAB increases as ripening progresses (Gantzias et al., 2020; Mugampoza et al., 2020; Settanni and Moschetti, 2010).

While *Lactococcus lactis* serves as the starter lactic acid bacteria (SLAB) for the manufacture of many cheese types, Lactobacilli constitute the majority of NSLAB in long time ripened cheeses (Broadbent et al., 2013; Calasso et al., 2020; Gobbetti et al., 2015). The NSLAB, mainly originating from raw milk and/or from the environment, contribute to the final characteristics of the cheese (Alessandria et al., 2016; de Pasquale et al., 2019; Saidi et al., 2020). The main NSLAB species predominating in cheese are *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus brevis*. Typical NSLAB cell densities in cheeses such as Cheddar cheese range from 10^2 to 10^4 cfu g⁻¹ during the first 5-10 days ripening and increase to about 10^7 to 10^8 cfu g⁻¹ after a few months ripening. Lactobacilli in cheese grow on the amino acids released from enzymatic hydrolysis of casein, as well as on the cell contents of the starter bacteria. NSLAB are tolerant to pH values as low as 4.9, salt up to 6 % and a wide range of temperature (2-53 C°) conditions (Gobbetti et al., 2015; Montel et al., 2014; Pangallo et al., 2019; Peterson and Marshall, 1990; Wouters et al., 2002).

The importance of autolysis of the starter lactic acid bacteria in cheese ripening is evident from the literature. This concept of cannibalism of SLAB by NSLAB was proposed by Thomas (1987) and it has been shown in other studies investigating the effect of sugars and amino acids in supporting NSLAB growth (Adamberg et al., 2005; Møller et al., 2020;

Ortakci et al., 2015; Saidi et al., 2020; Wallace and Fox, 1997). Czarán et al. (2018) suggested that the concentration of nutrients released from SLAB is an important factor influencing the rate of NSLAB growth during cheese ripening.

This study was performed in order to investigate the dynamics involved in the bacterial interaction between SLAB lysis and NSLAB growth using an *in vitro* model to simulate the conditions found during Cheddar cheese ripening. A starter-lysate prepared from a starter culture was investigated as a growth substrate for different NSLAB strains. The effect of the starter-lysate was modelled and evaluated, in order to get a better understanding of the mechanisms as well as generating insights for improving and accelerating cheese ripening.

2. Material and Methods

2.1 Starter-lysate preparation

A mesophilic homofermentative culture, type O, containing a combination of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* (Frozen-Direct Vat Set (F-DVS) of R-607, Chr. Hansen S/A, Hørsholm, Denmark) was used for preparation of the starter-lysate. The starter-lysate was obtained by enzymatic digestion of the starter culture applying treatment with lysozyme, phospholipase, proteinase, DNase and RNase. This protocol was designed to release of N-acetylmuramic acid, N-acetylglucosamine and amino acids from the cell wall, and ribose as well as nucleic acids from DNA and RNA. The starter culture (500 g) was melted and centrifuged (6,000 x g) for 10 min at 4 °C. The supernatant was discarded, and the initial volume was reached again by adding a 0.9 % sterile NaCl solution, the cells were resuspended by mixing 5 min on a vortex and centrifuged (6,000 x g for 10 min at 4 °C). The same procedure was performed three times and finally a 0.03 M sterile Tris-HCl (Sigma-Aldrich, Copenhagen, Denmark) buffer (pH 8.0) was added (8.33 mL g pellet⁻¹). After supplementing with 0.3 mg mL⁻¹ lysozyme (Sigma-Aldrich) and 5.0 µg mL⁻¹ phospholipase (Sigma-Aldrich), a three hours incubation took place with slow shaking at 37 °C. Subsequently, 6.0 µg mL⁻¹ DNase (Sigma-Aldrich) and 28 µg mL⁻¹ RNase (Sigma-Aldrich) were added and the described incubation with shaking at 37 °C for 2.5 h. Finally 75 µg mL⁻¹ proteinase K (Sigma-Aldrich) was added and the sample incubated at 37 °C for 14 h, and then heated at 80 °C for 30 min to inactivate the enzymes. After a last centrifugation step (6,000 x g for 30 min at 4 °C), the supernatant was stored at -20 °C and designated as “starter-lysate”. This starter-lysate was analysed for sugars and amino acids content. Determination of sugars was performed by HPLC – High Performance Liquid Chromatograph (Lloyd, 2008) and the most relevant sugar was

analysed with enzymatic assay (Megazyme, Co. Wicklow, Ireland), according to the manufacture's instructions. Amino acids content in the starter-lysate was obtained with UPLC – Ultra Performance Liquid Chromatograph (Møller et al., 2020).

2.2 Lactic acid bacteria strains and inoculum preparation

Fifteen non-starter lactic acid bacteria (NSLAB) were investigated and included *Lb. brevis* CCUG 30670^T, *Lb. casei* subsp. *lactis* DSM 20011^T, *Lb. corniformis* CCUG 30666^T, *Lb. curvatus* DSM 20019^T, *Lb. curvatus* CCUG 30669^T, *Lb. delbrueckii* subsp. *bulgaricus* DSM 20081^T, *Lb. delbrueckii* subsp. *delbrueckii* CCUG 34222^T, *Lb. fermentum* DSM 20052^T, *Lb. helveticus* CCUG 30139^T, *Lb. parabuchneri* DSM 20057^T, *Lb. paracasei* NCFB 151^T, *Lb. plantarum* subsp. *plantarum* ATCC 14917^T, *Lb. rhamnosus* DSM 20021^T, as well as *Lb. casei* KU-LbC1 and *Lb. rhamnosus* KU-LbP1 (isolated in this study from a vintage Cheddar cheeses according to the method applied by Møller et al. (2020)). In addition, the two strains of starter lactic acid bacteria (SLAB), isolated from the starter culture R-607 (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*) were also investigated. The inoculum of each of the 15 NSLAB and the two starter culture representatives were prepared individually. Stocks of the isolates (kept in 20 % glycerol at -81 °C) were thawed, mixed and 100 µL transferred to 10 mL media in 50 mL tubes, and incubated for 24 h, applying conditions for *Lactobacillus* (MRS broth at 30 °C) and *Lactococcus* (M17 broth with lactose at 25 °C) (OXOID, Hampshire, England). One mL of the grown culture was centrifuged (6,000 x g for 10 min at 4 °C) and the pellet with the cells was resuspended, washed with an equal volume of 0.9 % NaCl, and centrifuged (6,000 x g for 10 min at 4 °C). The washed, diluted culture was then used as an inoculum for the growth experiments.

2.3 Growth experiments

Growth experiments were performed in microtiter plates with a total volume of 250 μL in each well. The strains were individually inoculated (about $5.0 \log_{10} \text{ cfu mL}^{-1}$) in M17 broth (no lactose added), with or without 10 % starter-lysate (v/v), and incubated at 30 °C for 140 h. To avoid evaporation the surface of the wells was covered with sterile filtrated paraffin oil. The same experimental design was applied in a second trial, in which an inoculation level of $7.0 \log_{10} \text{ cfu mL}^{-1}$ was used. In a third trial the starter-lysate was also tested at the level of 33 % (v/v) in M17 broth containing 5 % NaCl (v/v), in order to simulate the salt-in-moisture level in Cheddar cheese. Triplicates of negative control (non-inoculated media) and of each of the tested conditions was monitored with an absorbance reader (Biotek Elx808, Vermont, USA) by measuring optical density (OD) at 600 nm every hour. Microtiter plate were shaken for 30 seconds prior to reading.

2.3 Modelling

To evaluate the impact of the tested conditions on the stages of the bacterial life cycle, it was essential to apply appropriate primary modelling. Therefore, the primary log-transformed Logistic model with delay (Dalgaard, 2009) was selected. Before fitting of the observed data, the average of the optical density measured in triplicate at 600 nm was multiplied by 10,000 and log transformed. By applying the Excel solver function, lag phase duration (h), maximum specific growth rate ($\mu_{\max}$, h^{-1}) and maximum population density ($N_{\max}$, $\text{OD}_{600\text{nm}}$) were then obtained. A new modelling approach was also proposed, to deal with the lack of fit, believed to be related to the nutrient limited conditions. Details of the alternative modelling approach are described in the Results and Discussion (Section 3.2.2).

2.4. Data analysis

Graphic evaluation and values of RMSE (Root-Mean-Square Error) were used to evaluate the quality of the modelling fits, in relation to the observed growth of the strains (Møller et al., 2016). Good graphic evaluation and low values of RMSE were considered for selecting the most appropriate values of lag phase duration, $\mu_{\max}$ and $N_{\max}$. Student's paired *t*-test was applied to identify if differences in these values were significant at the level of 0.05 %, using R studio software (Version 3.6.1), and the effect of the starter-lysate on the growth phases of the tested strains was investigated.

Acceptable Simulation Zone (ASZ) suggested by Oscar (2005) was applied as described by Møller et al. (2013; 2016) to evaluate the quality of the simulations obtained by applying the parameters from the modelling approach in a dataset different from that used in the fittings. According to Møller et al. (2013) and Velugoti et al. (2011) a deviation of ± 0.5

$\log_{10}$ cfu mL⁻¹ between the observed and simulated values is acceptable. Therefore, the $\log_{10}$ OD₆₀₀ variance between datasets with Nmin within 0.5 $\log_{10}$ cfu mL⁻¹ difference ($\pm 0.14 \log_{10}$ OD₆₀₀) was used to create the ASZ. For practical reasons and to avoid large deviation between the first and the second phase of the suggested two-phases Logistic approach, a baseline was used to correct the 2 $\log_{10}$ CFU mL⁻¹ difference between the fitted and simulated dataset. This baseline correction was performed by the addition of 0.37 to each of the simulated data points (which is the percentual difference of Nmin between the two datasets).

3 Results and Discussion

3.1 Graphic evaluation of the effect of starter-lysate

Not surprisingly, none of the two SLAB representatives (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*) was affected by the addition of the starter-lysate to the medium, as shown in Figure 1. On the other hand, 13 of the tested NSLAB had increased growth when starter-lysate was added. According to Figure 2, a similar effect of starter-lysate addition on growth pattern of the tested NSLAB was shown between: 1) *Lb. casei* KU-LbC1 (C) and *Lb. rhamnosus* LbR1 (O); 2) *Lb. coryniformis* CCUG 30666 (D), *Lb. delbrueckii* subsp. *bulgaricus* DSM 20081^T (G), and *Lb. delbrueckii* subsp. *delbrueckii* CCUG 34222^T (H); 3) *Lb. helveticus* CCUG 30139^T (J) and *Lb. plantarum* subsp. *plantarum* ATCC 14917^T (M); and 4) *Lb. parabuchneri* DSM 20057^T (K) and *Lb. paracasei* NCFB 151^T (L). Starter-lysate seem to have no effect on growth of the *Lb. curvatus* strains (E and F). Despite the effect of the addition of the starter-lysate, shown on growth of most of the investigated LAB, more than visual inspection of the growth curves was required to evaluate the dimension of this effect. Before performing this detailed analysis, modelling of the observed data was performed. The modelling makes the data comparable and allows investigation of the effect of the starter-lysate at each stage of the growth curves.

3.2 Modelling approach development

3.2.1 Fitting with the log-transformed Logistic model with delay

To allow a more detailed investigation of the starter-lysate addition, the log-transformed data for the 17 strains was initially fitted with the primary log-transformed Logistic model with delay (Dalgaard, 2009). As indicated with the high RMSE values (Table 1, RMSE (1)) and also exemplified in Figures 3A, 3C and 3E, the lack-of-fit was observed for most of the investigated growth curves, when applying the simple log-transformed logistic model with delay to the observed growth curves. Visual inspection of the curves when fitted to the simple log-transformed logistic model clearly indicated that the lack of fit was due to a biphasic growth behaviour.

The challenges in modelling biphasic growth has been reported by Guerra et al. (2010) when investigating *Enterococcus faecium* in realkalized fed-batch fermentations in whey, and many attempts have been suggested to overcome the lack-of-fit when applying traditional primary models to describe biphasic growth. As reported by Wilson et al. (2017) such models can improve the understanding of the life cycle of microorganisms, however it can be more complicated to use than conventional growth models, due to the need for high amounts of data, multiple growth phases and increased parameterization.

3.2.2 Dealing with the lack-of-fit

As suggested by Guerra et al. (2010) and Meyer (1994), in the case of biphasic growth, the most appropriate modelling approach is based on the sum of two simple growth phases. In search for a practical but an efficient and meaningful solution, a two-phases Logistic modelling approach is proposed in the present study.

The first step of the proposed two-phases Logistic modelling approach was to analyze the curves fitted with the one phase approach (Figures 3A, 3C and 3E). By graphic evaluation and considering the data point when consistent deviation of the residuals in ± 0.05 was shown, the curves were split and the same log-transformed Logistic model with delay (section 3.2.1) was applied for fitting each of the two sections of the observed curves, individually. Then, the fitted values from each of the sections were aligned in a single curve, the RMSE evaluated, the N_{min} and N_{max} determined and the sum of Lag phase durations and μ_{max} in the two phases was performed.

The graphic evaluation of the fittings are exemplified in Figures 3B, 3D and 3F. In addition, the much lower RMSE values in Table 1 (RMSE (2)) indicated a good description of the observed data by applying the proposed two-phase modelling approach. The improved fittings obtained with the proposed two-phases Logistic modelling approach was shown both when starter-lysate was added (Figures 3B and D) and when grown without starter-lysate (Figure 3F).

3.3 Detailed evaluation of the effect of starter-lysate

This evaluation focused on the growth phases of the 15 investigated NSLAB. To improve the robustness of the investigation, the graphic evaluation (Figures 2 and 3) was combined with the analysis of the parameter estimates in Table 1. Parameter estimates in Table 1

are the sum of the parameter estimates obtained from the first (parameters in Table 2) and second phases of the proposed modelling approach. The parameter estimates related to the second phase of the modelling can be accessed by subtracting the values in Table 2 from the values in Table 1.

Most of the tested NSLAB showed an increased in growth. While 12 strains had increased $\mu_{\max}$ values, 10 strains had increased $N_{\max}$ when starter-lysate was added. However, the degree of the effect varied between the species, with strains such as the cheese isolates *Lb. casei* KU-LbC1 and *Lb. rhamnosus* KU-LbR1 (Figures 2C, 2O and Table 1) and the *Lb. coryniformis* CCUG 30666^T (Figure 2D and Table 1) showing a high degree of the effect of the starter-lysate and the strains of *Lb. curvatus* showing much less effect (Figures 2E, 2F and Table 1). In fact, decline of the level of *Lb. curvatus* cells initiated after about 20 h of incubation and the rate of this decline was accelerated in presence of starter-lysate, especially after 110. Porcellato et al. (2015) have also showed decay of *Lb. curvatus* after extended periods of incubation. Despite the increase of only 3 % in the $\mu_{\max}$ of *Lb. parabuchneri* DSM 20057^T, its $N_{\max}$ was increased by 84 %, while for *Lb. coryniformis* CCUG 30666^T the increase of $N_{\max}$ was only 72 % regardless of the 100 % increase in $\mu_{\max}$ (Table 1). This indicates that while for some species of NSLAB the effect of starter-lysate was immediate and $N_{\max}$ reached at an earlier stage, for other NSLAB this process occurs gradually due to a slower cell adaptation to the extra nutrients, supplied by the starter-lysate. The same phenomena seems to occur not only between different species but also within strains of the same species. In Table 1, the parameter estimates and the shape of the curves obtained for the two strains of *Lb. delbrueckii* (Figures 2G and 2H) and the two strains of *Lb. rhamnosus* (Figures 2N and 2O) are different. When looking into the source of the obtained strains (Table 2), the combined

increase in $\mu_{\max}$ and $N_{\max}$, and thus effectiveness of the starter-lysate in increasing the growth of the strains, seems to be highest for the cheese isolates *Lb. casei* KU-LbC1 and *Lb. rhamnosus* KU-LbR1.

3.4 Effect of inoculation level

In Figure 4, the observed growth of *Lb. casei* KU-LbC1 (circular symbols) in absence (B) and in presence (E) of starter-lysate is shown at inoculation level of $7.0 \log_{10}$ CFU mL⁻¹. The parameter estimates obtained at the inoculum level of $5.0 \log_{10}$ CFU mL⁻¹ (Tables 1 and 2) and when inoculating *Lb. casei* KU-LbC1 at the level of $7.0 \log_{10}$ CFU mL⁻¹ (Table 3) were compared. At the levels of $7.0 \log_{10}$ CFU mL⁻¹ no lag phase was shown and the magnitude of the effect of starter-lysate on $N_{\max}$ was much lower. Contrary to what is shown for $\mu_{\max}$ when applying an inoculation level of $5.0 \log_{10}$ CFU mL⁻¹, no effect on $\mu_{\max}$ was shown with an inoculation level of $7.0 \log_{10}$ CFU mL⁻¹. Removing the lag phase and applying parameters (Tables 1 and 2) from the lower inoculation level (black lines in Figures 4A and 4D) on observed growth (circular symbols in Figures 4A and 4D) at $7.0 \log_{10}$ CFU mL⁻¹, it was shown that the shape of the curve was very similar in the absence of starter-lysate (Figure 4A), except for the underestimation of the exponential phase, because it occurred faster at an inoculation level of $7.0 \log_{10}$ CFU mL⁻¹. The same underestimation of the exponential phase was also shown in the presence of starter-lysate (black line in Figure 4D). Since a lower effect of starter-lysate was shown when applying a higher level of inoculation, the parameters obtained for $5.0 \log_{10}$ CFU mL⁻¹ (Tables 1 and 2) had a different shape (black line in Figure 4D) than the observed values (circular symbols in Figure 4D), due to an overestimation of the maximum population density.

3.5 Effect of starter-lysate concentration and NaCl

When comparing with the same conditions, in the absence of NaCl (Figures 4B and 4C in Table 3), the effect of NaCl was clear, resulting in a drastic reduction of the $\mu_{\max}$ and $N_{\max}$, even when 10 % of starter-lysate was present (grey and dashed black lines in Figure 5 and Table 3, respectively). When 33 % starter-lysate was added to the medium with NaCl (solid black line in Figure 5 and Table 3), $\mu_{\max}$ was higher and about the same as in the scenario with 10 % starter-lysate, but without NaCl (Fig 4-E and Fig 4-F in Table 3). However, even in presence of 33 % starter-lysate when NaCl was added, $N_{\max}$ was about 40 % lower than in the absence of NaCl. The effect of NaCl in promoting autolysis and consequently decay of optical density during incubation of *Lb. casei* was reported by Kang et al. (1998). The NaCl content also limited the time of stationary phase, causing the decline of *Lb. casei* KU-LbC1 after 28 h without starter-lysate (triangular symbols in Figure 5), or after 45 h in the presence of 10 % starter-lysate (squared symbols in Figure 5). The decline was not shown after 140 h (Figure 4) without NaCl, or before 60 h when 33 % of starter-lysate was added (circular symbols in Figure 5), even in presence of 5.0 % NaCl. This indicates that in the presence of higher concentrations of starter-lysate an increased growth may be expected, even under the limiting conditions as those found during cheese ripening. This is in agreement with Czarán et al. (2018) who hypothesized that the availability and the level of nutrients released from SLAB are the main factors determining the rate of NSLAB growth during Cheddar cheese ripening.

3.6 Composition of the starter-lysate

N-acetylglucosamine was present at the level of $2.47 \pm 0.01 \text{ g L}^{-1}$ in the starter-lysate.

Changes in levels of this sugar were then monitored (Figure 6), before (bars in light grey)

and after (bars in dark grey) 70 h incubation at 30 °C of *Lb. rhamnosus* KU-LbR1, in M17 broth (no lactose) without and with addition of 10 % starter-lysate. It was shown that the level of N-acetylglucosamine in the M17 broth without starter-lysate was about three times lower than in the M17 broth with starter-lysate (light grey bars in Figure 6), before incubation. While the level of N-acetylglucosamine was nearly exhausted in the M17 broth without starter-lysate after 70 h incubation (dark grey bars in Figure 6), the M17 broth with the starter-lysate had more than half of the initial concentration of N-acetylglucosamine still available, which may explain the increased N_{max} reached by most of the tested NSLAB (Figure 2). This is in agreement with the findings of Adamberg et al. (2005), who simulated in a carbohydrate-restricted medium the effect of N-acetylglucosamine, that could potentially be released from lysed bacterial cells. In the present study, the tested concentrations of 10 and 33 % starter-lysate added to the M17 broth 0.24 and 0.80 g L⁻¹ N-acetylglucosamine, respectively. These levels are below the N-acetylglucosamine levels of 2.5 and 10 g L⁻¹ investigated previously on growth of NSLAB (Adamberg et al., 2005). No relevant differences in the amino acids profile was shown between the M17 broth without (Figure 7A) or with 10 % starter-lysate (Figure 7B) before incubation, or after 70 h incubation of *Lb. rhamnosus* KU-LbR1 at 30 °C in M17 broth without (Figure 7C). The increased level of the ammonium ion could be due to the decarboxylation of amino acids, however, considering the high level of ammonium ion, it is more probably that this ion is the product of the metabolism of N-acetylglucosamine. Vesely et al. (2017) have reported that catabolism of N-acetylglucosamine releases ammonia, which is similar to growth on amino acids. Further investigation would be necessary in order to clarify this point. Nevertheless, these results indicate that N-acetylglucosamine content in the starter-lysate is the predominant factor in boosting the NSLAB growth.

3.7 Performance of the two-phases Logistic approach

The suggested biphasic modelling approach was challenged on data from a second trial (Figure 4 and Table 3), developed with *Lactobacillus casei* KU-LbC1 applying the same conditions as in the first trial, excepting for a higher inoculation level ($7.0 \log_{10}$ CFU mL⁻¹). The performance of the simulations obtained with the suggested approach applying the parameters of the fittings, shown in Tables 1 (sum of parameters from phases 1 and 2) and 2 (only parameters from phase 1) was evaluated through the acceptable simulation zone (ASZ) concept (Oscar, 2005). With the corrected baseline (as described in section 2.4), most of the simulated values (93.0 %) were within the ASZ (zone between dotted lines in Figure 4) when the availability of nutrients was low (Figure 4A). However, it is important to take into account that when the N_{min} of the observed data was $7.0 \log_{10}$ cfu mL⁻¹ the N_{max} was reached at an earlier stage and therefore the data points outside ASZ was underestimated during the exponential phase, which makes the simulation of a fail-dangerous nature. As pointed out by Oscar (2011), this fail-dangerous feature needs to be taken into consideration. The simulation performed on the same dataset but with addition of starter-lysate gave an even poorer description of the observed data (Figure 4D) with only 43.0 % of the simulated data points inside the ASZ. Nevertheless, the suggested two-phases approach gives a reasonable description of the observed data, according to the low RMSE values (Table 3) and the graphic evaluation both at reduced (Figures 4B and 4C) and at increased (Figures 4E and 4F) availability of nutrients, when applied for fitting purpose. The good performance of the model (Figures 4B and 4E) was achieved when using the split point between phase 1 and phase 2 (last data point in the fitting of the first phase of the approach, as shown in Table 2). This is particular relevant, considering that

the decision making for the split point is very time consuming, requiring fitting of the observed data with the traditional one-phase Logistic model, followed by graphic evaluation and defining of the persistent deviation of the residuals (± 0.05). As shown in (Figures 4C and 4F) and according to the RMSE values (Table 3) the improvement of the fittings is not always achieved (Figure 4F) or significant. Therefore, the split point indicated in Table 2 seems to be independent of the inoculation level, and may be useful when applying the two-phases Logistic approach on the conditions tested in this study. By fitting the observed data in a third trial (Figure 5), it was shown that besides the different initial density of LAB, the good fitting with the proposed approach was also achieved when an extra stress factor was included (5.0 % NaCl). Furthermore, the goodness of fit was shown to be independent of the level of nutrients as in the case where the level of nutrients was low (triangular symbols in Figure 5), or when starter-lysate was added at the level of 10 % (square in Figure 5) or 33 % (circular symbols in Figure 5), confirmed by graphic evaluation (lines in Figure 5) and the low values of RMSE (Table 3).

4. Conclusions

The suggested two-phases Logistic approach was successfully applied and allowed the evaluation of the effect of starter culture lysate on NSLAB growth. The starter-lysate boosted the growth of 13 of the tested NSLAB. This boosting effect was shown to be species as well as strain dependent. The effect of the starter-lysate was more prominent when NSLAB were inoculated at low levels. The starter-lysate was able to boost growth of NSLAB even when NaCl was present at high concentration. By increasing the level of the starter-lysate the boosting effect in NSLAB growth was maximized and it nearly annulled the stress effect of salt. Furthermore, the effect of the starter-lysate was greatest on the growth of *Lb. casei* KU-LbC1 and *Lb. rhamnosus* KU-LbR1, isolated from vintage Cheddar cheese. The presence of N-acetylglucosamine in the starter-lysate and its decline during NSLAB development indicates that this may be the predominant nutrient, responsible for the boosting effect on NSLAB growth. In conclusion, the developed modelling approach enabled investigating of the dynamics of starter-NSLAB interactions, which may allow the scientific community to generate new insights about the mechanisms involved in these bacterial interaction during cheese ripening, and support the industry to improve quality in cheese production.

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Tables

Table 1. Sum of growth parameters^{□,β} obtained modelling the effect of starter culture lysate on incubation^{*} of lactic acid bacteria (LAB)

LAB	Without starter-lysate ^α						With starter-lysate ^α					
	Lag phase ^β (h)	μ _{max} ^β (h ⁻¹)	Nmin (OD _{600nm})	Nmax (OD _{600nm})	RMSE (1)	RMSE (2)	Lag phase ^β (h)	μ _{max} ^β (h ⁻¹)	Nmin (OD _{600nm})	Nmax (OD _{600nm})	RMSE (1)	RMSE (2)
<i>Lc. lactis</i> subsp. <i>cremoris</i> (starter)	4.682	0.694	0.0007	0.1246	0.044	0.014	25.11 [∇]	0.709	0.0013	0.1235	0.051	0.027
<i>Lc. lactis</i> subsp. <i>lactis</i> (starter)	2.101	0.849	0.0014	0.1272	0.052	0.018	0.9 [*]	0.863	0.0005	0.1418	0.099	0.013
<i>Lb. brevis</i> CCUG 30670 [†]	1.953	0.412	0.0007	0.1524	0.032	0.01[*]	2.405 [∇]	0.508 [*]	0.0013	0.3105 [*]	0.053	0.014
<i>Lb. casei</i> subsp. <i>lactis</i> DSM 20011 [†]	0.000	0.243	0.0053	0.1549	0.032	0.00[*]	1.218 [∇]	0.324 [*]	0.0063	0.2163 [*]	0.060	0.006
<i>Lb. casei</i> (KU-LbC1)	3.887	0.399	0.0020	0.2108	0.111	0.014	3.848	0.590 [*]	0.0005	0.5659 [*]	0.047	0.020
<i>Lb. coryniformis</i> CCUG 30666 [†]	2.129	0.530	0.0007	0.1650	0.297	0.026	5.516 [∇]	1.062 [*]	0.0007	0.2842 [*]	0.030	0.023
<i>Lb. curvatus</i> CCUG 30669 [†]	3.690	0.899	0.0020	0.0230	0.27 [*]	0.171	0.938 [*]	0.717 [∇]	0.0001	0.0811	0.365	0.022
<i>Lb. curvatus</i> DSM 20019 [†]	2.709	0.875	0.0010	0.02 [∇]	0.26 [*]	0.025	1.536 [*]	0.590 [∇]	0.0013	0.0832	0.446	0.027
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> DSM 20081 [†]	4.000	0.449	0.0005	0.1589	0.037	0.038	3.757 [*]	0.711 [*]	0.0005	0.6644 [*]	0.050	0.025
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i> CCUG 34222 [†]	7.311	0.599	0.0003	0.1 [*]	0.059	0.039	3.930 [*]	0.690 [*]	0.0005	0.3981 [*]	0.048	0.020
<i>Lb. fermentum</i> DSM 20052 [†]	13.890	0.215	0.0010	0.03 [∇]	0.023	0.018	12.308 [*]	0.265 [*]	0.0010	0.1370 [*]	0.027	0.018
<i>Lb. helveticus</i> CCUG 30139 [†]	2.181	0.503	0.0007	0.2882	0.082	0.022	3.292 [∇]	0.706 [*]	0.0005	0.3271	0.031	0.018
<i>Lb. parabuchneri</i> DSM 20057 [†]	2.731	0.404	0.0010	0.0907	0.059	0.029	2.437 [*]	0.415	0.0017	0.1665 [*]	0.044	0.013
<i>Lb. paracasei</i> NCFB 151 [†]	2.327	0.351	0.0008	0.0495	0.028	0.026	3.681 [∇]	0.386 [*]	0.0012	0.0934	0.037	0.025
<i>Lb. plantarum</i> subsp. <i>plantarum</i> ATCC 14917 [†]	1.811	0.455	0.0007	0.2846	0.084	0.034	4.667 [∇]	0.743 [*]	0.0007	0.3191	0.028	0.018
<i>Lb. rhamnosus</i> DSM 20021 [†]	3.582	0.330	0.0013	0.3252	0.087	0.019	3.483	0.484 [*]	0.0012	0.4840 [*]	0.104	0.018
<i>Lb. rhamnosus</i> (KU-LbR1)	2.610	0.334	0.0019	0.2341	0.128	0.028	3.298 [∇]	0.518 [*]	0.0007	0.5629 [*]	0.190	0.019

[□] Lag phase duration (h), maximum specific growth rate (μ_{max}), minimum (Nmin) and maximum (Nmax) population density were obtained with the primary log transformed Logistic model with delay, fitting the averaged data obtained in triplicate (multiplied by 10,000 and log transformed) with the one phase approach (RMSE 1) and with the two phases approach (RMSE 2)

^{*} LAB were inoculated at the level of 5.0 log₁₀ cfu mL⁻¹ in M17 broth (no lactose) with and without 10 % starter culture lysate and incubated at 30 °C for 140 h. The 141 data points per growth curve were obtained by measuring the optical density every hour after shaking.

^α Values of lag phase, μ_{max}, Nmin and Nmax were fitted using the modelling approach with lower values of RMSE (highlighted in bold).

^β Lag phase duration and μ_{max} obtained by applying the two-phases approach are the sum of values obtained in each phase.

Differences in the parameter estimates identified as significant at the level of 0.05 % with the Student's paired *t*-test were classified as: ^(*) Positive effect of starter-lysate on growth of LAB; or

^(∇) Negative effect of starter-lysate on growth of LAB.

Table 2. Growth parameters from the first phase modelling of the data testing the effect of starter culture lysate on incubation of lactic acid bacteria (LAB)

LAB	Source	Without starter-lysate				With starter-lysate			
		Last data point ^α in the fitting	Lag phase ^β (h)	$\mu_{\max}^{\beta}$ (h ⁻¹)	Nmax ^β (OD _{600nm})	Last data point ^α in the fitting	Lag phase ^β (h)	$\mu_{\max}^{\beta}$ (h ⁻¹)	Nmax ^β (OD _{600nm})
<i>Lc. lactis</i> subsp. <i>cremoris</i>	Starter culture	25	4.682	0.685	0.1233	25	5.428 [∇]	0.709	0.1149 [∇]
<i>Lc. Lactis</i> subsp. <i>lactis</i>	Starter culture	30	2.088	0.840	0.1189	30	0.976 [*]	0.861	0.1310 [*]
<i>Lb. brevis</i> CCUG 30670 [†]	Human feces	27	1.953	0.447	0.1330	17	2.405 [∇]	0.433	0.1726 [*]
<i>Lb. casei</i> subsp. <i>lactis</i> DSM 20011 [†]	Cheese	33	0.000	0.241	0.116 [*]	20	1.218 [∇]	0.294	0.1106
<i>Lb. casei</i> (KU-LbC1)	Vintage Cheddar cheese	25	3.887	0.370	0.072 [*]	20	3.342 [*]	0.537 [*]	0.0618 [∇]
<i>Lb. coryniformis</i> CCUG 30666 [†]	Silage	32	2.129	0.379	0.145 [*]	25	5.516 [∇]	0.841 [*]	0.2422 [*]
<i>Lb. curvatus</i> CCUG 30669 [†]	Milk	20	3.690	0.915	0.1230	20	0.934 [*]	0.753 [∇]	0.0778 [*]
<i>Lb. curvatus</i> DSM 20019 [†]	Milk	20	2.808	0.890 [*]	0.0253	20	1.536 [*]	0.610 [∇]	0.0810 [*]
<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> DSM 20081 [†]	Bulgarian yoghurt	141	-	-	-	40	3.649	0.652 [*]	0.5164
<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i> CCUG 34222 [†]	Sour grain mash	103	3.242	0.555	0.1418	20	3.927 [∇]	0.638 [*]	0.2864 [*]
<i>Lb. fermentum</i> DSM 20052 [†]	Fermented beets	65	12.607	0.213	0.0335	49	12.253 [*]	0.265	0.1188 [*]
<i>Lb. helveticus</i> CCUG 30139 [†]	Emmental cheese	30	2.152	0.475	0.1589	35	3.663 [∇]	0.706 [*]	0.2745 [*]
<i>Lb. parabuchneri</i> DSM 20057 [†]	Tomato pulp	30	2.751	0.408	0.0873	40	2.437 [*]	0.418	0.1649 [*]
<i>Lb. paracasei</i> NCFB 151 [†]	Milk products	70	5.169	0.355	0.0486	34	3.934 [∇]	0.382	0.0724 [*]
<i>Lb. plantarum</i> subsp. <i>plantarum</i> ATCC 14917 [†]	Pickled cabbage	40	1.811	0.450	0.1568	40	4.667 [∇]	0.745 [*]	0.2772 [*]
<i>Lb. rhamnosus</i> DSM 20021 [†]	Unknown	40	3.582	0.305	0.1601	20	3.483	0.397 [*]	0.0849 [∇]
<i>Lb. rhamnosus</i> (KU-LbR1)	Vintage Cheddar cheese	40	2.611	0.261	0.0845	20	3.298 [∇]	0.460 [*]	0.0570 [∇]

^α Lag phase duration (h), maximum specific growth rate ($\mu_{\max}$), minimum (Nmin) and maximum (Nmax) population density were obtained with the primary log transformed Logistic model with delay, fitting the averaged data obtained in triplicate (multiplied by 10,000 and log transformed) with the first phase of the two phases approach.

[†] LAB were inoculated at the level of 5.0 log₁₀ cfu mL⁻¹ in M17 broth (no lactose), with and without 10 % starter culture lysate and incubated at 30 °C for 140 h. The 141 data points per growth curve were obtained by measuring the optical density every hour after shaking.

^α Selected by graphic performance + identification of the time point with persistent deviation of residual values (± 0.05). This data point indicates the start of the second phase of this two-phase modelling approach

^β Lag phase duration, $\mu_{\max}$ and Nmax values for the second phase of this two-phases approach can be obtained by abstracting the values in this table from the total values shown in Table 1.

Differences in the parameter estimates identified as significant at the level of 0.05 % with the Student's paired *t*-test were classified as: ^(*) Positive effect of starter-lysate on growth of LAB; or

^(∇) Negative effect of starter-lysate on growth of LAB.

Table 3. Challenging the biphasic modelling approach by testing presence of NaCl (5 %), different concentrations of starter lysate on growth of *Lactobacillus casei* KU-LbC1 inoculated at high level ($7.0 \log_{10}$ cfu mL⁻¹)

Fitting* code	NaCl added (%)	Starter-lysate level (%)	Time of incubation (h)	Phase 1					Phase 2				RMSE ^β
				Last data point in the fitting	Lag phase (h)	μmax (h ⁻¹)	Nmin (OD _{600nm})	Nmax (OD _{600nm})	First data point in the fitting	Lag phase (h)	μmax (h ⁻¹)	Nmax (OD _{600nm})	
Figure 4B	-	-	140	25 [∇]	-	0.390 ^a	0.0124	0.1453 ^a	25 [∇]	0.507	0.032 ^a	0.3786 ^{a,c}	0.0317 ^a
Figure 4C	-	-	140	16 ^α	-	0.439 ^a	0.0116	0.1292 ^b	16 ^α	0.665	0.029 ^a	0.3878 ^{a,c}	0.0303 ^a
Figure 4E	-	10	140	20 [∇]	0.602	0.342 ^b	0.0173	0.1497 ^a	20 [∇]	-	0.064 ^b	0.6330 ^b	0.0241 ^b
Figure 4F	-	10	140	15 ^α	-	0.352 ^b	0.0149	0.1372 ^c	15 ^α	-	0.060 ^b	0.6375 ^b	0.0259 ^b
Figure 5 (grey line)	5	-	70	25 ^α	0.171	0.198 ^c	0.0393	0.1671 ^d	25 ^α	0.016	-0.044 ^c	0.2305 ^d	0.0165 ^c
Figure 5 (dashed black line)	5	10	70	50 ^α	-	0.190 ^c	0.0297	0.1165 ^e	50 ^α	0.157	-0.081 ^d	0.3401 ^c	0.0308 ^a
Figure 5 (solid black line)	5	33	70	10 ^α	-	0.368 ^b	0.0210	0.1223 ^f	10 ^α	0.371	0.084 ^e	0.3950 ^a	0.0314 ^a

[□] Lag phase duration (h), maximum specific growth rate (μmax), minimum (Nmin) and maximum (Nmax) population density were obtained with the primary log transformed Logistic model with delay, fitting the averaged data obtained in triplicate (multiplied by 10,000 and log transformed) with the two phases approach.

[•] M17 broth (no lactose), with and without 10 or 33 % starter culture lysate, in absence or presence of NaCl (5 %), was incubated at 30 °C for 70 h (Fig 5) or 140 h (Fig 4). The data points were obtained by measuring the optical density every hour after shaking.

^{*} Same codes used in Figure 4 (B to F) and Figure 5 (lines), where the related growth curves are shown.

[∇] The same time point applied when modelling the dataset inoculated at the level of $5.0 \log_{10}$ cfu mL⁻¹ and shown in Table 2.

^α Selected by graphic performance + residual values indicating difference larger than ± 0.05 continuously in the remaining time points.

^β RMSE values were obtained with all data points of the entire curve, excluding the first data point in the fitting of the second phase of the modelling approach, to avoid repetition of this data point.

^{a, b, c} Within a same column, values of parameter estimates and RMSE with same letters indicates that the differences are not significant at the level of 0.05 %, according to the Student's paired *t*-test.

Figure 1 Growth of SLAB in presence (closed symbols in black) and absence (open symbols in grey) of 10 % starter lysate, added to the M17 broth (without lactose). Starter culture representatives, *Lactococcus lactis* subsp. *cremoris* (A) and *Lactococcus lactis* subsp. *lactis* (B), were individually inoculated at the level of about $5.0 \log_{10}$ CFU mL⁻¹. Optical density at 600 nm was measured for 140 h at 30 °C

Figure 2 Growth of NSLAB in presence (closed symbols in black) and absence (open symbols in grey) of 10 % starter culture lysate, added to the M17 broth (without lactose). *Lb. brevis* CCUG 30670^T (A), *Lb. casei* subsp. *lactis* DSM 20011^T (B), *Lb. casei* KU-LbC1 (C), *Lb. coryniformis* CCUG 30666^T (D), *Lb. curvatus* DSM 20019^T (E), *Lb. curvatus* CCUG 30669^T (F), *Lb. delbrueckii* subsp. *bulgaricus* DSM 20081^T (G), *Lb. delbrueckii* subsp. *delbrueckii* CCUG 34222^T (H), *Lb. fermentum* DSM 20052^T (I), *Lb. helveticus* CCUG 30139^T (J), *Lb. parabuchneri* DSM 20057^T (K), *Lb. paracasei* NCFB 151^T (L), *Lb. plantarum* subsp. *plantarum* ATCC 14917^T (M), *Lb. rhamnosus* DSM 20021^T (N) and *Lb. rhamnosus* KU-LbR1 (O). Each strain was individually inoculated at the level of about $5.0 \log_{10}$ CFU mL⁻¹. Optical density at 600 nm measured for 140 h at 30 °C was multiplied by 10,000 before log-transformed.

Figure 3 Observed (symbols in grey) and fitted (line in black) growth of NSLAB in M17 broth (no lactose) with 10 % (A to D) or without (E and F) starter lysate at 30 °C for 144 h (OD_{600nm}). The primary log transformed Logistic model with delay was applied in a single phase (A, C and E) or using the proposed two-phases approach (B, D and F). Optical Density values were multiplied 10,000 times and Log₁₀ transformed, in order to perform modelling. *Lactobacillus rhamnosus* DSM 20021 T (●, A and B), *Lactobacillus rhamnosus* KU-LbR1 (▲, C and D) and *Lactobacillus casei* KU-LbC1 (■, E and F) were individually inoculated at the level of about $5.0 \log_{10}$ CFU g⁻¹.

Figure 4 Observed (symbols in grey), simulated with parameters in Tables 1 and 2 (black line in A and D), and fitted (black line in B, C, E and F) growth of *Lactobacillus casei* KU-LbC1, inoculated at the level of $7.0 \log_{10}$ cfu mL⁻¹ in M17 broth (no lactose) without (A, B and C) and with (D, E and F) starter lysate, during 140 h at 30 °C. The acceptable simulation zone (ASZ) concept was applied considering $OD \pm 0.14 \log_{10}$ OD₃₀₀ (limited by the dotted grey lines in A and D). Simulations performed testing absence (A) and presence (D) of starter lysate generated RMSE values of 0.264 and 0.376, respectively. Fittings were performed applying the same end point in phase 1 as indicated in Table 2 (B and E) or corrected (C and F) according graphic evaluation and residuals with difference below ± 0.05 .

Figure 5 Observed (symbols) and fitted (lines) growth of *Lactobacillus casei* KU-LbC1 (inoculated at $7.0 \log_{10}$ CFU mL⁻¹) in absence (triangular symbols and continuous grey line) or in presence of 10 % (squared symbols and dashed black line) or 33 % (circular symbols and continuous black line) starter culture lysate, added to the M17 broth (without lactose). NaCl was added to the level of 5.0 % to simulate conditions found in Cheddar cheese. Averaged of triplicate values of optical density obtained at 600 nm was measured for 70 h at 30 °C. The averaged data points were multiplied by 10,000 before log transformed, and then modelled with the two-phases approach.

Figure 6 Concentration of N-acetylglucosamine (g L⁻¹) in M17 broth without or with 10 % lysate. Samples inoculated with *Lactobacillus rhamnosus* KU-LbR1 were analysed before (light grey bars) and after (dark grey bars) 71 h at 30 °C with enzymatic assay. The results are shown as averaged triplicates and error bars are shown in black.

Figure 7 Amino acids profile (Absorbance Unit at 280 nm) before incubation of M17 broth without (A) and with (B) 10 % starter lysate, or after 70 h incubation of *Lb. rhamnosus* KU-LbR1 at 30 °C in M17 broth without (C) or with 10 % starter lysate (D)

Highlights

- An enzymatic protocol to digest starter culture was developed and applied
- The effect of the obtained lysate was tested on growth of 15 non-starter and 2 starter lactic acid bacteria (SLAB)
- Lack-of-fit by applying log-transformed Logistic model was shown due to biphasic growth
- A two-phases Logistic modelling approach was proposed and successfully applied
- N-glucosamine from SLAB cell wall seem to be the responsible for the increased growth of most tested non-starter LAB
- The effect of starter lysate was shown to be species and strain dependent

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Figure 1

Figure 2

A **B** **C** **D** **E** **F** **Time (h)**

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7