TITLE PAGE

- Korean Journal for Food Science of Animal Resources -

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title	Mathematical models for ~~Modeling~~ the biofilm formation of *Geobacillus* and *Anoxybacillus* on stainless steel surface in whole milk
Running Title (within 10 words)	Biofilm modeling of thermophilic bacteria
Author	Basar Karaca[1], Sencer Buzrul[2], Arzu Coleri Cihan[1]
Affiliation	1 Department of Biology, Ankara University, Ankara, Turkey 2 Department of Food Engineering, Konya Food and Agriculture University, Konya, Turkey
Special remarks – if authors have additional information to inform the editorial office	
ORCID (All authors must have ORCID) https://orcid.org	Basar Karaca (https://orcid.org/0000-0001-6943-8965) Sencer Buzrul (https://orcid.org/0000-0003-2272-3827) Arzu Coleri Cihan (https://orcid.org/0000-0002-7289-6251)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This research was supported by The Scientific and Technical Research Council of Turkey (TÜBİTAK), project no.116Z422.
Author contributions (This field may be published.)	BK carried out the experiment. SB made the modeling. BK and SB wrote the manuscript with support from ACC. ACC supervised the study. All authors discussed the results and commented on the manuscript, and they also read and approved the final manuscript.
Ethics approval (IRB/IACUC) (This field may be published.)	This manuscript does not require IRB/IACUC approval because there are no human and animal participants.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the <u>corresponding</u> author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Sencer Buzrul
Email address – this is where your proofs will be sent	sencer.buzrul@gidatarim.edu.tr
Secondary Email address	sencer.buzrul@gmail.com
Postal address	Konya Food & Agriculture University, Melikşah Mah., Beyşehir Cad., No:9, 42080, Meram, Konya/Turkey
Cell phone number	+90 532 558 4611
Office phone number	+90 332 223 5360
Fax number	+90 332 223 5490

Abstract Biofilm formation of *Geobacillus thermodenitrificans*, *Geobacillus thermoglucosidans* and *Anoxybacillus flavithermus* in milk on stainless steel were monitored at 55, 60, and 65 ℃ for various incubation times. Although species of *Geobacillus* showed a rapid response and produced biofilm within 4 h on stainless steel, a delay (lag time) was observed for *Anoxybacillus*. A hyperbolic equation and a hyperbolic equation with lag could be used to describe the biofilm formation of *Geobacillus* and *Anoxybacillus*, respectively. The highest biofilm formation amount was obtained at 60 ℃ for both *Geobacillus* and *Anoxybacillus*. However, the biofilm formation rates indicated that the lowest rates of formation were obtained at 60 ℃ for *Geobacillus*. Moreover, biofilm formation rates of *G. thermodenitrificans* (1.2-1.6 $\log_{10}$CFU/mL·h) were higher than *G. thermoglucosidans* (0.4-0.7 $\log_{10}$CFU/mL·h). Although *A. flavithermus* had the highest formation rate values (2.7-3.6 $\log_{10}$CFU/mL·h), this was attained after the lag period (4 or 5 h). This study revealed that modeling could be used to describe the biofilm formation of thermophilic bacilli in milk.

Keywords *Anoxybacillus*, dairy industry, *Geobacillus*, predictive microbiology, thermophilic bacteria

Introduction

Biofilms are highly organized, microbial communities that can develop on biotic or abiotic surfaces (Costerton et al., 1987; Costerton et al., 1999). Microbial biofilms can be found almost everywhere, and also in industrial and clinical environments (Tsai, 2005). Biofilms are a severe problem for human health (for only pathogenic microorganisms) and industry because they are highly resistant to antimicrobial agents, sanitizers, and biocides, and are particularly difficult to eliminate after the maturation phase (Costerton et al., 1987; Cvitkovitch and Ellen, 2003; Mah et al., 2003).

Microorganisms found on moist surfaces in food processing environments can easily attach to many surfaces to form microcolonies and produce biofilms (Wirtanen et al., 1996). The development of biofilms in food processing environments leads to continuous contamination of products. Food biofilms may contain both pathogenic microorganisms that can cause infectious diseases and spoilage microorganisms that decline the food quality (Boulange-Peterman, 1996). Microorganisms in biofilms can be protected from sanitation agents used in clean-in-place (CIP) procedures because the possibility of survival for the cells in biofilms is higher than the planktonic counterparts. Inadequate routine sanitation procedures against food biofilms lead to shorter shelf-life of foods and the spread of foodborne diseases (Bower et al., 1996). Also, biofilm-associated extracellular polymeric substances termed as the matrix that holds the cells in biofilms together cannot be removed by sanitation procedures, and enable the development of biofilms for newly arrived microorganisms (Stewart et al., 1997). The formation of biofilm may also hinder the heat transfer and cause corrosion on metal surfaces where the products are processed (Chmielewski and Frank, 2003).

Thermophilic bacilli such as *Anoxybacillus flavithermus* and *Geobacillus* spp. are contaminants for the dairy industry (Burgess et al., 2009). Although *G. stearothermophilus* is one of the most common *Geobacillus* species in dairy product manufacture, *G.*

thermodenitrificans, and *G. thermoglucosidans* may also pose risks for this industry. *G. thermodenitrificans* can be a contaminant for heat-treated food products and can produce biofilm in simulated dairy conditions (Manachini et al., 2000; Karaca et al., 2019). *G. themoglucosidans* can be isolated from the end product in the units where dairy products are processed, and it is known as a problematic biofilm former (Zhao et al., 2012; Cho et al., 2018).

These thermophilic bacilli are non-pathogenic; however, their presence in dairy products may be indicative of poor hygiene, and high numbers are unacceptable to food quality and market sales. The development of thermophilic bacilli in products leads to a significant decrease in the quality of the product due to acid and enzyme production (Marchand et al., 2012). Also, the spores of obligate thermophiles are more resistant to heat than the spores of mesophilic bacteria in milk flora (Sadiq et al., 2016). Spores of heat resistant thermophiles cannot be inactivated by almost any process (Cho et al., 2018). The durable biofilms of thermophilic bacilli also cause the constantly multiplying bacteria, spores, and heat resistant enzymes to be released into the dairy units (Sadiq et al., 2017). Product processing conditions in the dairy industry are capable of selectively promoting the development of thermophilic bacilli. These bacilli can quickly multiply in sections where temperatures reach 40-68 ℃ in dairy production facilities (Flint et al., 2001). Besides, they are challenging to eliminate because they are spore formers. They also tend to grow very rapidly (generation time of approximately 15-20 min) and are capable of quickly forming biofilms (Ronimus et al., 2003; Scott et al., 2007).

It is known that routine sanitation strategies for eliminating, preventing, or delaying thermophilic bacilli biofilm formation in dairy environments may not be sufficient. In addition, it is known that the application of sodium hydroxide, preferred in routine sanitation processes in the product processing units in the dairy industry, is not sufficient for the removal of

Anoxybacillus and *Geobacillus* contaminants (Wedel et al., 2019). In order to develop better control mechanisms, the link between the production of thermophilic biofilms and the conditions of the dairy environment where the products are processed needs to be better understood (Parkar et al., 2003; Parkar et al., 2004; Bremer et al., 2006; Marchand et al., 2012). Predictive microbiology allows defining the behavior of microorganisms under defined conditions, but only if the responses of microorganisms to environmental factors can be repeated. The prediction of the growth of microorganisms affected by different environmental factors can be beneficial for evaluating the food safety and shelf life of food products (McMeekin et al., 1993). In order to benefit from predictive microbiology applications in the food industry, there is a need for appropriate mathematical models that consistently define microbial behavior. There are several preferred sigmoid equations and various models for the development kinetics of microorganisms. Each of these models differs in terms of "ease of use" and the number of parameters in the equation. Comparisons of mathematical and statistical suitability criteria of different growth models are essential for the construction of more useful models (Zwietering et al., 1990; Buchanan et al., 1997; Baty and Delignette-Muller, 2004; López et al., 2004).

Temperature and incubation time are the most important parameters that should be taken into consideration in order to estimate the biofilm development of thermophilic bacilli in the dairy environment. ~~Important parameters, such as incubation time and temperature, should be taken into consideration in order to estimate the biofilm development of thermophilic bacilli in the dairy environment.~~ Modeling could be a powerful technique by means of studying the effects of primary conditions such as temperature and time on thermophilic bacilli biofilms and reconsidering process conditions in terms of minimizing thermophilic biofilm risks. Thus, the objective of this study was to describe the biofilm formation of *Geobacillus* and *Anoxybacillus* in whole milk on stainless steel surfaces at different temperature levels for

various incubation times by using mathematical models.

Materials and methods

Bacterial strains

G. thermodenitricifcans DSM 465^{T}, *G. thermoglucosidans* B84a and *A. flavithermus* DSM 2641^{T} strains were provided from Ankara University, Microbiology Research Laboratory of Biology Department, Turkey. These bacteria are influential biofilm formers in dairy products (Karaca et al. 2019). All reference strains were stored at –86 ℃ in MI broth [composed of 0.5% peptone (Sigma, Missouri, USA), 0.3% yeast extract (Merck, Darmstadt, Germany), 0.3% K2HPO4 (Sigma, Missouri, USA), 0.1% KH2PO4 (Sigma, Missouri, USA)] cultures supplemented with 20% glycerol (Suzuki et al., 1976).

Culture enrichment procedures

Culture enrichment procedures were performed before the experiments, as described by Kilic et al. (2017). This inoculation process was crucial in terms of stimulating biofilm formation of the thermophilic bacilli. Briefly, a colony of each thermophilic bacilli culture on tryptic soy agar (TSA; Merck, Darmstadt, Germany) were transferred into tryptic soy broth (TSB; Merck, Darmstadt, Germany) and incubated at 55 ℃ for 18 h (170 rpm). These cultures were then inoculated into fresh TSB and grown at 55 ℃ for an additional 6 h.

Determination of biofilm production responses of *G. thermodenitrificans* DSM 465 ^{T}, *G. thermoglucosidans* B84a, and *A. flavithermus* DSM 2641^{T}

The biofilms were sampled and screened at three temperatures (55, 60 and 65 ℃) for different incubation times (up to 144 h) to determine the biofilm production responses on 316 L type stainless steel surfaces. The biofilms were sampled with 10% reconstituted dry whole milk (Sigma-Aldrich, USA) which had been autoclaved at 121 ℃ for 5 min before (Somerton

et al., 2015).

The study was performed based on a 6-well microtiter plate layout. As an abiotic surface, specially cut stainless steel (316 L) surfaces were preferred (R: 7 mm, total surface area; 3.08 cm^2). These surfaces were treated with some cleaning and sterilization procedures such as detergent, acetone treatments, rinsing, and autoclaving in order to remove possible organic residues. The surfaces were initially treated with isopropanol overnight and agitated with a chlorinated detergent (Presept effervescent disinfectant tablets, Johnson & Johnson, Paranaque City, Philippines) for 30 min. The coupons were then rinsed with deionized water and autoclaved before use. Inoculation preparation of the thermophilic bacilli was carried out, as previously stated (Kilic et al., 2017). Sterile surfaces were planted into each well of the microtitre plate in duplicate. The wells were then filled with 5 mL of sterile standard whole milk, and active cultures were inoculated into these contents (4% v/v; approximately 10^7 CFU/mL). The plates were sealed to hinder evaporation and incubated at given incubation temperatures under static conditions. At the end of each incubation period, the wells were emptied under aseptic conditions, and the surfaces removed. The surfaces rinsed with sterile physiological saline (0.9% NaCl) to remove planktonic counterparts. The surfaces were placed in a sterile plastic tube containing 5 mL of physiological saline and 3 g of glass beads (R: 3 mm) to detach the biofilm cells. The tubes were then vortexed for 2 min at maximum intensity. For total bacterial counts, ten-fold dilutions in physiological saline were prepared, and each dilution was dropped in 10 µL onto TSA (Tryptic Soy Broth; Merck, Germany) agar plates. The plates were incubated at 55 °C for 24 h before colony counting. The results were calculated as colony-forming units per unit area (CFU/cm^2) and then converted to the logarithmic base ($log_{10}CFU/cm^2$). The colony-forming unit detection limit of the preferred method for counting biofilm cells is approximately 1.5 $log_{10}CFU/cm^2$. All the experiments were done at least in duplicate (Burgess et al., 2014; Karaca et al., 2019). The sampled

thermophilic biofilms on stainless steel surfaces were also confirmed by Confocal Scanning Laser Microscopy (Carl Zeiss Microscopy, Thornwood, NY, US). It was possible to analyze biofilm samples of thermophilic bacilli in standard whole milk. It was also clearly observed that the current biofilm dispersing method used was efficient in harvesting the biofilm cells of thermophilic bacilli, and the efficacy of the method was confirmed by the crystal violet method (results not shown).

Modeling

The biofilm formation data of *G. thermodenitrificans* and *G. thermoglucosidans* was described by using the hyperbolic equation [Eq.(1)]:

$$\log_{10}N(t) = \frac{\log_{10}N_{max}\cdot t}{t_h + t} \quad (1)$$

where $N(t)$ is the number of bacteria in CFU/cm^2 on stainless steel surface at a time t, N_{max} is the maximum cell number attained during the stationary period, and t_h is the time to reach $\log_{10}N_{max}/2$. It was assumed that when t = 0 $\log_{10}N(t) = 0$ indicating that number of cells attached initially on the surface was low in numbers.

Since the lag time was observed, different models were used for *A. flavithermus*. The first model was hyperbolic equation with lag [Eq.(2)]:

$$\text{If } t \leq t_{lag} \qquad \log_{10}N(t) = 0$$

$$\text{If } t > t_{lag} \qquad \log_{10}N(t) = \frac{\log_{10}N_{max}\cdot t}{t_h + t} \quad (2)$$

where t_{lag} is the lag time in h.

The second model was the Gompertz equation [Eq.(3)] proposed by Zwietering et al. (1990):

$$\log_{10}N(t) = \log_{10}N_0 + A \cdot exp\left\{-exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} \quad (3)$$

where A is the maximum cell number in $\log_{10}$CFU/cm^2 attained during the stationary period, μ_m is the maximum biofilm formation rate in $\log_{10}$CFU/cm^2·h, and λ is the lag time in h.

Although this model is widely used to describe the microbial growth curves, it could also be possible to use the modified Gompertz equation [Eq.(3)] to describe the biofilm formation of bacteria (Speranza et al., 2011; Karaca et al., 2013).

The third model was the Baranyi [Eq.(4)] which model consists of two rate equations (Baranyi and Roberts, 1994):

$$\frac{dN(t)}{dt} = \frac{q(t)}{1+q(t)} \cdot \mu_{max} \cdot N(t) \cdot \left\{1 - \left[\frac{N(t)}{N_{max}}\right]^{m}\right\} \quad (4)$$

where $\frac{dq(t)}{dt} = \mu_{max} \cdot q(t)$, m is the curvature or shape parameter which is, in general, assumed to be 1 for simplicity, and N_{max} is the maximum cell density. The term $q(t)/[1+q(t)]$ is associated with the lag time (λ) through the introduced parameter $h_0 = \mu_{max} \cdot \lambda$ which appears in the solution of the rate equation (Peleg, & Corradini 2011). Therefore, it could be possible to obtain both a maximum biofilm formation rate (μ_{max}) and lag time (λ) by solving these two differential equations.

The last model used was the three-phase linear model [Eq.(5)] proposed Buchanan et al. (1997):

$$\begin{gathered} \text{If } t \le \lambda \quad \log_{10}N(t) = \log_{10}N_0 \\ \text{If } \lambda < t < t_{max} \quad \log_{10}N(t) = \log_{10}N_0 + \mu \cdot (t - \lambda) \\ \text{If } t \ge t_{max} \quad \log_{10}N(t) = \log_{10}N_{max} \quad or \quad \log_{10}N(t) = \log_{10}N_0 + \mu \cdot (t_{max} - \lambda) \end{gathered} \quad (5)$$

where t_{max} is the time to reach maximum population density ($\log_{10}N_{max}$), and μ is the biofilm formation rate.

Model evaluation

Non-linear regression was performed by using SigmaPlot 2000 version 12.00 (Chicago, IL, USA). The goodness-of-fit of the models was evaluated by using the adjusted coefficient of determination (R^2_{adj}), and root mean square error (RMSE) values.

Results

Biofilm formation of *G. thermodenitrificans* DSM 465^T and *G. thermoglucosidans* B84a

The biofilm formation data of *G. thermodenitrificans* and *G. thermoglucosidans* indicated that rapid biofilm formation occurred in the first few hours. As time passed, the biofilm formation rate decreased and became zero. A suitable model for this initially fast biofilm-producing followed by a stationary period can be the hyperbolic equation [Eq.(1)].

Fig. 1. and Fig. 2. show both the biofilm formation data and model fits of *G. thermodenitrificans* and *G. thermoglucosidans*, respectively. A rapid initial biofilm formation rate was observed for G. *thermodenitrificans*, i.e., more than 3 $\log_{10}$CFU/cm^2 was obtained on stainless steel within 4 h (Fig. 1.). The biofilm rate was slower for *G. thermoglucosidans* compared to G. *thermodenitrificans*: more than 3 $\log_{10}$CFU/cm^2 was obtained on stainless steel within 8 h (Fig. 2.).

Fig. 1. & 2.

The goodness-of-fit of the model and model parameters are given in Table 1. It could be said that the model with a relatively high adjusted coefficient of determination ($R^2_{adj} \geq 0.87$) and relatively low root mean square error (RMSE $\leq$ 0.39) values could be used to describe the biofilm formation data of *Geobacillus* spp. The highest $\log_{10}N_{max}$ observed at 60 ℃ for both *G. thermodenitrificans* and *G. thermoglucosidans* were 5.2 and 5.8 $\log_{10}$CFU/cm^2, respectively indicating *Geobacillus* spp. had higher biofilm production at 60 ℃ than those of 55 and 65 ℃. On the other hand, higher counts were observed at 65 ℃ compared to 55 ℃ for *G. thermodenitrificans*. In contrast, just the opposite was obtained for *G. thermoglucosidans* (see Fig. 1. and 2, and $\log_{10}N_{max}$ values in Table 1). It could also be possible to calculate the biofilm formation rate by assuming a linear relationship for the rapid initial stage and by using the parameters given in Table 1. Since t_h is the time to reach $log_{10}N_{max}/2$, biofilm formation rates

can be calculated as $\log_{10}N_{max}/(2\times t_h)$. The calculated formation rates are listed in Table 2. Note that biofilm-producing rates for *G. thermodenitrificans* were much higher than the biofilm-producing rates of *G. thermoglucosidans*, and highest biofilm-producing rates were observed at 65 ℃ for both bacteria. The formation of the high amount of biofilm did not necessarily indicate a higher biofilm formation rate since the highest biofilm amount was observed at 60 ℃ for both bacteria (Table 1). However, the biofilm formation rate was the lowest at this temperature (Table 2).

Tables 1 & 2

Biofilm formation of *A. flavithermus* DSM 2641^T

The same hyperbolic trend was also observed biofilm formation of *A. flavithermus* except that there was a lag time for the formation. The very same model [Eq.(1)] with lag time integrated [Eq.(2)] was also used to describe the biofilm formation of *A. flavithermus* since hyperbolic growth with lag was observed.

Table 3 shows the R^2_{adj} and RMSE values of the models used for describing the biofilm formation of *A. flavithermus*. Although all models produced reasonable fits, the hyperbolic equation with lag was superior based on R^2_{adj} and RMSE values. Note that the modified Gompertz [Eq.(3)], the Baranyi [Eq.(4)], and three-phase linear [Eq.(5)] models produced almost the same fits (results not shown). Moreover, Baranyi model had the convergence failure at 55 ℃, which was not surprising since the biofilm formation data of *A. flavithermus* is not the same as the expected microbial growth: after the lag period, a rapid biofilm formation was observed.

Table 3

Fig. 3 shows the fit of the hyperbolic equation with lag [Eq.(2)] and the modified Gompertz equation [Eq.(3)] to the biofilm formation data of *A. flavithermus* in whole milk on stainless

steel. Since Gompertz [Eq.(3)], Baranyi [Eq.(4)] and three-phase linear [Eq.(5)] models were overlapped, only the fit of Gompertz [Eq.(3)] are shown in Fig. 3.

Fig. 3.

Comparison of the parameters of both models revealed (Table 4) that although similar parameter values were obtained, the hyperbolic equation with lag [Eq.(2)] had the highest maximum biofilm cell number, Gompertz equation [Eq.(3)] had the highest biofilm formation rate. In contrast, the three-phase linear had the lowest rate. All the models had almost identical lag time values (Table 4). Moreover, calculated formation rates from Eq.(2) (3.59, 2.7 and 2.8 $\log_{10}$CFU/cm^2·h at 55, 60 and 65 ℃, respectively) were also similar to that of obtained from Gompertz equation (Table 4). Biofilm formation rates of *A. flavithermus* were much higher than the biofilm formation rates of *G. thermodenitrificans* and *G. thermoglucosidans*, indicating that after the lag period *A. flavithermus* could proliferate on stainless steel.

Table 4

The highest biofilm cell number was obtained at 60 ℃ followed by 65 and 55 ℃ (see Fig. 3. and also see parameters in Table 4). Similarly, the same bacteria in whole milk had higher biofilm ~~forming~~ formation on stainless steel (about 4 $\log_{10}$CFU/cm^2) at 65 ℃ than that of 55 ℃ (about 2 $\log_{10}$CFU/cm^2) (Karaca et al., 2019). It should be noted that t_h was defined as the time to reach $\log_{10}N_{max}/2$ in h; however, since there was lag time for *A. flavithermus* $t_{lag} + t_h$ was required to reach the half of the maximum cell number. Hence, 5.5, 5.1, and 4.9 h were needed to reach 1.8, 3.1, and 2.4 $\log_{10}$CFU/cm^2 at 55, 60, and 65 ℃, respectively.

Discussion

Although the attachment of different bacteria to stainless steel surfaces at different temperatures has been shown, the biofilm formation of thermophilic bacilli under various conditions is still limited. The genus *Geobacillus* and *Anoxybacillus* can adhere to various

surfaces such as polyvinyl chloride, polypropylene, polystyrene, polycarbonate, glass, and stainless steel, and form biofilm on these surfaces. Among them, stainless steel is widely used material by the dairy industry (Karaca et al., 2019). Furthermore, residuals of milk during processing may remain on different parts of the stainless steel equipment and hence forms a thin layer. This layer, which is rich in nutrients, makes the stainless steel surfaces more susceptible to bacterial adhesion and biofilm formation (Silva et al., 2018). Therefore, the biofilm formation of these bacteria in whole milk on stainless steel was investigated in this study. A recent study indicated that both *Anoxybacillus* and *Geobacillus* in whole milk produced a high amount of biofilm (> 4 $\log_{10}$CFU/cm^2) on stainless steel at 65 ℃ while at 55 ℃ higher formation was observed (> 4 $\log_{10}$CFU/cm^2) on glass surfaces (Karaca et al. 2019). In this study, a new temperature level (60 ℃) was added, and the highest amount of biofilm was observed at this temperature (Fig. 1 and 2, and Table 1).

On the other hand, since microbial growth models such as Gompertz, Baranyi, and three-phase linear models could also be used to describe such data (data with the lag), these models were also tried. Although the Gompertz equation [Eq.(3)] is widely used to describe the microbial growth curves, it could also be possible to use to describe the biofilm formation of bacteria (Sperenza et al., 2011; Karaca et al., 2013).

There is a contradiction in the literature as to which model is the most suitable for describing the microbial growth data, and the choice of a model in predictive food microbiology is often subjective. However, there are many studies regarding the consistency and applicability of the mentioned models for the microbial growth prediction. Gompertz, Baranyi, Richards, logistic, and three-phase linear models are the most widely used models (López et al., 2004; Coroller, 2012; Jewell, 2012; Huang, 2013) and these models could be used for biofilm development modeling as well. Tsai (2015) described the accumulation of microorganisms on surfaces in water distribution systems underflow with a logistic model. The attachment patterns of

foodborne pathogens such as *Listeria monocytogenes*, *Shigella boydii*, *Staphylococcus aureus*, and *Salmonella* Typhimurium was estimated by using the modified Gompertz model under the effect of NaCl treatment by Xu et al. (Xu et al., 2010; Karaca et al., 2013). Response surface modeling is another commonly used method to mimic potential industrial food-processing conditions for evaluating the physiological requirements of biofilm formation (Goeres et al., 2005; Sperenza et al., 2011). In this study, however, the hyperbolic equation with lag was the best model among the alternatives to describe the biofilm formation of *A. flavithermus* since the highest R^2_{adj}, and lowest RMSE values were obtained.

This study showed that mathematical modeling could be a useful tool to describe the biofilm formation of thermophilic bacilli in milk on stainless steel. The hyperbolic equation for *Geobacillus* and hyperbolic equation with lag for *Anoxybacillus* could successfully be used to describe the biofilm formation. It should be noted that the findings of this study may not be generalized to the genera *Geobacillus* and *Anoxybacillus* since biofilm formation can be intensely strain specific even within a single species. However, the procedure can be extended to different bacteria in different foods on various surfaces. Further studies may also focus on dynamic rather than static conditions. Moreover, modeling and predicting the biofilm formation under dynamic conditions may open new doors and would be beneficial for the food industry.

References

Baranyi J, Roberts TA. 1994. A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol 23:277-294.

Boulange-Peterman, L 1996. Processes of bioadhesion on stainless steel surfaces and cleanability: a review with special reference to the food industry. Biofouling 10:275-300.

Bower CK, McGuire J, Daeschel MA. 1996. The adhesion and detachment of bacteria and spores on food contact surfaces. Trends Food Sci Technol 7:152-157.

Bremer PJ, Fillery S, McQuillan AJ. 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. Int J Food Microbiol 106:254-262.

Buchanan RL, Whiting RC, Damert, WC. 1997. When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiol 14:313-326.

Burgess SA, Brooks JD, Rakonjac J, Walker KM, Flint SH. 2009. The formation of spores in biofilms of *Anoxybacillus flavithermus*. J Appl Microbiol 107:1012-1018.

Burgess SA, Lindsay D, Flint SH. 2014. Biofilms of thermophilic bacilli isolated from dairy processing plants and efficacy of sanitizers. In *Microbial Biofilms*. pp. 367-354. Humana Press, New York, NY.

Chmielewski RAN, Frank JF. 2003. Biofilm formation and control in food processing facilities. Compr Rev Food Sci 2:22-32.

Cho TJ, Kim HW, Kim NH, Park SM, Kwon JI, Kim YJ, Rhee MS. 2018. New insights into the thermophilic spore-formers in powdered infant formula: implications of changes in microbial composition during manufacture. Food Control 92:464-470.

Coroller L, Kan-King-Y, D, Leguerinel I, Mafart P, Membré JM. 2012. Modelling of growth, growth/no-growth interface and nonthermal inactivation areas of *Listeria* in foods. Int J Food Microbiol 152:139–152

Costerton, JW, Cheng, KJ, Geesey, GG, Ladd, TI, Nickel, JC, Dasgupta, M, Marrie, TJ. 1987. Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435-464.

Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318-1322.

Cvitkovitch DG, Li YH, Ellen RP. 2003. Quorum sensing and biofilm formation in Streptococcal infections. J Clin Investig 112:1626-1632.

Flint S, Palmer J, Bloemen K, Brooks J, Crawford R. 2001. The growth of *Bacillus stearothermophilus* on stainless steel. J Appl Microbiol 90:151-157.

Goeres DM, Loetterle LR, Hamilton MA, Murga R, Kirby DW, Donlan RM. 2005. Statistical assessment of a laboratory method for growing biofilms. Microbiol 151:757-762.

Huang L. 2013. Optimization of a new mathematical model for bacterial growth. Food Control 32:283-288.

Jewell K. 2012. Comparison of 1-step and 2-step methods of fitting microbiological models. Int J Food Microbiol 160:145-161.

Karaca B, Buzrul S, Tato V, Akçelik N, Akçelik M. 2013. Modeling and Predicting the Biofilm Formation of Different *Salmonella* Strains. J Food Safety 33:503-508.

Karaca B, Buzrul, S, Coleri Cihan A. 2019. *Anoxybacillus* and *Geobacillus* biofilms in the dairy industry: effects of surface material, incubation temperature and milk type. Biofouling 35:551-560.

Kilic T, Karaca B, Ozel BP, Ozcan B, Cokmus C, Coleri Cihan A. 2017. Biofilm characteristics and evaluation of the sanitation procedures of thermophilic *Aeribacillus pallidus* E334 biofilms. Biofouling 33:352-367.

López S, Prieto M, Dijkstra J, Dhanoa MS, France J. 2004. Statistical evaluation of mathematical models for microbial growth. Int J Food Microbiol 96:289-300.

Manachini PL, Mora D, Nicastro G, Parini C, Stackebrandt E, Pukall R, Fortina MG. 2000. *Bacillus thermodenitrificans* sp. nov., nom. rev. Int J Syst Evol Microbiol 50:1331-1337.

Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'toole GA. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature 426:306-310.

373 Marchand S, De Block, J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L. 2012. Biofilm
374 formation in milk production and processing environments; influence on milk quality and
375 safety. Compr Rev Food Sci F 11:133-147.
376 McMeekin TA, Olley J, Ross T. 1993. Predictive Microbiology: Theory and Application, John
377 Wiley & Sons Ltd, Taunton, UK.
378 Parkar SG, Flint SH, Brooks JD. 2003. Physiology of biofilms of thermophilic bacilli—
379 potential consequences for cleaning. J Ind Microbiol Biotech 30:553-560.
380 Parkar SG, Flint SH, Brooks JD. 2004. Evaluation of the effect of cleaning regimes on biofilms
381 of thermophilic bacilli on stainless steel. J Appl Microbiol 96:110-116.
382 Peleg M, Corradini MG. 2011. Microbial growth curves: what the models tell us and what they
383 cannot. Critt Rev Food Sci Nutr 51:917-945.
384 Ronimus RS, Parker LE, Turner N, Poudel, S, Rückert A, Morgan HW. 2003. A RAPD-based
385 comparison of thermophilic bacilli from milk powders. Int J Food Microbiol 85:45-61.
386 Sadiq FA, Li Y, Liu T, Flint S, Zhang G, Yuan L, He G. 2016. The heat resistance and spoilage
387 potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from
388 Chinese milk powders. Int J Food Microbiol 238:193-201.
389 Sadiq FA, Flint S, Yuan L, Li Y, Liu T, He G. 2017. Propensity for biofilm formation by aerobic
390 mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders.
391 Int J Food Microbiol 262:89-98.
392 Scott SA, Brooks JD, Rakonjac J, Walker KM, Flint SH. 2007. The formation of thermophilic
393 spores during the manufacture of whole milk powder. Int J Dairy Technol 60:109-117.
394 Silva HO, Lima JAS, Aguilar CEG, Rossi GAM, Mathias LA, Vidal AMC. 2018. Efficiency
395 of Different Disinfectants on *Bacillus cereus* sensu stricto Biofilms on Stainless-steel
396 Surfaces in Contact with Milk. Front Microbiol 9:2934.
397 Somerton B, Lindsay D, Palmer J, Brooks J, Flint S. 2015. Changes in sodium, calcium, and

magnesium ion concentrations that inhibit *Geobacillus* biofilms have no effect on *Anoxybacillus flavithermus* biofilms. Appl Environ Microbiol 81:5115-5122.

Speranza B, Corbo MR, Sinigaglia M. 2011. Effects of nutritional and environmental conditions on *Salmonella* sp. biofilm formation. J Food Sci 76:M12-M16.

Stewart PS, Camper AK, Handran SD, Huang CT, Warnecke M. 1997. Spatial distribution and coexistence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in biofilms. Microbiol Ecol 33:2-10.

Suzuki Y, Kishigami T, Abe S. 1976. Production of extracellular alpha-glucosidase by a thermophilic *Bacillus* species. Appl Environ Microb 31:807-812.

Tsai YP. 2005. Impact of flow velocity on the dynamic behaviour of biofilm bacteria. Biofouling 21:267-277.

Watnick P, Kolter R 2000. Biofilm, city of microbes. J Bacteriol 182:2675-2679.

Wedel C, Wenning M, Dettling A, Scherer S, Hinrichs J. 2019. Resistance of thermophilic spore formers isolated from milk and whey products towards cleaning-in-place conditions: Influence of pH, temperature and milk residues. Food Microbiol 83:150-158.

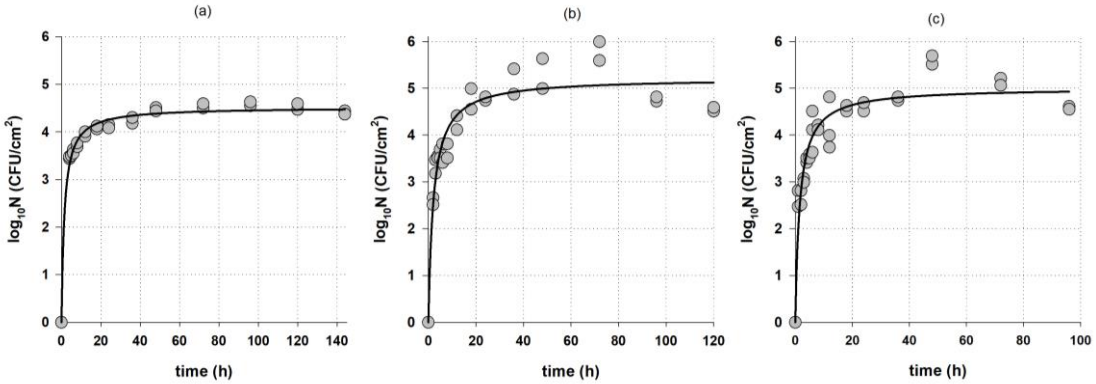
Wirtanen G, Husmark U, Matilla-Sandholm T. 1996. Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. J Food Prot 59:727-733.

Xu H, Zou Y, Lee HY, Ahn J. 2010. Effect of NaCl on the biofilm formation by foodborne pathogens. J Food Sci 75:M580-M585.

Zhao Y, Caspers MP, Abee T, Siezen RJ, Kort R. 2012. Complete genome sequence of *Geobacillus thermoglucosidans* TNO-09.020, a thermophilic sporeformer associated with a dairy-processing environment. J Bacteriol 194:4118-4118.

Zwietering MH, Jongenburger I, Rombouts FM, Van't Riet KJAEM. 1990. Modeling of the bacterial growth curve. Appl Environ Microbiol 56:1875-1881.

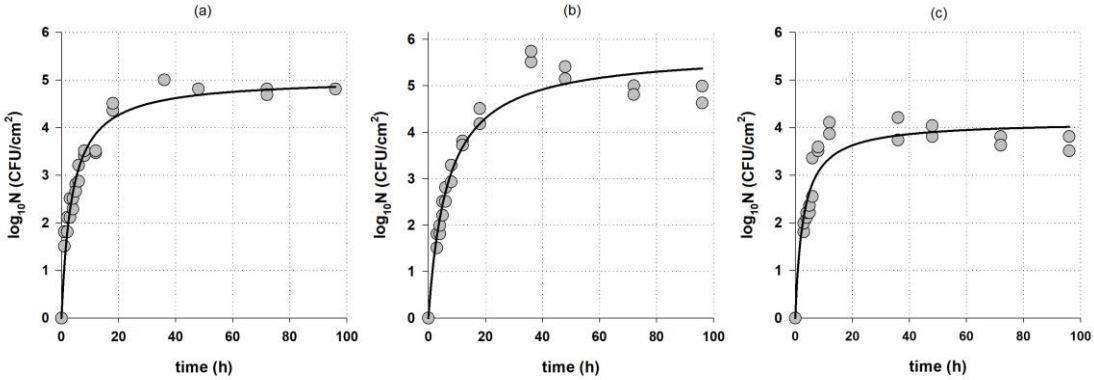
423 **Figure legends**



424

425 **Fig. 1** Biofilm formation data of *G. thermodenitrificans* DSM 465 ^{T} (grey circles) in whole milk
426 at 55 ℃ (a), 60 ℃ (b), and 65 ℃ (c). The solid black line indicates the fit of the hyperbolic
427 equation [Eq.(1)].

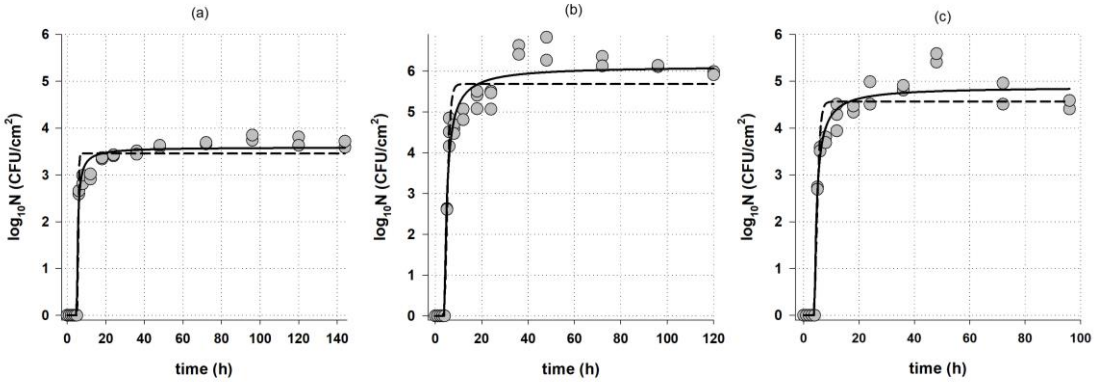
428



429

430 **Fig. 2** Biofilm formation data of *G. thermoglucosidans* B84a (grey circles) in whole milk at
431 55 ℃ (a), 60 ℃ (b), and 65 ℃ (c). The solid black line indicates the fit of the hyperbolic
432 equation [Eq.(1)].

433



434

435 **Fig. 3** Biofilm formation data of *A. flavithermus* DSM 2641^T (grey circles) in whole milk at
436 55 ℃ (a), 60 ℃ (b), and 65 ℃ (c). Black solid and black dashed lines indicate the fits 479 of
437 the hyperbolic equation with lag [Eq.(2)] and modified Gompertz equation [Eq.(3)],
438 respectively.

Table 1 Parameters ± standard errors of the fit of the hyperbolic equation [Eq. (1)] together with adjusted coefficient of determination (R^2_{adj}) and root mean square error (RMSE) values.

T (°C)	$log_{10}N_{max}$ (log_{10}CFU/cm^2)		t_h (h)		R^2_{adj}		RMSE	
	G. thermodenitrificans	*G. thermoglucosidans*	*G. thermodenitrificans*	*G. thermoglucosidans*	*G. thermodenitrificans*	*G. thermoglucosidans*	*G. thermodenitrificans*	*G. thermoglucosidans*
55	4.52 ± 0.03	5.04 ± 0.14	1.47± 0.09	3.63 ± 0.36	0.99	0.95	0.11	0.29
60	5.21 ± 0.12	5.75 ± 0.19	2.14± 0.27	6.73 ± 0.77	0.90	0.94	0.38	0.37
65	5.01± 0.12	4.13 ± 0.16	1.57± 0.21	2.79 ± 0.52	0.89	0.87	0.38	0.39

Table 2 Biofilm formation rate (μ) values calculated by using the parameters of the hyperbolic equation [Eq. (1)] i.e., $log_{10}N_{max}$ and t_h given in Table 1.

T (°C)	μ (log_{10}CFU/cm^2·h)	
	G. thermodenitrificans	*G. thermoglucosidans*
55	1.54	0.69
60	1.22	0.43
65	1.59	0.74

Table 3 Coefficient of determination (R^2_{adj}) and root mean square error (RMSE) values for hyperbolic equation with lag [Eq.(2)], Gompertz equation [Eq.(3)], Baranyi model [Eq.(4)] and three phase linear model [Eq.(5)].

T (°C)	R^2_{adj}				RMSE			
	Hyperbolic with lag	Gompertz	Baranyi	Three phase linear	Hyperbolic with lag	Gompertz	Baranyi	Three phase linear
55	0.99	0.98	—[a]	0.98	0.16	0.24	—	0.24
60	0.98	0.95	0.95	0.95	0.39	0.55	0.58	0.57
65	0.98	0.96	0.95	0.96	0.28	0.42	0.45	0.45

[a] Baranyi model did not converge.

Table 4 Parameters ± standard errors of the fit of hyperbolic equation with lag [Eq.(2)], Gompertz equation [Eq.(3)], Baranyi model [Eq.(4)] and three phase linear model [Eq.(5)].

T (°C)	Hyperbolic with lag	Gompertz	Baranyi	Three phase linear
55	$log_{10}N_{max}$ = 3.59 ± 0.04 log_{10}CFU/cm^2 t_h = 0.50 ± 0.07 h t_{lag} = 4.99 ± 0.02 h	A = 3.46 ± 0.05 log_{10}CFU/cm^2 μ_m = 3.64 ± 1.70 log_{10}CFU/cm^2·h λ = 5.20 ± 0.37 h	—[a]	$log_{10}N_{max}$ = 3.46[b] μ = 3.11 ± 0.55 log_{10}CFU/cm^2·h λ = 5.16 ± 0.25 h, t_{max} = 6.27 ± 3.39 h
60	$log_{10}N_{max}$ = 6.13 ± 0.11 log_{10}CFU/cm^2 t_h = 1.12 ± 0.15 h t_{lag} = 3.99 ± 0.05 h	A = 5.68 ± 0.12 log_{10}CFU/cm^2 μ_m = 2.45 ± 0.47 log_{10}CFU/cm^2·h λ = 3.99 ± 0.25 h	$log_{10}N_{max}$ = 5.64 ± 0.13 log_{10}CFU/cm^2 μ_{max} = 2.33 ± 0.39 log_{10}CFU/cm^2·h λ = 4.00 ± 0.26 h	$log_{10}N_{max}$ = 5.64[b] μ = 2.23 ± 0.26 log_{10}CFU/cm^2·h λ = 3.94 ± 0.17 h, t_{max} = 6.47 ± 0.19 h
65	$log_{10}N_{max}$ = 4.88 ± 0.09 log_{10}CFU/cm^2 t_h = 0.87 ± 0.12 h t_{lag} = 3.99 ± 0.04 h	A = 4.57 ± 0.10 log_{10}CFU/cm^2 μ_m = 2.28 ± 0.45 log_{10}CFU/cm^2·h λ = 3.96 ± 0.22 h	$log_{10}N_{max}$ = 4.56 ± 0.11 log_{10}CFU/cm^2 μ_{max} = 1.89 ± 0.32 log_{10}CFU/cm^2·h λ = 3.87 ± 0.24 h	$log_{10}N_{max}$ = 4.57[b] μ = 1.78 ± 0.22 log_{10}CFU/cm^2·h^{-1} λ = 3.82 ± 0.18 h, t_{max} = 6.40 ± 0.21 h

[a] Baranyi model did not converge.
[b] Calculated from $\mu \cdot (t_{max} - \lambda)$