1 **Title:** Cardinal models to describe the effect of temperature and pH on the growth of

2 *Anoxybacillus flavithermus* & *Bacillus licheniformis.*

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9 **Abstract**

10 *Anoxybacillus flavithermus* and *Bacillus licheniformis* are among the predominant spore-

11 formers of heat-processed foods. To our knowledge, no systematic analysis of growth kinetic

12 data of *A. flavithermus* or *B. licheniformis* is currently available. In the present study, the

13 growth kinetics of *A. flavithermus* and *B. licheniformis* in broth at various temperature and pH

14 conditions were studied. Cardinal models were used to model the effect of the above-

15 mentioned factors on the growth rates. The estimated values for the cardinal parameters

16 $T_{min}, T_{opt}, T_{max}$, pH_{min} and $pH_{1/2}$ for *A. flavithermus* were 28.70 ± 0.26, 61.23 ±0.16 and

17 71.52 ± 0.32 °C 5.52 ± 0.01 and 5.73 ± 0.01, respectively, while for *B. licheniformis* were 11.68

18 ± 0.03, 48.05 ± 0.15, 57.14 ± 0.01 °C, 4.71 ± 0.01 and 5.670 ± 0.08, respectively. The growth

19 behaviour of these spoilers was also investigated in a pea beverage at 62 and 49 °C,

20 respectively, to adjust the models to this product. The adjusted models were further validated

21 at static and dynamic conditions and demonstrated good performance with 85.7 and 97.4 %

22 of predicted populations for *A. flavithermus* and *B. licheniformis*, respectively, being within the

23 -10 % to 10 % relative error (RE) zone. The developed models can be useful tools in assessing

24 the potential of spoilage of heat-processed foods including plant-based milk alternatives.

25 **Keywords**

26 *Anoxybacillus flavithermus; Bacillus licheniformis*; cardinal model; growth kinetics; climate

27 change; plant-based milk alternatives

1. Introduction

29 Thermophilic and thermotolerant spore-forming bacteria are responsible for spoilage of a wide

30 range of non-refrigerated heat-processed food products, including milk and dairy products.

31 Among the different species found in dairy powders, *Geobacillus stearothermophilus*,

32 *Anoxybacillus flavithermus* and *Bacillus licheniformis* are the predominant spore-formers

33 (Delaunay et al., 2019; Lindsay et al., 2022). *A. flavithermus* is a facultative thermophilic spore-

34 forming bacterium used by the dairy powders industry as a poor hygiene indicator (Remize,

35 2017). Its optimum temperature of growth, around 60 °C, can be reached in different sections

36 of the production line, including the preheating phase in milk evaporators (Flint et al., 2011).

37 *A. flavithermus* is rarely found in raw and pasteurized milk, with a prevalence from 0.8 to 1.6

38 and 1.6%, respectively (Chauhan et al., 2013; Kable et al., 2019). However, during summer

39 season *A. flavithermus* has been isolated from 40% of the selected samples of raw milk (Kable

40 et al., 2019). Regarding its enzymatic activity, *A. flavithermus* has been found to produce

41 lipase and β-galactosidase, which may lead to significant spoilage of dairy products (Lücking

42 et al., 2013; Sadiq et al., 2016).

43 Besides the facultative thermophilic spore-forming bacilli, moderate thermophilic and

44 thermotolerant bacilli are equally important for the food industry. Among these groups,

45 *B. licheniformis*, is considered of great interest for the dairy industry due to its ability to survive

46 and multiply at moderate and high temperatures and at the same time form heat resistant

47 spores (Ivy et al., 2012; Li et al., 2019). Many studies have reported the presence of

48 *B. licheniformis* both in bulk tank milk kept under refrigerated temperature and ultrafiltration

49 plants (Boor et al., 2017; Eijlander et al., 2019). Interestingly, *B. licheniformis* has also been

50 associated with food poisoning (Fernández-No et al., 2011; Mikkola et al., 2000; Salkinoja-

51 Salonen et al., 1999). Regarding food spoilage, some strains of *B. licheniformis* are capable

52 of producing a slimy extracellular substance that can affect the quality of pasteurised milk and

53   cream (Gilmour and Rowe, 1990), while the majority of strains demonstrate β-galactosidase,

54   lipolysis, and proteolysis activity (De Jonghe et al., 2010; Lücking et al., 2013).

55   The potential of thermophilic and thermotolerant spore-forming bacteria, including

56   *A. flavithermus* and dairy originated strains of *B. licheniformis*, to form biofilms has been

57   demonstrated in many studies (Burgess et al., 2009; Flint et al., 2011; Seale et al., 2008;

58   Ostroy et al., 2019). Temperature applied during processes and the presence of divalent

59   cations in milk have been found to positively influence the formation of biofilms (Burgess et

60   al., 2014, 2009), indicating that dairy manufacturing plants could be an ideal harbour for

61   entrapped spores. Thus, thermophilic bacilli could be found in various final dairy and non-dairy

62   products due to formation of biofilms in the lines.

63   In the past few years, consumers' demand for plant-based alternatives has increased

64   worldwide due to special dietary needs and behavioural changes. According to Nielsen data,

65   plant-based consumption across Europe has increased by 49% in two years, reaching total

66   sales values of €3.6 billion in 2020 (Nielsen, 2021). Following this tremendous growth, the

67   dairy (food) industry has focused on producing many innovative plant-based milk alternatives

68   from nuts, cereals and legumes, such as oat, soy and pea.

69   Considering that soil constitutes the harbour of spore-forming bacteria (Carlin, 2011), plant-

70   based proteins used as raw material might be contaminated with various thermophilic and

71   thermotolerant bacilli. Plant-based milk alternatives are usually low acid products and are

72   therefore sterilized by ultra-high temperature (UHT) treatment (Sethi et al., 2016), which is

73   primarily designed to eliminate spores of *Clostridium botulinum* (Membré and van Zuijlen,

74   2011). Nevertheless, this thermal process has been proven insufficient in eliminating spores

75   of thermophilic and thermotolerant spore-forming bacteria that exhibit extreme heat

76   resistance, such as *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* (Alonso

77   et al., 2021; André et al., 2016; Burgess et al., 2010; Wells-Bennik et al., 2016). The surviving

78   spores may cause spoilage issues in non-refrigerated food products when the products

79   storage temperature exceed their minimum growth temperature for a certain time and allow

80 germination and outgrowth. While the minimum growth temperature is usually relatively high,

81 these products are distributed and stored at ambient temperature. Current environmental

82 conditions, particularly temperature, generally do not allow extensive growth of thermophilic

83 bacilli and therefore ensure their microbiological stability (Misiou et al., 2021a). However,

84 several studies have already highlighted the emerging risk of spoilage in hot climate regions

85 and temperate climates, due to climate change and global warming (Kakagianni and

86 Koutsoumanis, 2018; Misiou et al., 2021b; Misiou and Koutsoumanis, 2021).

87 In order to assess the potential risk of spoilage, the growth behaviour of thermophilic bacteria

88 as a function of the environment should first be described. Predictive microbiology modelling

89 can be used as a tool to predict the bacterial behaviour as function of extrinsic and intrinsic

90 factors, such as temperature and pH. In this sense, several secondary and primary models

91 have already been developed for thermophilic spore-forming bacteria. More specifically, the

92 effects of temperature and pH on the maximum specific growth rate (μ_{max}) of *Geobacillus*

93 *stearothermophilus*, *Bacillus coagulans* and *Alicyclobacillus acidoterrestris* has been

94 described by means of cardinal parameter models (Mtimet et al., 2015; Kakagianni et ai.,

95 2018; Misiou et al., 2021a; Misiou et al., 2021b). Although *A. flavithermus* and *B. licheniformis*

96 are responsible for the spoilage of a wide range of non-refrigerated foods, the scientific

97 literature lacks quantitative kinetic data and models that could be used by the food industry to

98 predict their growth.

99 Against this background, the objective of the present study was to model the effect of

100 temperature and pH on the growth of *Anoxybacillus flavithermus* and *Bacillus licheniformis*

101 and validate the developed models against observed growth in a pea-based milk alternative

102 under static and dynamic temperature conditions.

103 **2. Materials and methods**

104 **2.1 Bacterial strain and culture conditions**

105 Type strain *B. licheniformis* DSM 13 and stain *A. flavithermus* DSM 21510 were used in the

106 present study. The strains were maintained as working culture onto Microbank™ porous beads

107 (Pro-Lab Diagnostics, Ontario, Canada) and stored at -20 °C. All experiments were performed

108 using the strains harvested in their stationary phase. Therefore, *A. flavithermus* strain was

109 cultured by inoculation of 10 mL Brain Heart Infusion (BHI; Thermofisher™ Diagnostics

110 CM1135B Waltham, MA, U.S) with a bead from the working culture, incubating aerobically at

111 55 °C for 8 h. After 8 h, 0.1 mL of the sub-culture were transferred into a fresh 9.9 mL BHI

112 tube and incubated at the same conditions for 18 h. The same procedure was followed for

113 *B. licheniformis* strain, with the exemption that the culture was inoculated into Tryptone Soy

114 Broth (TSB; Thermofisher™ Diagnostics, CM0129B) and incubated at 37 °C. The initial

115 concentration of the suspension of both microorganisms was determined by spread plating on

116 tryptone soy agar (TSA; Thermofisher™ Diagnostics, CM0131B).

117 **2.2 Growth experiments in broth**

118 The *A. flavithermus* working cultures were decimally diluted in BHI for temperature

119 experiments or pH-adjusted BHI, based on the experimental set-up for the pH experiments, to

120 a concentration of approximately 10^6 CFU/mL. The same was applied for *B. licheniformis* with

121 the exemption that cultures were diluted in TSB or pH-adjusted TSB, respectively.

122 *2.2.1* **Effect of temperature on maximum specific growth rate** (μ_{max})

123 The effect of temperature on the maximum specific growth rate (μ_{max}) of *A. flavithermus* was

124 studied in BHI with pH 7.4 ± 0.2. In order to cover the growth range to the greatest possible

125 extent, a step of 2 °C was used and 17 temperature levels between 33 and 68 °C were

126 selected. Concerning the effect of temperature on (μ_{max}) of *B. licheniformis*, experiments were

127 conducted in TSB with pH 7.2 ± 0.2 at 22 temperature levels between 15 and 57 °C.

128 *2.2.2* **Effect of pH on maximum specific growth rate** (μ_{max})

129 The effect of pH on maximum specific growth rates (μ_{max}) of *A. flavithermus* and

130 *B. licheniformis* was evaluated in BHI and TSB, respectively, at suboptimal temperature

131 conditions (55 and 43 °C, respectively), following the recommendation of Le Marc et al., 2021.

132 The pH adjustment was performed by adding NaOH 1 N (HC73325137, Merck, Darmstadt,

133 Germany) or HCl 1 N (HC67744657, Merck) to sterilized BHI or TSB by using a pre-sterilized

134 0.22 µm filter unit until the pH reached the necessary level; from 4.8 to 7.0 with a step of 0.2.

135 The pH measurements were performed at 25 °C with a pH meter with a glass electrode

136 (SevenMulti, Metter Toledo, Ohio, USA).

137 *2.2.3* **Automated optical density measurements**

138 To estimate the maximum specific growth rate (μ_{max}) values reached in the different pH levels

139 and temperatures between 30 and 59 °C, the automated turbidimetric system BioScreen C

140 (Oy Growth Curves Ab Ltd., Raisio, Finland) was used, following the method described by

141 Lianou and Koutsoumanis, 2011. The method adjusted as follows. Except for the first and fifth

142 rows that will receive the inoculum, the BioScreen plate wells were prefilled with 180 µL of the

143 appropriate broth, based on the experimental set-up. The first and fifth rows were inoculated

144 with 200 µL of 10^6 CFU/mL from the 26-h culture, prepared as described in **Section 2.1**. To

145 avoid evaporation, the outer columns of the plate were filled with 200 µL of the selected broth

146 but were not used for sample inoculation. Decimal dilutions of the inoculum were performed

147 across the BioScreen plate by transferring 20 µL from one well to the other, while 20 µL of the

148 fifth dilution was discarded to keep the same culture volume (180 µL). Thus, the range of initial

149 concentrations obtained throughout the BioScreen plate was approximately 10^6 - 10^2 CFU/mL.

150 Optical density (OD) measurements were automatically measured at standard time intervals

151 (10 min) using the wideband filter (600 nm) of the instrument, at temperatures between 30 and

152 59 °C, for a total period during which a considerable OD increase (0.2) for all five decimally

153 diluted cultures was observed. For the assessment of the effect of pH and on μ_{max}

154 experiments were conducted at sub-optimal growth temperature for each microorganism (55

155 and 43 °C), based on the results obtained for the temperature experiments. The BioScreen

156 plates were agitated for 30 sec to stop 5 sec prior to the OD measurements in all experiments.

157 One independent experiment was conducted for each selected pH level, and eight samples

158 (e.g., quadruple wells of five serially diluted cultures) were analysed (n=8), while for each

159 temperature level one independent experiment with 16 samples was analysed (n=16).

160 *2.2.4* **Manual optical density measurements and plate count experiments**

161 BioScreen C instrument can operate up to 59 °C. However, thermophilic bacteria can grow to

162 much higher temperature levels. Since a precise harmonized methodology for the estimation

163 of cardinal values does not exist, two different protocols were applied in parallel for

164 temperature levels above 59 °C.

165 In the first protocol, the BioScreen plate inoculated with *A. flavithermus* was incubated into a

166 high-precision oven (Heratherm oven, ThermoScientific^{TM}) set to the studied temperature. The

167 temperature was monitored during incubation using electronic temperature-monitoring

168 devices (iButton; iButtonLink, Thermochron, Whitewater, WI, USA). The BioScreen plate was

169 prepared as described in **Section 2.2.3** and transferred manually to the BioScreen C, where

170 OD measurements were taken every 0.3 h for a total period such that a considerable OD

171 increase for all five decimally diluted cultures was observed.

172 In the second protocol, the BioScreen plate was inoculated with 200 µL of 10^2 CFU/well,

173 except for the wells in the outer columns, and incubated in the same oven. 0.1 mL were taken

174 every half an hour from a different well. After the appropriate serial decimal dilutions, samples

175 were spread plated on TSA agar plates and incubated aerobically at 55 °C for 24 h. Following

176 the plate count method, one experiment per temperature level was conducted (n=1).

177 ***2.3 Growth experiments in plant-based milk alternative***

178 The growth kinetic behaviour of *A. flavithermus* and *B. licheniformis* was investigated

179 individually in a shelf stable, ready-to-eat food product, namely a pea-based beverage. This

180 product was selected based on the high frequency and high level of contamination of pea

181 isolates with thermoresistant and thermophilic spores, compared to other plant-based raw

182 materials (NIZO, 2022). The pea-based beverage contained 2.1 g proteins, 1.4 g fat and 5.7

183 g carbohydrates per 100 mL. The initial pH and water activity values of this product were 7.01

184 $\pm$ 0.01 and 0.996 $\pm$ 0.02, respectively. For this set of experiments, the samples were

185 artificially and independently contaminated with vegetative cells of *B. licheniformis* and

186 *A. flavithermus.* The inoculum of both microorganisms was prepared as described in **Section**

187 **2.1**. Portions of 200 mL of the samples were transferred into pre-sterilized 250 mL Duran

188 bottles and pre-heated in a water bath (ThermoScientific™, Haake w26 Fisons, Digitana AG)

189 at the selected temperature; 62 °C for *A. flavithermus* and at 49 °C for *B. licheniformis*,

190 respectively. After the appropriate dilutions of the inoculum, samples were aseptically

191 inoculated with approximately 10^2 - 10^3 CFU/mL and submerged again in the pre-heated water

192 bath at static conditions. The selected temperatures constitute the optimum temperature for

193 *A. flavithermus* and *B. licheniformis* growth, respectively, based on the optical density

194 experiments performed in broth. Three independent experiments were performed for each

195 microorganism (n=3).

196 For the enumeration of *B. licheniformis* and *A. flavithermus* concentration, samples were taken

197 every 30 min for the first four hours and every 1 h for the rest of the experimental duration.

198 Appropriate serial decimal dilutions of the samples were performed in TS solution before

199 surface plating on TSA plates. Before counting, plates were incubated aerobically at 37 and

200 55 °C for 24 h, respectively. At the same time span, monitoring of pH and visual observation

201 of the macroscopic changes in the structure of the samples were performed. The data derived

202 from this set of experiments were used to estimate the maximum specific growth rates of *B.*

203 *licheniformis* and *A. flavithermus* and then deduce their optimum specific growth rates in the

204 tested product (see **Section 2.4**).

205 The developed growth models of *A. flavithermus* and *B. licheniformis* were validated in the

206 pea beverage under static conditions at 55 and 42 °C, respectively. The validation

207 temperatures were selected, taking into account the temperature applied during UHT products'

208 quality control to detect insterilty defaults caused by thermophilic spore-forming bacteria,

209   based on the recommendations (Cordier, 1990), and the applied industrial practices. The

210   developed models were further validated under non-isothermal conditions. The temperature

211   profiles were designed taking into account, the "worst case scenario" during distribution and

212   storage at a retail and domestic level. Hence, experiments were performed within a

213   temperature range from 12 to 55 °C. More specifically, the pea beverages inoculated with *A.*

214   *flavithermus* were stored at 25 °C for 12 h, 55 °C for 5 h, 30 °C for 24 h and 37 °C for 24 h,

215   while samples inoculated with *B. licheniformis* were stored at 12 °C for 12 h, 55 °C for 5 h, 30

216   °C for 24 h and 37 °C for 24 h. The temperature fluctuation was electronically monitored using

217   cox tracer data loggers (Cox Tracer, Cox Technologies, Belmont, NC, USA). Temperature

218   data were obtained with a time interval of 15 min. Samples were prepared and treated

219   following the same procedure as the one applied for the development of the model. For each

220   microorganism one independent experiment with two technical replicates was performed

221   (n=2).

222   ***2.4 Data analysis***

223   Regarding the OD method experiments, the detection times of the five serial decimal dilutions

224   were plotted against the natural logarithm of their initial concentrations and the μ_{max} values

225   were determined by linear regression, according to the following equation as proposed by

226   Dalgaard and Koutsoumanis, 2001:

227
$$lnN_i = k - \mu_{max} \cdot DT_i \tag{1}$$

228   where DT_i is being generally defined as the time required for OD increase to 0.20 to be

229   observed.

230   For the plate count method experiments, the μ_{max} values were determined by fitting the

231   primary model of Baranyi and Roberts, 1994 (Eq. (2) and (3)) to the data by using the DMFit

232   Microsoft Excel add-in downloadable from the www.combase.cc.

233
$$\frac{dN(t)}{dt} = \frac{Q(t)}{Q(t)+1} \cdot \mu_{max} \cdot \left(1 - \frac{N(t)}{N_{max}}\right) \cdot N(t) \tag{2}$$

$$234 \qquad \frac{dQ(t)}{dt} = \mu_{max} \cdot Q(t) \qquad\qquad (3)$$

235

236 where N_t is the number of cell concentration at time t (CFU/mL); μ_{max} is the maximum specific

237 growth rate of the cell population (1/h); N_{max} is the maximum cell density (CFU/mL).

238 The parameter Q denotes the concentration of a substance critical to growth, which is

239 assumed to be synthesised during the lag phase, and is related to the physiological parameter

240 h_0 as follows:

$$241 \qquad h_0 = \ln\left(1 + \frac{1}{Q_0}\right) = \mu_{max} \cdot \lambda$$

242 The effects of temperature and pH on μ_{max} of each microorganism were modelled individually

243 using the Cardinal Model for temperature (Rosso et al., 1993) and pH (Aryani et al., 2015):

$$244 \qquad \mu_{max} = \mu_{opt-T} \cdot CM(T) \qquad\qquad (4)$$

245 with

$$246 \qquad CM(T) = \begin{cases} 0, & T \le T_{min} \\ \frac{(T-T_{min})^2(T-T_{max})}{(T_{opt}-T_{min})[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)]}, & T_{min} \le T \ge T_{max} \\ 0, & T \ge T_{max} \end{cases} \qquad (5)$$

247 and

$$248 \qquad \mu_{max} = \mu_{opt-pH} \cdot CM(pH) \qquad\qquad (6)$$

249 with

$$250 \qquad CM(pH) = 1 - 2^{\frac{(pH-pH_{min})}{(pH_{min}-pH_{1/2})}} \qquad\qquad (7)$$

251

252 Where μ_{opt} is the value for the maximum specific growth rate when $T = T_{opt}$ and $pH = pH_{opt}$,

253 T_{min}, T_{opt} and T_{max} are the theoretical minimum, optimum and maximum values of T enabling

254 growth, pH_{min} is the theoretical minimum and $pH_{1/2}$ is the pH at which the μ_{max} is the half of

255 the μ_{opt}.

256 The cardinal values (T_{min}, T_{opt}, T_{max}, pH_{min} and $pH_{1/2}$) were determined by fitting the obtained

257 μ_{max} values for *A. flavithermus* and *B. licheniformis* to the above model using the CardinalFit

258 software (Cadavez and Gonzales-Barron, 2020). The goodness of fit was evaluated by a

259 graphical comparison between the observed and the predicted values, the coefficient of

260 determination (R^2) and Root Mean Square Error (RMSE) (Ratkowsky, 2004).

261 The combined effect of temperature and pH on μ_{max}, was predicted based on the following

262 equation, as proposed by Rosso et al., 1995:

263 $$\mu_{max} = \mu_{opt} \cdot CM(T) \cdot CM(pH) \tag{8}$$

264

265 The above secondary model was adjusted for the tested plant-based alternative as following:

266

267 $$\mu_{max} = \mu_{ref} \cdot \frac{CM(T) \cdot CM(pH)}{CM(T_{ref}) \cdot CM(pH_{ref})} \tag{9}$$

268

269 Where rate μ_{ref} is the maximum specific growth rate in the plant-based alternative estimated

270 based on the growth data obtained from the experiments in this product at static conditions

271 and *CM(T_{ref}), CM(pH_{ref})* are the cardinal model parameters (Eq. (5) and (7), respectively) for

272 the reference conditions (pH=7.01 and T=62 °C for *A. flavithermus* and T=49 °C for *B.*

273 *licheniformis*.. To estimate the growth kinetic parameters, the primary model of Baranyi and

274 Roberts, 1994 (Eq. (2) and (3)) was fitted to the above-mentioned data by using the DMFit

275 add-in in Microsoft Excel.

276 For the prediction of growth of *A. flavithermus* and *B. licheniformis* at 55 and 42 °C,

277 respectively, the parameter h_0 referring to the "work" required by the cells to adjust to the new

278 environment (Baranyi and Roberts, 1994), was estimated as the product of λ* μ_{max} based on

279 the values of the growth kinetic parameters obtained from the experiments performed in the

280 pea-based milk alternative (at 62 and 49 °C). Since h_0 constitutes a "characteristic" of the

281 cell culture and remains constant and independent from the storage temperature when the

282 pre-history of the culture is the same, (Baranyi and Roberts, 1995, 1994; Gougouli and

283 Koutsoumanis, 2016; Koutsoumanis et al., 2006; Pin and Baranyi, 2006) the parameter was

284 set on the same value for all predictions of growth.

285 The growth predictions of *A. flavithermus* and *B. licheniformis* in the selected food products

286 were estimated using the multiplicative secondary model (Eq. (8)) and primary model (Eq. (2)

287 and (3)). The prediction of growth at dynamic temperature conditions drew on the assumption

288 that the growth rate is adapted directly to the new temperature environment after a

289 temperature change and the equations (2) and (3) were numerically integrated based on time.

290 For the calculation of the "momentary" μ_{max} the multiplicative secondary model (Eq. (8)) was

291 used. The parameter μ_{opt} used in the secondary model (Eq. (8)) was calculated for the pea-

292 based milk alternative through the primary model (Eq. (2) and (3)), based on the estimated

293 μ_{max} determined from fitting the primary model (Eq. (2) and (3)) into the growth data obtained

294 from the experiments that were performed under static conditions.

295 The performance of the developed models in both static and dynamic temperature conditions

296 was evaluated using the percent relative errors (Koutsoumanis et al., 2006):

297
$$\% \ Relative \ Error \ (RE) = \frac{\left(Log(N_{t_{observed}}) - Log(N_{t_{predicted}})\right)}{Log\left(N_{t_{observed}}\right)} \times 100 \tag{10}$$

298 **3. Results**

299 ***3.1 Growth experiments in broth***

300 The effect of temperature (range: 33-68 °C) and pH (range: 5.6-7.0) on *A. flavithermus* growth

301 rate is presented in **Figures 1 and 2**. For the effect of temperature the growth rate increased

302 from 0.118 1/h (± 0.004) at T=33 °C to a maximum value of 3.393 1/h (± 0.09) at T=60 °C,

303 while at T levels above 60 °C a gradual reduction of μ_{max} was observed down to 2.319 1/h (±

304 0.10) at 68 °C. Accordingly, the μ_{max} (1/h) when studying the pH effect, increased from 0.847

305   1/h (±0.002) at pH 5.6 to a maximum value of 1.805 1/h (± 0.04) at pH 6.4 where it reached

306   the plateau.

307   The effect of temperature (range: 15-57 °C) and pH (range: 4.8-7.0) on *B. licheniformis* growth

308   rate is presented in **Figures 3 and 4**. The growth rate increased from 0.068 1/h (± 0.003) at

309   T=15 °C to a maximum value of 3.083 1/h (± 0.04) at T=49 °C, while at T levels above this

310   level, a gradual reduction of μ_{max} was observed down to 0.178 1/h (± 0.009) at 57 °C.

311   Accordingly, for the effect of pH, the μ_{max} (1/h) increased from 0.516 1/h (±0.01) at pH 4.8

312   and reached a plateau at 6.4 with a maximum value of 1.662 1/h (± 0.03).

313   The above experimental data (μ_{max}) were modelled as a function of temperature and pH using

314   CM for both microorganisms separately. The graphical evaluation of the fitting (**Figure 1**-**4**),

315   along with the statistical factors (**Table 1**) indicated a satisfactory performance of all models

316   in describing the effect of temperature and pH on the μ_{max} of *A. flavithermus* and

317   *B. licheniformis*. **Table 1** shows the estimated values for the cardinal parameters T_{min},

318    $T_{opt} \ and \ T_{max}$ of *A. flavithermus* and *B. licheniformis* being 28.70, 61.23 and 71.52 °C and

319   11.68, 48.05 and 57.14 °C, respectively. Likewise, the estimated values for the parameters

320   pH_{min} and $pH_{1/2}$ of *A. flavithermus* and *B. licheniformis* were 5.52 and 5.73 and 4.71 and 5.67,

321   respectively. The goodness of fit was demonstrated graphically and by using the low values

322   of Root Mean Square Error (RMSE) (**Table 1**).

323   ***3.2 Model development and validation in plant-based milk alternative***

324   In an attempt to adjust the models, the growth kinetics of *A. flavithermus* and *B. licheniformis*

325   were investigated in a pea-based milk alternative.

326   **Figures 5a** and **5b** depict representative fittings of the primary model (Eq. (2) and (3)) to the

327   growth data obtained in the pea beverage under static conditions at 62 °C for *A. flavithermus*

328   and at 49 °C for *B. licheniformis,* respectively. As illustrated in **Figures 5a** and **5b,** the

329   observed growth of *A. flavithermus* and *B. licheniformis* in the same plant-based milk

330   alternative showed a lag phase in both cases, estimated to 1.08 ± 0.15 and 1.36 ± 0.02 h,

331   respectively. Based on the latter observation, for predictions of *A. flavithermus* and

332   *B. licheniformis* growth in plant-based milk alternatives the parameter h_0 was set at 2.78 and

333   3.76, respectively. The maximum specific growth rates (μ_{max}) of *A. flavithermus* and

334   *B. licheniformis* in the pea beverage were estimated at 2.555 ± 0.09 and 2.229 ± 0.13 1/h,

335   respectively, while the optimum rates (μ_{opt}) were determined at 2.581 ± 0.09 and 2.764 ± 0.13

336   1/h, respectively. The maximum population concentration (N_{max}) was set at $10^{6.8}$ CFU/mL for

337   prediction of *A. flavithermus* growth and at $10^{7.5}$ CFU/mL for prediction of *B. licheniformis*

338   growth, based on the observed growth data obtained from the experiments performed for the

339   model development (see **Figure 5a** and **5b**).

340   In order to test the performance of the developed models in plant-based milk alternatives, the

341   model predictions were validated against the observed growth of *A. flavithermus* and

342   *B. licheniformis* at two static temperatures that were not used during the model development

343   stage, namely 55 and 42 °C, respectively, as well as under non-isothermal conditions. The

344   growth was predicted by using a combination of the secondary model (Eq. (4)) with the Baranyi

345   and Roberts primary model (Eq. (2) and (3)), following the method described in **Section 2.4.**

346   The performance of the model was first graphically evaluated as depicted in **Figures 6** - **9.** As

347   illustrated in **Figure 6**, *A. flavithermus* in the selected plant-based milk alternative exhibited

348   an approximately 5 log increase when exposed at 55 °C for 10 h; that was predicted by the

349   model. *B. licheniformis* reached its maximum concentration when the tested products were

350   stored at 42 °C for 10 h (**Figure 7**), and it was accurately predicted by the model. **Figures 8**

351   and **9** depict the observed growth of *A. flavithermus* and *B. licheniformis*, respectively, along

352   with the predicted growth under non-isothermal conditions. The developed models predicted

353   growth satisfactory under dynamic temperatures suggesting that the assumptions made for

354   growth prediction were valid. The results showed that the bacterium adapts instantaneously

355   to the new environment without presenting any additional lag phase and grow with the

356   expected μ_{max}. Even after a storage period of about 12 h at temperatures below T_{min}, both

357   bacteria were able to initiate growth when temperature increased to levels within the biokinetic

358   range with a lag phase and a growth rate very close to those predicted by the model. The

359   performance of the models was further evaluated through the estimation of the percent relative

360   error (%RE). Based on the combined growth data obtained from the validation experiments,

361   %RE values were estimated for each developed model. As presented in **Figure 10**, for

362   *A. flavithermus* 85.7% of the predictions were within -10 and 10 % RE zone, while for

363   *B. licheniformis* percentage of predictions within the same zone was 97.4 %. In addition, for

364   both models none of the predictions were outside the -20 and 20 % RE zone.

365   **4. Discussion**

366   The estimated cardinal values of the temperature and pH of *B. licheniformis* are comparable

367   with the values reported by Trunet et al., 2015, who modelled the recovery of heat-treated

368   *Bacillus licheniformis* spores at suboptimal temperature and pH using growth limits. In

369   particular, the authors reported slightly higher optimum (49.01°C), maximum temperature

370   values (57.87°C) and lower minimum pH values (4.63) compared to the ones obtained in the

371   present study (see **Table 1**). The above differences could be attributed mainly to strain

372   variability (Lianou and Koutsoumanis, 2013), since in the Trunet et al.'s study a different strain

373   of *B. licheniformis* (Ad978) was used. At the same time the observed difference might also be

374   explained by the different model fitted to the data. The experiments for the estimation of

375   cardinal pH values presented in this study were conducted at a temperature lower than the

376   optimum one since temperature has a markable effect on the estimation of pH_{min} parameter

377   (Le Marc et al., 2002; Martinez-Rios et al., 2019). The above-described relationship between

378   temperature and minimum pH values has already been highlighted for mesophilic *B. cereus*

379   sensu *lato* (Le Marc et al., 2021). However, in the present study the effect of and extrinsic (T)

380   and intrinsic factors (pH) on the μ_{max} was studied individually without including any interaction

381   term, based on the gamma concept and the hypothesis that each factor has an independent

382   effect on the growth rate (Zwietering et al., 1992).

383   To our knowledge, this study provides the first attempt to estimate the cardinal temperature

384   and pH values of *A. flavithermus* DSM 21510. However, the estimated cardinal temperature

385   values are in line with the temperature growth range (30-72 °C) and the optimum temperature

386   range (60-65 °C) of *A. flavithermus* DSM 2641 reported by Ellis and Magnuson, 2012.

387   Interestingly, the results of the present study indicated that both optical density and the plate

388   count method provide comparable μ_{max} values, since the μ_{max} values obtained through the

389   plate count method fall within the "cloud" of μ_{max} values obtained through optical density

390   method (data not shown). The above observation suggests that experiments at high

391   temperature (above 59 °C) for the estimation of cardinal temperature values of thermophilic

392   bacteria can be conducted using any of the above methodologies.

393   The accuracy of the developed models in the plant-based milk alternative was graphically

394   evaluated by comparing the observed with predicted values. In addition, the performance of

395   the models was statistically evaluated by using the percent relative error (% RE). Considering

396   that % RE for *A. flavithermus* and *B. licheniformis* models ranged between -15.46 and 13.24

397   % and from -12.73 to 9.79 %, respectively, the ability of the models to predict growth was

398   deemed satisfactory. Moreover, no specific over- or underestimating trend was observed.

399   Food spoilage challenges emerge from innovative raw materials and food products. In similar

400   vein, the vast majority of plant-based proteins used as raw materials are known for their poor

401   solubility properties during dissolution which can hamper the effectiveness of the heat process

402   (Akharume et al., 2021; Sim et al., 2021), allowing the survival of spores. The models

403   developed in the present study can be used to evaluate the risk of microorganisms surviving

404   the heat process to grow to levels that can cause spoilage. In particular, the developed models

405   can be incorporated in a quantitative microbial spoilage risk assessment model to estimate

406   the growth during transportation and storage and the associated risk of spoilage. While the

407   effect of temperature and pH on the growth rate of these microorganisms has been described,

408   the quantitative risk assessment model can be also applied to control growth, by providing

409   alternatives that can mitigate the risk of spoilage.

410   For the evaluation of the risk, it is of great importance to investigate the prevalence and

411   contamination level of several thermophilic and thermotolerant bacteria in plant-based

412   proteins that are not currently available in the literature. The NIZO institute reported that less

413   than 100 CFU/g might be found in pea proteins (NIZO, 2021). When a low number of surviving

414   spores are expected in the products, cell heterogeneity should be taken into consideration as

415   a significant variability source (Baranyi and Pin, 2004; Kakagianni et al., 2017; Koutsoumanis

416   and Lianou, 2013). Nonetheless, the present deterministic form of the model does not take

417   into account the variability of individual spore behaviour. Future data and information on

418   individual spore heterogeneity as affected by the heat treatment can allow a stochastic

419   modelling approach and provide more precise growth predictions (Koutsoumanis & Lianou,

420   2013). This data shall include the likelihood of individual spore outgrowth in a finished product

421   after exposure to the heat treatment. In addition, the performance of the models developed in

422   the present study can be further improved by taking into account strain variability. Considering

423   the diversity of strains that may be present in the final product, "feeding" the models with

424   additional research data on the intra-species differences in the growth kinetics of *A.*

425   *flavithermus* and *B. licheniformis* are certainly expected to increase their accuracy in predicting

426   the risk of spoilage (Lianou and Koutsoumanis, 2013).

427   **5. Conclusions**

428   To our knowledge, this is the first study on the development and validation of a predictive

429   model for the effect of temperature and pH on the growth of *Anoxybacillus flavithermus* and

430   *Bacillus licheniformis* in plant-based milk alternatives. Validation studies showed that the

431   developed models satisfactorily predicted the growth behaviour of both spoilage bacteria in

432   the selected pea beverage. Hence, the models developed in the present study could be

433   applied for an effective spoilage risk assessment to ensure microbial stability of plant-based

434   milk alternatives.

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CRediT authorship statement

Ourania Misiou: Conceptualization, Data curation, Investigation, Writing - Original Draft, Visualisation. **Mariem Ellouze**: Conceptualization, Methodology, Supervision, Writing - Review & Editing. **Konstantinos Koutsoumanis**: Conceptualization, Funding acquisition, Methodology, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known conflict of financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Table 1. Estimated values and fitting statistics for the cardinal parameter model describing the effect of temperature and pH on the maximum specific growth rate (μ_{max}) of *Anoxybacillus flavithermus* DSM 21510 and *Bacillus licheniformis* DSM 13 in brain heart infusion and tryptone soy broth, respectively.

Parameter	Estimated value[a]	95 % Confidence Limits Upper	Lower	RMSE[b]	R^{2c}
Anoxybacillus flavithermus					
Temperature cardinal values					
μ_{opt-T} (pH= 7.4) (1/h)	3.471 ± 0.04				
T_{min}	28.70 ± 0.26	28.69	28.71		
T_{opt}	61.23 ± 0.16	58.05	65.00	0.08	0.974
T_{max}	71.52 ± 0.32	71.51	71.53		
pH cardinal values					
μ_{opt-pH} (T= 55 °C) (1/h)	3.242 ± 0.06				
pH_{min}	5.52 ± 0.01	5.51	5.53	0.07	0.946
$pH_{1/2}$	5.73 ± 0.01	5.72	5.73		
Bacillus licheniformis					
Temperature cardinal values					
μ_{opt-T} (pH= 7.2) (1/h)	2.836 ± 0.03				
T_{min}	11.68 ± 0.26	11.67	11.69		
T_{opt}	48.05 ± 0.15	45.50	51.19	0.09	0.972
T_{max}	57.14 ± 0.01	57.13	57.15		
pH cardinal values					
μ_{opt-pH} (T= 43 °C) (1/h)	3.195 ± 0.15				
pH_{min}	4.71 ± 0.01	4.70	4.71	0.07	0.969
$pH_{1/2}$	5.67 ± 0.08	5.652	5.69		

[a] ±: Standard Error
[b] RMSE: Root Mean Square Error
[c] R^2: Coefficient of determination
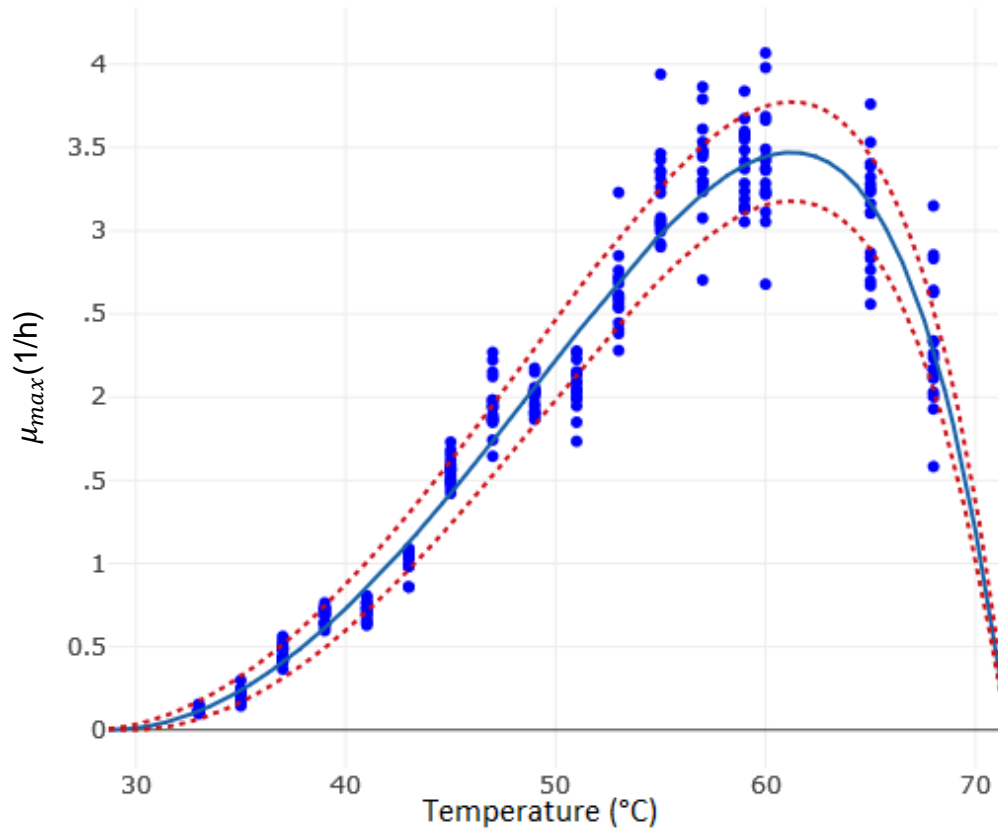
Figure 1. Effect of temperature on the maximum specific growth rate (μ_{max}) of *Anoxybacillus flavithermus* DSM 21510 in brain heart infusion broth. Data are fitted to the Cardinal Model (—). Points (○) represent observed values of the μ_{max}. The dashed red lines (--) indicate the 95% confidence limits.
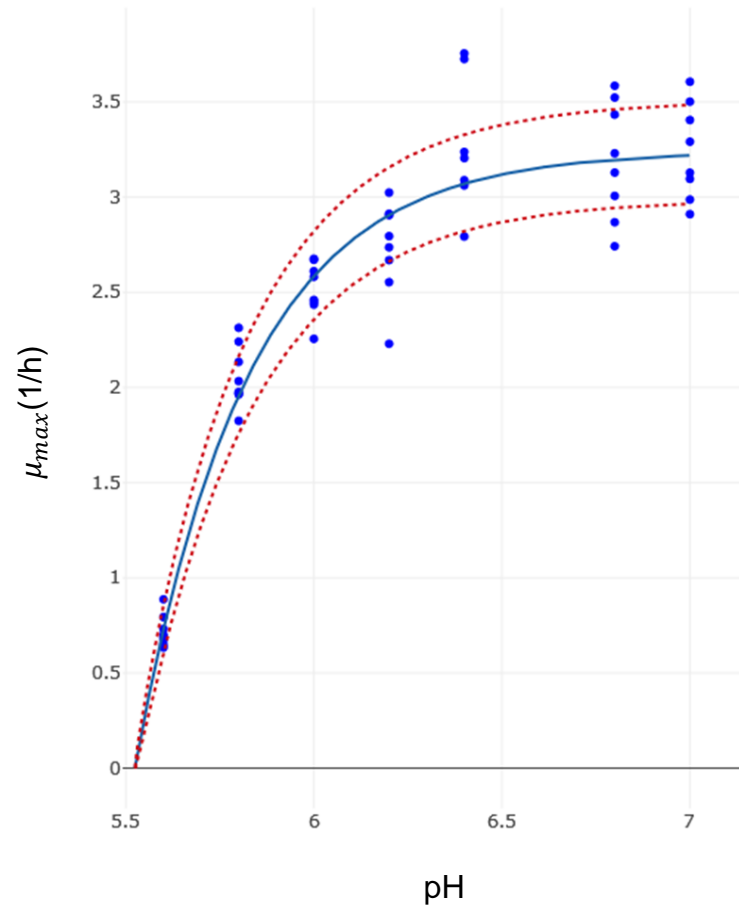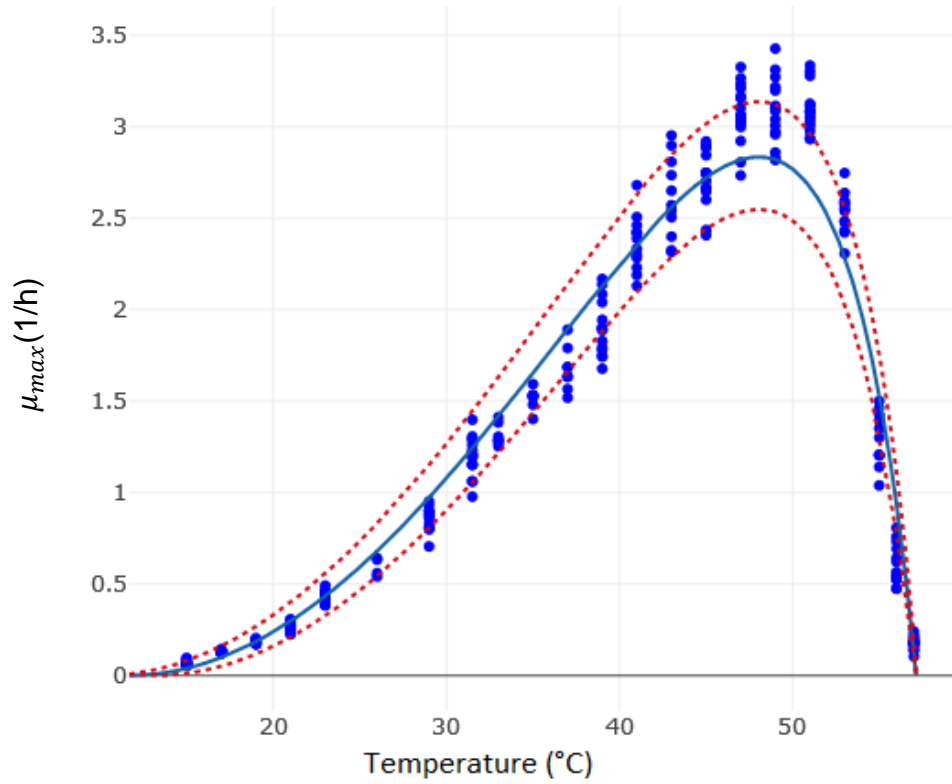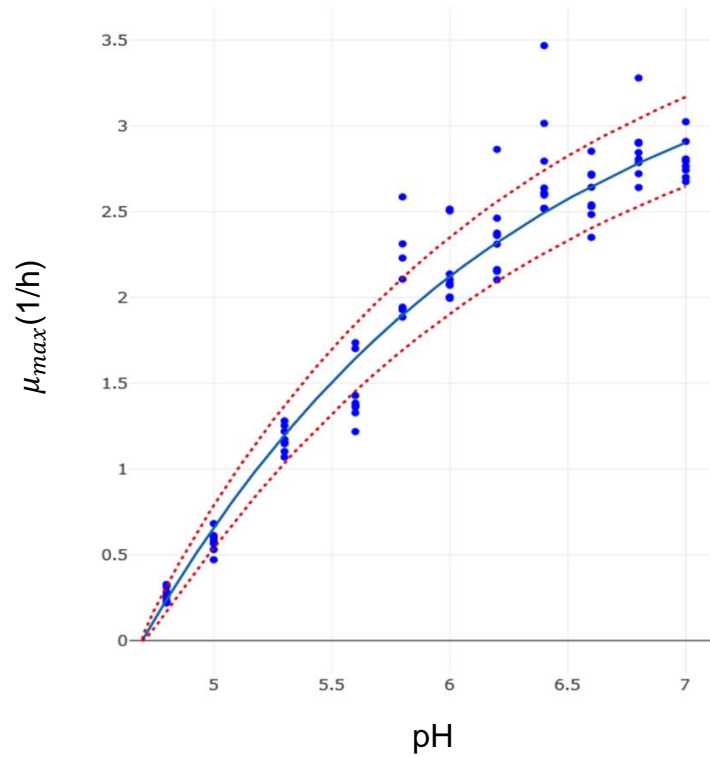
Figure 2. Effect of pH on the maximum specific growth rate (μ_{max}) of *Anoxybacillus flavithermus* DSM 21510 in brain heart infusion broth. Data are fitted to the Cardinal Model (—). Points (○) represent observed values of the μ_{max}. The dashed red lines (--) indicate the 95% confidence limits.

Figure 3. Effect of temperature on the maximum specific growth rate (μ_{max}) of *Bacillus licheniformis* DSM 13 in tryptone soy broth. Data are fitted to the Cardinal Model (—). Points (○) represent observed values of the μ_{max}. The dashed red lines (--) indicate the 95% confidence limits.

Figure 4. Effect of pH on the maximum specific growth rate (μ_{max}) of *Bacillus licheniformis* DSM 13 in tryptone soy broth. Data are fitted to the Cardinal Model (—). Points (○) represent observed values of the μ_{max}. The dashed red lines (--) lines the 95% confidence limits.
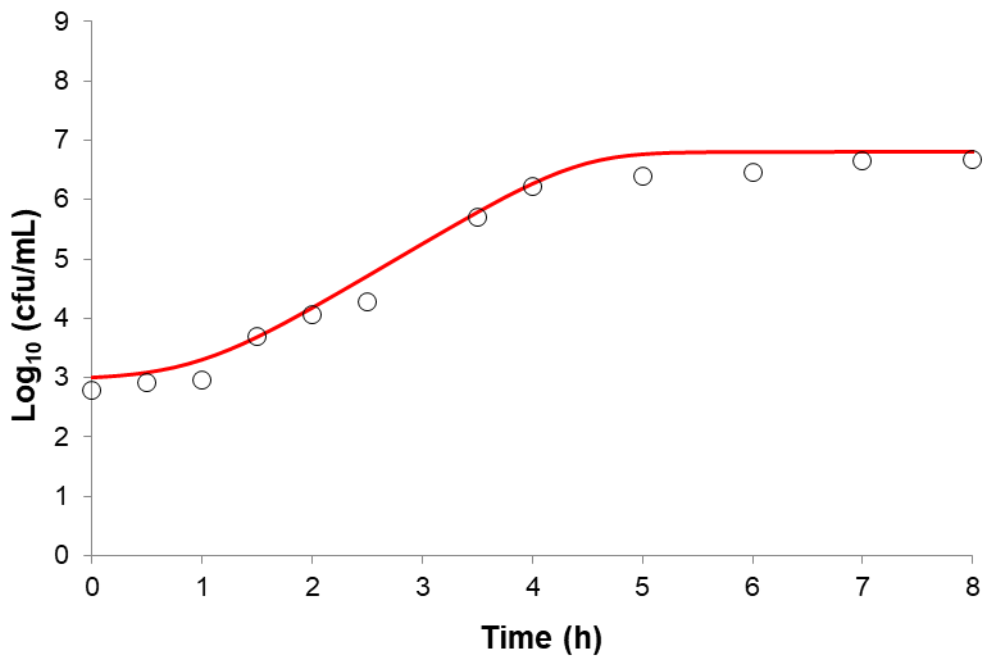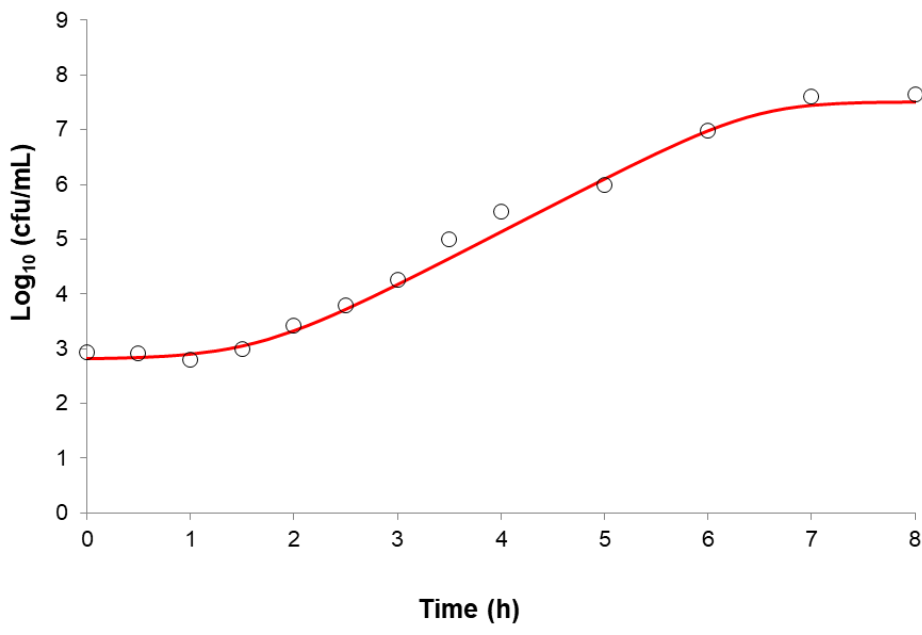
Figure 5. Growth kinetics of *Anoxybacillus flavithermus* DSM 21510 (a) and *Bacillus licheniformis* DSM 13 (b) in plant-based milk alternative during storage at optimum growth temperature at 62 and 49 °C, respectively. The red solid lines (-) indicate the fitting of the Baranyi and Roberts model to the growth data. One representative fitting of each spoilage bacterium is shown.
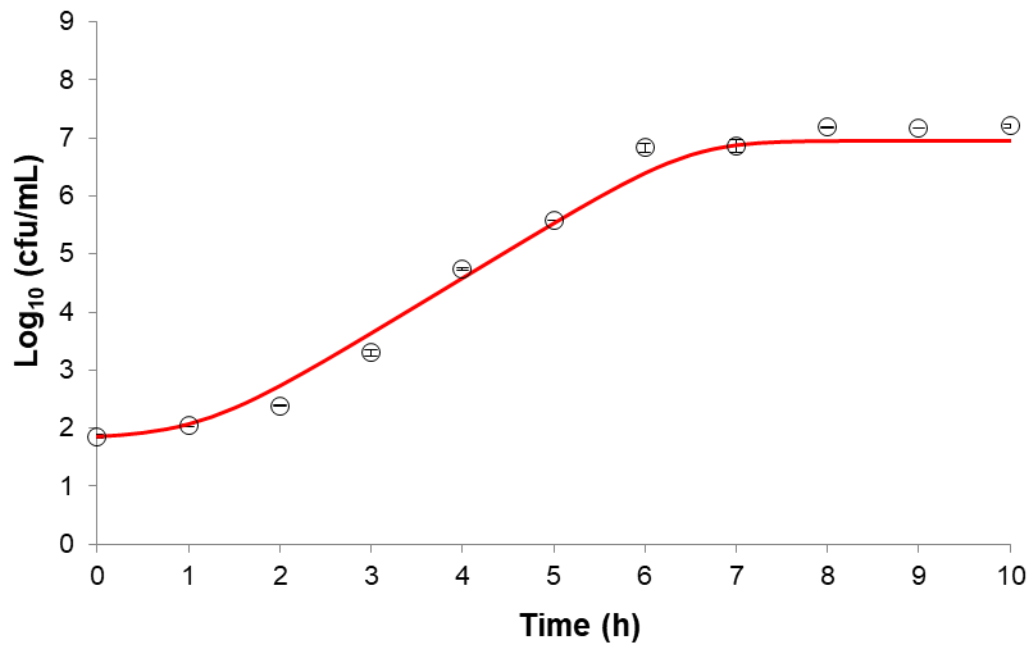
Figure 6. Comparison between observed (○) and predicted growth (-) of *Anoxybacillus flavithermus* DSM 21510 with time (h) in plant-based milk alternative when stored at 55 °C. Mean values ± standard deviation of one independent experiment with two replicates are shown.
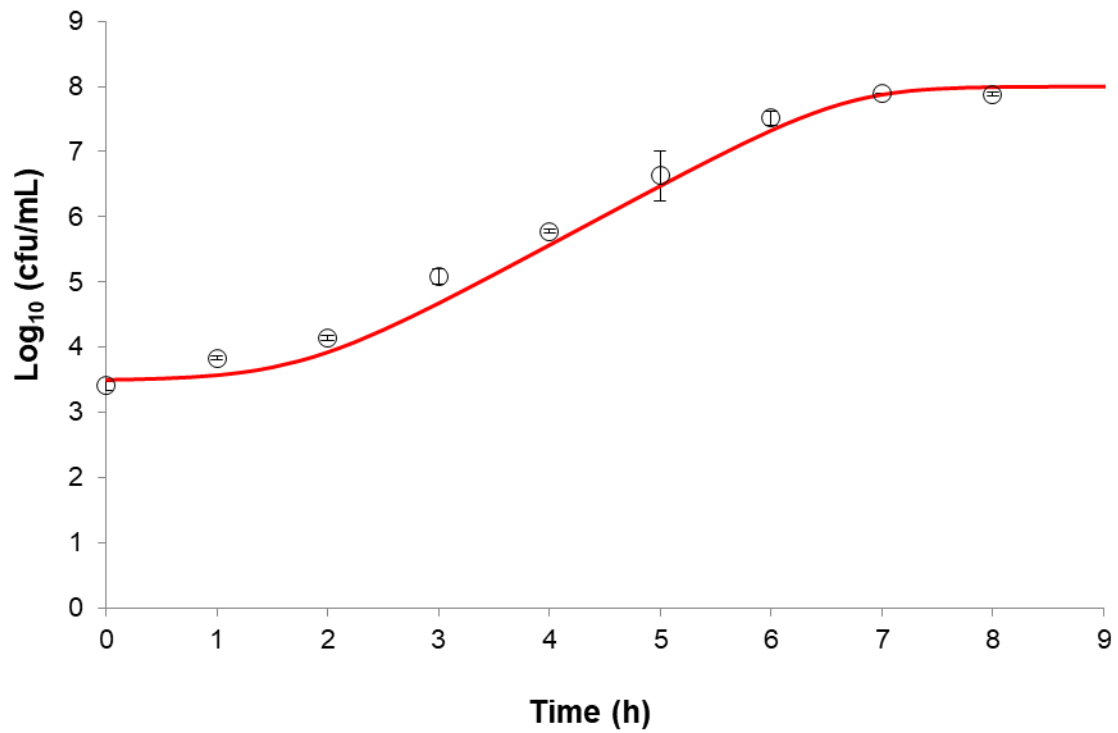
Figure 7. Comparison between observed (○) and predicted growth (-) of *Bacillus licheniformis* DSM 13 in plant-based milk alternative stored at 42 °C. Mean values ± standard deviation of one independent experiment with two replicates are shown.
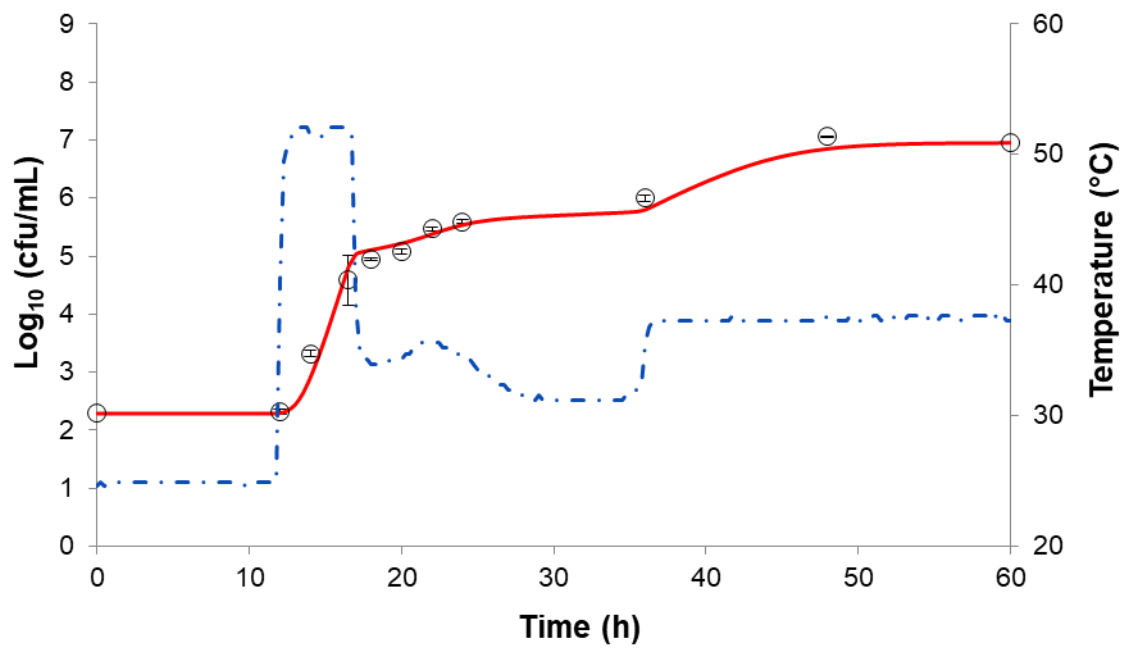
Figure 8. Comparison between observed (○) and predicted growth (-) of *Anoxybacillus flavithermus* DSM 21510 with time (h) in plant-based milk alternative stored at 25 °C for 12h, 55 °C for 5 h, 30 °C for 24 h and 37 °C for 24 h. Dashed dot blue line (--) indicates temperature changes. Mean values ± standard deviation of one independent experiment with two replicates are shown.
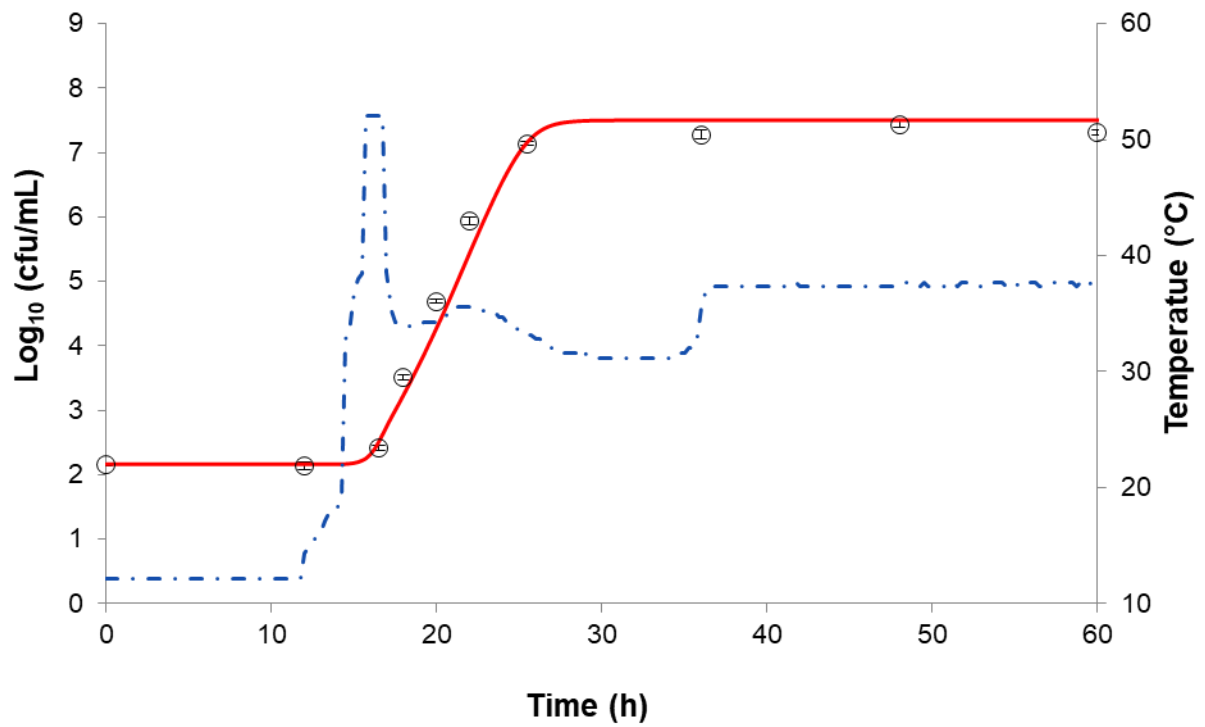
Figure 9. Comparison between observed (○) and predicted growth (-) of *Bacillus licheniformis* DSM 13 with time (h) in plant-based milk alternative stored at 12 °C for 12h, 55 °C for 5 h, 30 °C for 24 h and 37 °C for 24 h. Dashed dot blue line (--) indicates temperature changes. Mean values ± standard deviation of one independent experiment with two replicates are shown.
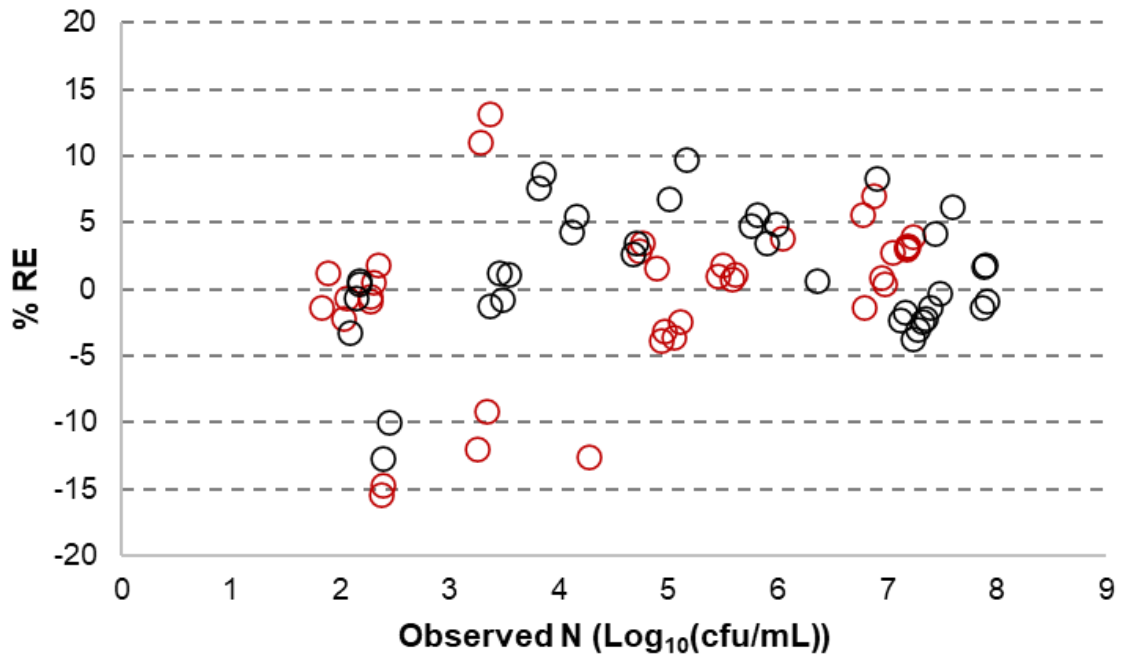
Figure 10. Relative error values (%RE) (Eq. (10)) for the comparison between observed and predicted population (Log CFU/mL) of *Anoxybacillus flavithermus* DSM 21510 (○) and *Bacillus licheniformis* DSM 13 (○) in plant-based milk alternative stored under static and dynamic temperature conditions.