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<b>Running Title (within 10 words)</b>	Biofilm modeling of thermophilic bacteria
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8

9 **Abstract** Biofilm formation of *Geobacillus thermodenitrificans*, *Geobacillus*  
10 *thermoglucosidans* and *Anoxybacillus flavithermus* in milk on stainless steel were monitored  
11 at 55, 60, and 65 °C for various incubation times. Although species of *Geobacillus* showed a  
12 rapid response and produced biofilm within 4 h on stainless steel, a delay (lag time) was  
13 observed for *Anoxybacillus*. A hyperbolic equation and a hyperbolic equation with lag could  
14 be used to describe the biofilm formation of *Geobacillus* and *Anoxybacillus*, respectively. The  
15 highest biofilm formation amount was obtained at 60 °C for both *Geobacillus* and  
16 *Anoxybacillus*. However, the biofilm formation rates indicated that the lowest rates of formation  
17 were obtained at 60 °C for *Geobacillus*. Moreover, biofilm formation rates of *G.*  
18 *thermodenitrificans* (1.2-1.6 log<sub>10</sub>CFU/mL·h) were higher than *G. thermoglucosidans* (0.4-  
19 0.7 log<sub>10</sub>CFU/mL·h). Although *A. flavithermus* had the highest formation rate values (2.7-  
20 3.6 log<sub>10</sub>CFU/mL·h), this was attained after the lag period (4 or 5 h). This study revealed that  
21 modeling could be used to describe the biofilm formation of thermophilic bacilli in milk.

22  
23 **Keywords** *Anoxybacillus*, dairy industry, *Geobacillus*, predictive microbiology,  
24 thermophilic bacteria

## 25 **Introduction**

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

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

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

50 *thermodenitrificans*, and *G. thermoglucosidans* may also pose risks for this industry. *G.*  
51 *thermodenitrificans* can be a contaminant for heat-treated food products and can produce  
52 biofilm in simulated dairy conditions (Manachini et al., 2000; Karaca et al., 2019). *G.*  
53 *thermoglucoisidans* can be isolated from the end product in the units where dairy products are  
54 processed, and it is known as a problematic biofilm former (Zhao et al., 2012; Cho et al.,  
55 2018).

56 These thermophilic bacilli are non-pathogenic; however, their presence in dairy products  
57 may be indicative of poor hygiene, and high numbers are unacceptable to food quality and  
58 market sales. The development of thermophilic bacilli in products leads to a significant  
59 decrease in the quality of the product due to acid and enzyme production (Marchand et al.,  
60 2012). Also, the spores of obligate thermophiles are more resistant to heat than the spores of  
61 mesophilic bacteria in milk flora (Sadiq et al., 2016). Spores of heat resistant thermophiles  
62 cannot be inactivated by almost any process (Cho et al., 2018). The durable biofilms of  
63 thermophilic bacilli also cause the constantly multiplying bacteria, spores, and heat resistant  
64 enzymes to be released into the dairy units (Sadiq et al., 2017). Product processing conditions  
65 in the dairy industry are capable of selectively promoting the development of thermophilic  
66 bacilli. These bacilli can quickly multiply in sections where temperatures reach 40-68 °C in  
67 dairy production facilities (Flint et al., 2001). Besides, they are challenging to eliminate  
68 because they are spore formers. They also tend to grow very rapidly (generation time of  
69 approximately 15-20 min) and are capable of quickly forming biofilms (Ronimus et al., 2003;  
70 Scott et al., 2007).

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

75 *Anoxybacillus* and *Geobacillus* contaminants (Wedel et al., 2019). In order to develop better  
76 control mechanisms, the link between the production of thermophilic biofilms and the  
77 conditions of the dairy environment where the products are processed needs to be better  
78 understood (Parkar et al., 2003; Parkar et al., 2004; Bremer et al., 2006; Marchand et al.,  
79 2012). Predictive microbiology allows defining the behavior of microorganisms under defined  
80 conditions, but only if the responses of microorganisms to environmental factors can be  
81 repeated. The prediction of the growth of microorganisms affected by different environmental  
82 factors can be beneficial for evaluating the food safety and shelf life of food products  
83 (McMeekin et al., 1993). In order to benefit from predictive microbiology applications in the  
84 food industry, there is a need for appropriate mathematical models that consistently define  
85 microbial behavior. There are several preferred sigmoid equations and various models for the  
86 development kinetics of microorganisms. Each of these models differs in terms of "ease of use"  
87 and the number of parameters in the equation. Comparisons of mathematical and statistical  
88 suitability criteria of different growth models are essential for the construction of more useful  
89 models (Zwietering et al., 1990; Buchanan et al., 1997; Baty and Delignette-Muller, 2004;  
90 López et al., 2004).

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

100 various incubation times by using mathematical models.

## 101 **Materials and methods**

### 102 **Bacterial strains**

103 *G. thermodenitrificans* DSM 465<sup>T</sup>, *G. thermoglucosidans* B84a and *A. flavithermus*  
104 DSM 2641<sup>T</sup> strains were provided from Ankara University, Microbiology Research Laboratory  
105 of Biology Department, Turkey. These bacteria are influential biofilm formers in dairy products  
106 (Karaca et al. 2019). All reference strains were stored at -86 °C in MI broth [composed  
107 of 0.5% peptone (Sigma, Missouri, USA), 0.3% yeast extract (Merck, Darmstadt, Germany),  
108 0.3% K<sub>2</sub>HPO<sub>4</sub> (Sigma, Missouri, USA), 0.1% KH<sub>2</sub>PO<sub>4</sub> (Sigma, Missouri, USA)] cultures  
109 supplemented with 20% glycerol (Suzuki et al., 1976).

110

### 111 **Culture enrichment procedures**

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

118

### 119 **Determination of biofilm production responses of *G. thermodenitrificans* DSM 465<sup>T</sup>, 120 *G. thermoglucosidans* B84a, and *A. flavithermus* DSM 2641<sup>T</sup>**

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

125 et al., 2015).

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

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

156

### 157 **Modeling**

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

$$160 \quad \log_{10}N(t) = \frac{\log_{10}N_{max} \cdot t}{t_h + t} \quad (1)$$

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

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

$$167 \quad \begin{aligned} &\text{If } t \leq t_{lag} && \log_{10}N(t) = 0 \\ 168 \quad &\text{If } t > t_{lag} && \log_{10}N(t) = \frac{\log_{10}N_{max} \cdot t}{t_h + t} \end{aligned} \quad (2)$$

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

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

$$171 \quad \log_{10}N(t) = \log_{10}N_0 + A \cdot \exp \left\{ -\exp \left[ \frac{\mu_m e}{A} (\lambda - t) + 1 \right] \right\} \quad (3)$$

172 where  $A$  is the maximum cell number in  $\log_{10}$ CFU/cm<sup>2</sup> attained during the stationary period,  
 173  $\mu_m$  is the maximum biofilm formation rate in  $\log_{10}$ CFU/cm<sup>2</sup>·h, and  $\lambda$  is the lag time in h.



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

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

$$179 \quad \frac{dN(t)}{dt} = \frac{q(t)}{1+q(t)} \cdot \mu_{max} \cdot N(t) \cdot \left\{ 1 - \left[ \frac{N(t)}{N_{max}} \right]^m \right\} \quad (4)$$

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

186 The last model used was the three-phase linear model [Eq.(5)] proposed Buchanan et al.  
 187 (1997):

$$188 \quad \begin{aligned} &\text{If } t \leq \lambda \quad \log_{10}N(t) = \log_{10}N_0 \\ &\text{If } \lambda < t < t_{max} \quad \log_{10}N(t) = \log_{10}N_0 + \mu \cdot (t - \lambda) \\ &\text{If } t \geq t_{max} \quad \log_{10}N(t) = \log_{10}N_{max} \quad \text{or} \quad \log_{10}N(t) = \log_{10}N_0 + \mu \cdot (t_{max} - \lambda) \end{aligned} \quad (5)$$

190 where  $t_{max}$  is the time to reach maximum population density ( $\log_{10}N_{max}$ ), and  $\mu$  is the biofilm  
 191 formation rate.  
 192

193

### 194 **Model evaluation**

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

198

## 199 **Results**

### 200 **Biofilm formation of *G. thermodenitrificans* DSM 465<sup>T</sup> and *G. thermoglucosidans*** 201 **B84a**

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

206 Fig. 1. and Fig. 2. show both the biofilm formation data and model fits of *G.*  
207 *thermodenitrificans* and *G. thermoglucosidans*, respectively. A rapid initial biofilm formation  
208 rate was observed for *G. thermodenitrificans*, i.e., more than 3 log<sub>10</sub>CFU/cm<sup>2</sup> was obtained on  
209 stainless steel within 4 h (Fig. 1.). The biofilm rate was slower for *G. thermoglucosidans*  
210 compared to *G. thermodenitrificans*: more than 3 log<sub>10</sub>CFU/cm<sup>2</sup> was obtained on stainless  
211 steel within 8 h (Fig. 2.).

#### 212 **Fig. 1. & 2.**

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

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

### 231 **Tables 1 & 2**

#### 232 **Biofilm formation of *A. flavithermus* DSM 2641<sup>T</sup>**

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

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

### 245 **Table 3**

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

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

### 250 **Fig. 3.**

251  
252 Comparison of the parameters of both models revealed (Table 4) that although similar  
253 parameter values were obtained, the hyperbolic equation with lag [Eq.(2)] had the highest  
254 maximum biofilm cell number, Gompertz equation [Eq.(3)] had the highest biofilm formation  
255 rate. In contrast, the three-phase linear had the lowest rate. All the models had almost identical  
256 lag time values (Table 4). Moreover, calculated formation rates from Eq.(2) (3.59, 2.7 and 2.8  
257  $\log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$  at 55, 60 and 65 °C, respectively) were also similar to that of obtained from  
258 Gompertz equation (Table 4). Biofilm formation rates of *A. flavithermus* were much higher than  
259 the biofilm formation rates of *G. thermodenitrificans* and *G. thermoglucosidans*, indicating that  
260 after the lag period *A. flavithermus* could proliferate on stainless steel.

### 261 **Table 4**

262  
263 The highest biofilm cell number was obtained at 60 °C followed by 65 and 55 °C (see Fig.  
264 3. and also see parameters in Table 4). Similarly, the same bacteria in whole milk had higher  
265 biofilm forming formation on stainless steel (about 4  $\log_{10}\text{CFU}/\text{cm}^2$ ) at 65 °C than that of 55 °C  
266 (about 2  $\log_{10}\text{CFU}/\text{cm}^2$ ) (Karaca et al., 2019). It should be noted that  $t_h$  was defined as the time  
267 to reach  $\log_{10}N_{max}/2$  in h; however, since there was lag time for *A. flavithermus*  $t_{lag} + t_h$  was  
268 required to reach the half of the maximum cell number. Hence, 5.5, 5.1, and 4.9 h were needed  
269 to reach 1.8, 3.1, and 2.4  $\log_{10}\text{CFU}/\text{cm}^2$  at 55, 60, and 65 °C, respectively.

270

## 271 **Discussion**

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

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

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

292 There is a contradiction in the literature as to which model is the most suitable for describing  
293 the microbial growth data, and the choice of a model in predictive food microbiology is often  
294 subjective. However, there are many studies regarding the consistency and applicability of the  
295 mentioned models for the microbial growth prediction. Gompertz, Baranyi, Richards, logistic,  
296 and three-phase linear models are the most widely used models (López et al., 2004; Coroller,  
297 2012; Jewell, 2012; Huang, 2013) and these models could be used for biofilm development  
298 modeling as well. Tsai (2015) described the accumulation of microorganisms on surfaces in  
299 water distribution systems underflow with a logistic model. The attachment patterns of

300 foodborne pathogens such as *Listeria monocytogenes*, *Shigella boydii*, *Staphylococcus aureus*,  
301 and *Salmonella* Typhimurium was estimated by using the modified Gompertz model under the  
302 effect of NaCl treatment by Xu et al. (Xu et al., 2010; Karaca et al., 2013). Response surface  
303 modeling is another commonly used method to mimic potential industrial food-processing  
304 conditions for evaluating the physiological requirements of biofilm formation (Goeres et al.,  
305 2005; Sperenza et al., 2011). In this study, however, the hyperbolic equation with lag was the  
306 best model among the alternatives to describe the biofilm formation of *A. flavithermus* since  
307 the highest  $R^2_{adj}$ , and lowest RMSE values were obtained.

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

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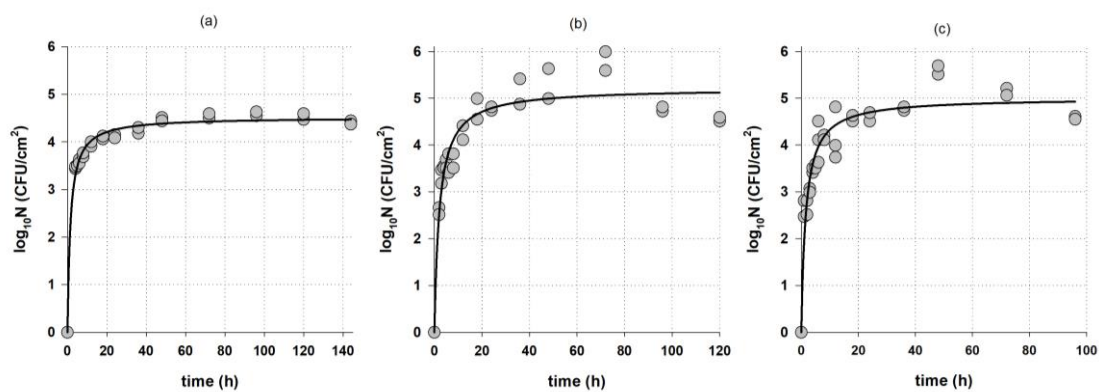
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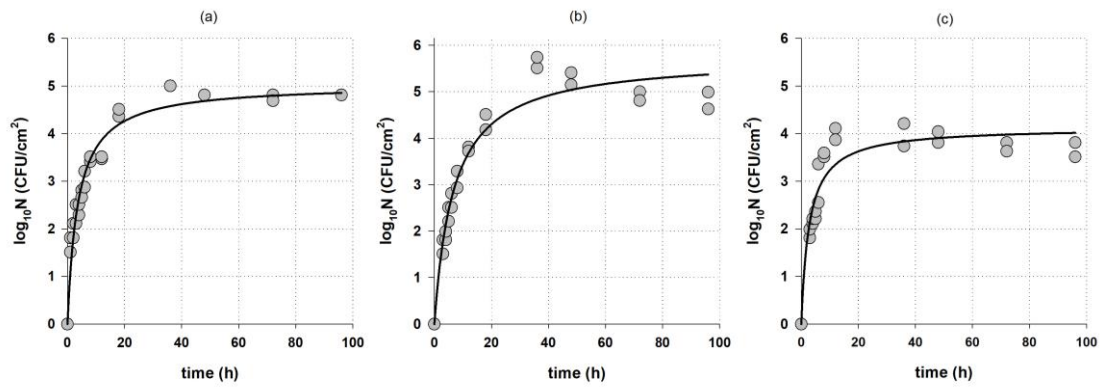
423 **Figure legends**



424

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

428



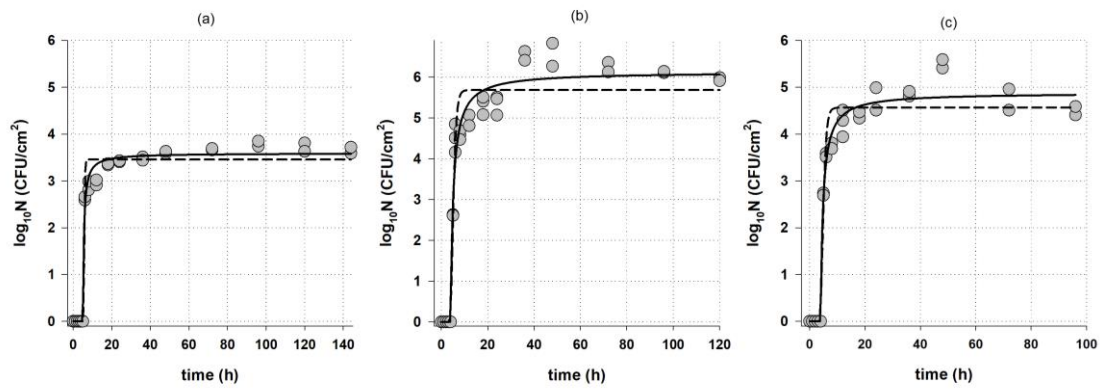
429

430 **Fig. 2** Biofilm formation data of *G. thermoglucosidans* B84a (grey circles) in whole milk at

431 55 °C (a), 60 °C (b), and 65 °C (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<sup>T</sup> (grey circles) in whole milk at  
 436 55 °C (a), 60 °C (b), and 65 °C (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  $\pm$  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)	$\log_{10}N_{max}$ ( $\log_{10}\text{CFU}/\text{cm}^2$ )		$t_h$ (h)		$R^2_{adj}$		RMSE	
	<i>G. thermodenitrificans</i>	<i>G. thermoglucosidans</i>	<i>G. thermodenitrificans</i>	<i>G. thermoglucosidans</i>	<i>G. thermodenitrificans</i>	<i>G. thermoglucosidans</i>	<i>G. thermodenitrificans</i>	<i>G. thermoglucosidans</i>
55	4.52 $\pm$ 0.03	5.04 $\pm$ 0.14	1.47 $\pm$ 0.09	3.63 $\pm$ 0.36	0.99	0.95	0.11	0.29
60	5.21 $\pm$ 0.12	5.75 $\pm$ 0.19	2.14 $\pm$ 0.27	6.73 $\pm$ 0.77	0.90	0.94	0.38	0.37
65	5.01 $\pm$ 0.12	4.13 $\pm$ 0.16	1.57 $\pm$ 0.21	2.79 $\pm$ 0.52	0.89	0.87	0.38	0.39

**Table 2** Biofilm formation rate ( $\mu$ ) 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)	$\mu$ ( $\log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ )	
	<i>G. thermodenitrificans</i>	<i>G. thermoglucosidans</i>
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	— <sup>a</sup>	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

<sup>a</sup> Baranyi model did not converge.

**Table 4** Parameters  $\pm$  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	$\log_{10}N_{max} = 3.59 \pm 0.04 \log_{10}\text{CFU}/\text{cm}^2$ $t_h = 0.50 \pm 0.07 \text{ h}$ $t_{lag} = 4.99 \pm 0.02 \text{ h}$	$A = 3.46 \pm 0.05 \log_{10}\text{CFU}/\text{cm}^2$ $\mu_m = 3.64 \pm 1.70 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 5.20 \pm 0.37 \text{ h}$	— <sup>a</sup>	$\log_{10}N_{max} = 3.46^b$ $\mu = 3.11 \pm 0.55 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 5.16 \pm 0.25 \text{ h}, t_{max} = 6.27 \pm 3.39 \text{ h}$
60	$\log_{10}N_{max} = 6.13 \pm 0.11 \log_{10}\text{CFU}/\text{cm}^2$ $t_h = 1.12 \pm 0.15 \text{ h}$ $t_{lag} = 3.99 \pm 0.05 \text{ h}$	$A = 5.68 \pm 0.12 \log_{10}\text{CFU}/\text{cm}^2$ $\mu_m = 2.45 \pm 0.47 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 3.99 \pm 0.25 \text{ h}$	$\log_{10}N_{max} = 5.64 \pm 0.13 \log_{10}\text{CFU}/\text{cm}^2$ $\mu_{max} = 2.33 \pm 0.39 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 4.00 \pm 0.26 \text{ h}$	$\log_{10}N_{max} = 5.64^b$ $\mu = 2.23 \pm 0.26 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 3.94 \pm 0.17 \text{ h}, t_{max} = 6.47 \pm 0.19 \text{ h}$
65	$\log_{10}N_{max} = 4.88 \pm 0.09 \log_{10}\text{CFU}/\text{cm}^2$ $t_h = 0.87 \pm 0.12 \text{ h}$ $t_{lag} = 3.99 \pm 0.04 \text{ h}$	$A = 4.57 \pm 0.10 \log_{10}\text{CFU}/\text{cm}^2$ $\mu_m = 2.28 \pm 0.45 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 3.96 \pm 0.22 \text{ h}$	$\log_{10}N_{max} = 4.56 \pm 0.11 \log_{10}\text{CFU}/\text{cm}^2$ $\mu_{max} = 1.89 \pm 0.32 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}$ $\lambda = 3.87 \pm 0.24 \text{ h}$	$\log_{10}N_{max} = 4.57^b$ $\mu = 1.78 \pm 0.22 \log_{10}\text{CFU}/\text{cm}^2 \cdot \text{h}^{-1}$ $\lambda = 3.82 \pm 0.18 \text{ h}, t_{max} = 6.40 \pm 0.21 \text{ h}$

<sup>a</sup> Baranyi model did not converge.

<sup>b</sup> Calculated from  $\mu \cdot (t_{max} - \lambda)$