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Running head: Growth dynamics of ropy slime producing *Leuconostoc mesenteroides*

Research note

Characterization and growth under different storage temperatures of ropy slime-producing *Leuconostoc mesenteroides* isolated from cooked meat products

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Keywords: Ropy slime, *Leuconostoc mesenteroides*, meat spoilage, growth curves

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ABSTRACT

The presence of lactic acid bacteria (LAB) can be detrimental when the abundant growth of slime-producing strains (*Lactobacillus* spp. and *Leuconostoc* spp.) causes spoilage of meat products. Two strains of LAB were isolated from vacuum packed, cooked hams, which had been withdrawn from the market for the so-called «*ropy slime*» defect and identified as *Leuconostoc mesenteroides*. In an attempt to define the behaviour of ropy slime-producing bacteria, two strains of *L. mesenteroides* were incubated in MRS broth at different storage temperatures and conditions of thermal abuse (4, 12, 20, 30, 37, 44 °C). Both strains showed a lack of growth at 44°C, a good level of development at 30 and 37°C and evident growth ability at low temperatures with a long stationary phase. In particular, the bacterial concentration at 4°C was above 10^5 cfu ml^{-1} after over 120 days of incubation. This research demonstrates that the refrigeration temperature for cooked meat products does not constitute a hurdle for ropy slime-producers and their subsequent ability to spoil.

HIGHLIGHTS

- Lactic acid bacteria can be detrimental when slime-producing strains cause meat spoilage.
- *L. mesenteroides* from cooked ham investigated at different temperature.
- Refrigeration temperature is not a hurdle for slime-producer strains

Meat spoilage is one of the most important causes of food waste and market rejection of meat products *(12, 38)*. The shelf-life of meat depends on the type of bacteria initially present and their ability to grow on food. Favourable growth conditions during storage, such as storage temperature, should be determined for each bacterial genus involved *(16, 22)*. Knowledge of bacterial growth kinetics is, therefore, considered the first step to prevent the development of spoilage microbiota on meat products *(8, 37)*. Although a large variety of species can be isolated from meat and meat products, the majority of attention has focused on lactic acid bacteria (LAB), widely found in nature and in the processing plants *(30, 34)*. They are «Generally Regarded As Safe» (GRAS) micro-organisms *(18, 20, 30, 32)* and are not only traditionally applied to the manufacture of fermented meat products *(4, 5, 7, 9, 48)*, such as salami, but are also used as biopreservatives *(9, 43, 44, 46)*. However, some strains of LAB are defined as specific spoilage organisms (SSO) and represent the major cause of spoilage of vacuum and modified-atmosphere packed, cooked meat products *(25, 30, 31, 35)*. Typical, detectable, organoleptic alterations are: off-flavours, discoloration, gas production, an excessive decrease in pH and slime formation, resulting in the reduction of the product's shelf-life *(2)*. Spoilage can occur within the shelf-life period and this requires producers to make withdrawals *(17)*. Even though these bacteria are usually below detection level (<10 cfu g^{-1}) after packaging, the products may spoil quickly at a later stage *(13, 27, 39)*. Among the sensory changes, the accumulation of ropy slime on the surface of cooked meat products is probably one of the most important and least tackled defects *(28, 34)*. Ropy slime-producing LAB are able to grow and survive at refrigeration temperatures, competing with other bacteria in meat products and meat processing plants *(24, 41)*. Consequently, the use of low temperatures in the preparation and storage of meat products may not prevent the formation of ropy-slime, even though refrigeration storage temperatures determine a longer shelf-life of the product *(19)*. The constant bacterial growth is favoured by the slime formation, which creates a barrier between the surrounding environment and competitors *(45)*. The slime is due to long-chain, high-molecular-mass, viscosifying or gelling, exocellular polysaccharides *(10)*.

Despite the many studies on the control of *Leuconostoc* spp. spoilage that have been carried out by the academia and the industry in recent years *(11, 20, 23, 27, 36)*, there has been no agreed methodology nor qualified studies for the evaluation of growth dynamics of *L. mesenteroides*. This study was therefore intended to evaluate the behaviour of two slime-producing *L. mesenteroides* strains at different storage temperatures and thermal abuse conditions, isolated from vacuum packed cooked hams, which had been withdrawn from the market due to the so-called ropy slime defect.

MATERIALS AND METHODS

Origin of *L. mesenteroides* strains. Strains were isolated from commercial cooked ham presenting slime on the surface, during a thorough study on *Leuconostoc* spp. contamination of cooked meat products. Samples were taken from a batch of commercial, cooked ham, withdrawn from the market due to the so-called ropy slime defect in order to isolate and identify the causative agent. Cooked ham had been produced by a large Italian company that exports products to the US market. The production is done according to a traditional recipe, after selecting fresh pork thighs which have previously been defatted and deboned.

Isolation and identification of *L. mesenteroides*. Approximately 25 cm^2 of ham were aseptically swabbed and transferred to 225 ml of sterile, buffered, peptone water (PW, Oxoid, Milan, Italy, CM1049), and homogenized at room temperature in a stomacher (PBI International) for 1 min at low speed and 1 min at high speed. Serial decimal dilutions in buffered PW were prepared and triplicate 1 ml or 0.1 ml samples of appropriate dilutions were poured or spread on de Man, Rogosa, Sharpe (MRS) Agar (Oxoid, CM0361), added with Vancomycin (20 μg/ml) to create a selective media for vancomycin-resistant bacteria *(29, 40)* and incubated for 48 hours at 30°C in microaerophilic conditions and further purified by four steps of sequential streaking on MRS-Va agar. Circular shaped colonies, with a convex elevation, 3-4 mm high, 4-6 mm in diameter, and a smooth surface, an even margin and opaque density, were presumed to be *Leuconostoc* spp. and

95 subcultured on MSE Agar (Mayeux, Sandine and Elliker, Biolife, Milan, Italy) and incubated at
96 30°C for 48 hours. Colonies grown on MSE Agar with a gelatinous appearance were primarily
97 characterized by means of Gram staining, cell morphology, catalase reaction of 3% hydrogen
98 peroxide (H_2O_2) and oxidase reactions. Gas production from glucose was evaluated on MRS broth
99 (Oxoid, CM0359) with a Durham tube, after incubation in air at 30°C for 48 hours. Bacterial
100 motility was detected using the hanging drop method.
101 Gram positive, catalase negative and oxidase negative, gas-producing, non-motile bacteria were
102 transferred to MRS broth and incubated at 30°C for 24 hours and then stored as stock cultures
103 at -80°C for further examination.

104 **Isolation and purification of DNA, oligonucleotide primers and identification by**
105 **sequencing**. Template DNA was obtained using the method described by Cenci Goga et al. *(6)*.
106 Primer sequences, lengths of PCR amplification products and amplification conditions are listed in
107 Table 1. Briefly, genus-specific primers Lu1r and Lu2 were used (MWG Biotech, Ebersberg, DE)
108 *(47)* followed by *L. mesenteroides* identification by primers that target the 16S rRNA gene: L.mesF
109 and L.mesR (MWG Biotech) *(3, 26)*. Universal primers Y1 and Y2 for bacterial 16S rRNA gene
110 were used as a positive control to ensure that the template DNA was correctly amplified *(47)*. PCR
111 products were visualized after agarose gel electrophoresis under UV illumination *(15)*. The
112 universal primers for eubacteria P27f and P1495r were used to amplify a 16S rRNA gene segment
113 *(9, 33)*. The amplicons were sent to the Microgem Lab (University of Naples, Italy) for purifying
114 and sequencing. Isolates were allocated to a given species on percentages of sequencing identity
115 and on visual inspection of the concordance, using the DIALIGN software
116 (**http://dialign.gobics.de/chaos-dialign-submission**).

117 **Growth dynamics of *L. mesenteroides*.** Each strain examined for its growth curve was
118 transferred in MRS broth (Oxoid, CM0359) tubes and incubated at 30°C for 48 hours to get a
119 concentration of approximately 10^8 cfu ml^{-1}. A dilution series to get a final concentration of 10^4 cfu

120 ml^{-1} was performed in MRS broth before incubation at different temperatures for appropriate
121 periods. A Sanyo MIR-153 incubator (Moriguchi-City Osaka, Japan) was used to maintain storage
122 temperatures of 4, 12, 20, 30, 37 and 44°C. Samples were taken in triplicate at 6, 12, 24, 36, and
123 48 h and then, every 24 h until the bacterial concentration fell below the detection limit (10 cfu
124 ml^{-1}). At each sampling point, 1 ml of sample was transferred aseptically to 9 ml of Maximum
125 Recovery Diluent (Oxoid, CM0733) for serial decimal dilutions. Duplicate samples of appropriate
126 dilutions were poured or spread plated on MRS Agar (Oxoid, CM1153) and incubated at 30°C for
127 48 hours in jars (Oxoid) under microaerophilic conditions. Growth rates based on the viable counts
128 on MRS agar were determined with a polynomial curve fitting. The sensitivity of the spread plate
129 was 10^2 cfu ml^{-1} and of the pour plate was 10 cfu ml^{-1}. The 95% confidence limit, as given by the
130 classic formula $2s=2\sqrt{x}$ *(1)*, ranged between ±37% and ±12% (*i.e.* plates with a number of cfu
131 ranging from 30 to 300). Consequently, plates with less than 30 cfu were not considered for data
132 analysis and when this applied to the lowest dilution, the results were recorded as <30 for the pour
133 plate and <300 for the spread plate *(4)*.
134 **Analysis of the results**. Each triplicate tube was examined in duplicate for each sampling,
135 and all values were converted to log for microbiological analyses and analysed using GraphPad
136 InStat, version 3.0b for Mac OS X. A Canonical Discriminant Analysis (CDA) was performed
137 using 4 parameters to show the temperature action on the 2 strains: the intercept of the fourth-
138 degree polynomial equation (a_0); day (D_{max}) and log (Y_{max}) at peak; growth rate (b).
139 A fourth-degree polynomial equation was used as an empirical model to fit the experimental data.
140 [1] $y=a_0+a_1x+a_2x^2+a_3x^3+a_4x^4$
141 where: y = log population; x = time from 0 to 180 days; a_0, a_1, a_2, a_3, a_4 = coefficients of polynomial
142 determined by the function «PROC REG» regression procedure in SAS 9.4 *(42)*. The first
143 derivative of [1] gives the maximum value for log population and days (Y_{Max} D_{max}), which

144 corresponds to the peak point of the growth curve. The growth rate from day 0 to D_{max} was
145 calculated as the slope of a linear equation as in [2].
146 [2] $b=(y-a)/x$
147 where: y = log concentration; x = time from 0 to D_{max} days; a = intercept or initial concentration for
148 d=0. Data analysis was performed by SAS/STAT in SAS 9.4 *(42)* using a regression model (REG).
149 The canonical correlation is a multivariate analysis of correlation. Canonical is the statistical term
150 for analysing latent variables (which are not directly observed) that represent multiple variables
151 (which are directly observed). A Canonical Discriminant Analysis (CDA) is a dimension-reduction
152 technique related to principal component analysis and canonical correlation. In this study CDA
153 finds linear combinations (canonical variables) of the quantitative variables (different temperatures)
154 that have the highest possible multiple correlations with the groups and provide maximal separation
155 between groups in much the same way that principal components summarize total variation. The
156 canonical variable can show substantial differences between the groups, even though none of the
157 original variables do.

158 **RESULTS**

159 **Isolation and identification of *L. mesenteroides***. According to the phenotypic and
160 genotypic findings, two strains of ropy slime-producing were identified as *L. mesenteroides*. The
161 isolates collected were Gram positive and catalase negative, vancomycin resistant coccoid bacteria,
162 producing gas and acid by fermenting glucose. The identification via PCR and DNA sequencing
163 defined the strains as *L. mesenteroides*. The two strains of ropy slime-producing *L. mesenteroides*
164 were, therefore, identified and classified as 649 and 650 (Laboratory collection ID).

165 **Growth dynamics of *L. mesenteroides* 649 and 650**. Growth curves are graphically
166 represented in Figure 1. Both strains of *L. mesenteroides* (649 and 650) started from an initial
167 concentration of approximately 10^4 cfu ml^{-1}.
168 The highest population level (3.58 log cfu ml^{-1} and 3.94 log cfu ml^{-1} for *L. mesenteroides* 649 and
169 650, respectively) was reached after 4 hours at 44°C.The bacterial concentration was below the

detection limit after 24 and 48 hours for *L. mesenteroides* 650 and 649, respectively. At 37°C, the maximum population (8 log cfu ml^{-1}) was observed after 2.5 and 2.6 days for *L. mesenteroides* 650 and 649, respectively. After 6 days of storage, cell concentration was still over 5 log cfu ml^{-1} and then rapidly fell below the detection limit.

At 30°C, the highest population level (9.22 log cfu ml^{-1} and 9.40 log cfu ml^{-1} for *L. mesenteroides* 649 and 650) occurred after 48 hours. Bacterial concentration was below the detection limit after 8 days and 13 days for *L. mesenteroides* 649 and 650, respectively.

At 20°C, the maximum population (8.53 log cfu ml^{-1} and 8.56 log cfu ml^{-1} for *L. mesenteroides* 649 and 650 respectively) was reached after 8,8 days and 7,7 days for *L. mesenteroides* 649 and 650. At 12°C, the maximum population (8,1 log cfu ml^{-1} and 8.16 log cfu ml^{-1} for *L. mesenteroides* 649 and 650, respectively) was reached after 15.4 days and 13.7 days for *L. mesenteroides* 649 and 650; the stationary phase was maintained for two weeks. Then, bacterial concentration was below the detection limit after 120 days of incubation for both strains. At 4°C, the maximum population (8,9 log cfu ml^{-1} and 8.97 log cfu ml^{-1} for *L. mesenteroides* 649 and 650, respectively) was reached after 42.8 days and 44.7 days for *L. mesenteroides* 649 and 650. Bacterial concentration was maintained over 5 log cfu ml^{-1} after 120 days of incubation for both strains.

Figures 1 and 2 show the effect of the different storage temperatures on the growth of two *L. mesenteroides* strains.

Canonical Discriminant Analysis. Table 2 indicates the estimated days of the growth peak (D_{max}), and log (Y_{max}) at peak and the growth rate (b) to reach the maximum viable cell concentration. With the exception of the abuse temperature of 44°C with an immediate decrease in the population, the maximum growth rate detected was at 30°C. The maximum length of complete inhibition was described during each temporal slot. However, viable cells were still detectable at 4°C. Table 3 shows the polynomial, descriptive parameters for both strains and the growth

194 temperature. Figure 2 (CDA) shows the effect of the temperature on the two strains. The first
195 canonical variable explained 99% of the among-class separation and all 4 parameters contributed
196 significantly. Four groups (12ºC-20ºC-30ºC; 37ºC; 4ºC and 44ºC) are clearly identified, mostly by
197 D_{max} and Y_{max}. The univariate statistics result in R^2 values ranging from 0.903 for Y_{max} to 0.999 for
198 D_{max} and each variable is significant above the 0.005 level.

199 **DISCUSSION**

200 The microbiota of many valuable foods, which are susceptible to bacterial spoilage, is usually
201 dominated by LAB *(14)*. Refrigerated meat products can show several defects, such as
202 discoloration, gas production, off-odours, off-flavours, a decrease in pH and slime production *(19,*
203 *22)*. Kalschne, D. L. et al. *(22)* observed the formation of milky exudates at 45 days of storage. The
204 initial LAB population was approximately 1.98 log cfu g^{-1}, and after 45 days of storage raised
205 values of 7.59 log cfu g^{-1} at 4 °C and 8.25 log cfu g^{-1} at 8 °C. Therefore, *Lactobacillus curvatus,*
206 *Lactobacillus sakei* were identified as the dominant spoilage bacteria of sliced, vacuum-packed,
207 cooked ham after 45 days of storage and *Leuconostoc mesenteroides* as a minor component *(22)*.
208 Raimondi at al (2019) described the microbiota of sliced, cooked ham, packaged in a modified
209 atmosphere: a few days after packaging, the LAB population of the samples was 2.9 log cfu g^{-1} on
210 average, whereas the amount increased to 7.7 log cfu g^{-1} in the samples from the end of their shelf-
211 life and in those rejected due to spoilage phenomena.
212 The results of our research, on the other hand, give a detailed description of the growth behaviour of
213 two *L. mesenteroides* strains isolated from cooked meat products. The optimum growth temperature
214 in MRS broth is 30°C, with a short lag phase followed by fast growth. Strain 649 reached the
215 highest concentration (9.22 log cfu ml^{-1}) on day 2.2 and 650 (9.40 log cfu ml^{-1}) on day 1.9. On the
216 contrary, no growth was recorded at 44°C, whereas the ability to grow at refrigeration temperatures
217 was well documented at 12°C and 4°C. Low storage temperatures determined a slowing down of
218 bacterial growth. However, the rate was steady and very high concentrations (up to 8.9 log cfu ml^{-1})

were reached (strain 649 on day 42.8 and 650 on day 44.7 at 4°C). These findings show that starting from an initial concentration of 4 log cfu ml^{-1}, the maximum population concentration reached over 8 log cfu ml^{-1} for all storage temperature considered (except for 44°C). However, the length of the lag phase stretched to a greater or lesser extent. In addition, it demonstrated that viable cells are still detectable after several days of incubation at refrigeration temperature (>5 log cfu ml^{-1} on day 120) and this supports the hypothesis of the abundant bacterial growth and subsequent accumulation of slime on the surface of meat products, even though the cold chain is maintained throughout product shelf life. CDA (Figure 2) showed that four groups (12ºC-20ºC-30ºC; 37ºC; 4ºC and 44ºC) are clearly identified and that the growth dynamics of *L. mesenteroides* at 4ºC and 12ºC are more similar to the growth dynamics at 20ºC and 30ºC (fastest growth rate and highest bacterial concentration in cfu ml^{-1}) than the abuse at 40ºC. From the bacterial growth perspective, the refrigeration at 4ºC or at a minor temperature abuse of 12ºC is worse than a mismanagement at 37ºC or at 44ºC.

This research highlighted the growth ability of two strains of *L. mesenteroides* at refrigeration temperatures. Their capability to grow and persist at refrigeration temperatures makes the exponential increase of their total population possible, by exploiting the nutrients and producing abundant exopolysaccharides to create a favourable surrounding environment. The latter is facilitated by the low concentration of the competitive microbiota present on the product following heat treatment *(21)*. Maintenance of the cold chain, of paramount importance for food safety, appears not to be an obstacle for the growth of *L. mesenteroides*, given the ability of these microorganisms to grow well at refrigeration temperatures.

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376

377 **FIGURE LEGEND**

378 FIGURE 1. Growth curves of strains 649 (___) and 650 (---) at 4-12-20-30-37-44°C (log cfu ml^{-1},

379 average of three replications).

380 FIGURE 2. Axis 1-2 by canonical discriminant analysis of the effect of temperature on the growth

381 of *L. mesenteroides* strains 649 and 650.

382

TABLE 1. Primer sets used in this study.

DNA target	Primer	Sequence (5'-3')	Size (bp)	PCR conditions	References
Bacterial 16S rRNA	P27f P1495r	GAG AGT TTG ATC CTG GCT CAG CTA CGG CTA CCT TGT TAC GA	1100	1 cycle at 95 °C for 5', 35 cycles at 94 °C for 30'', 50 °C for 45'' and 72 °C for 2' and final extension at 72 °C for 10'	*(9, 33)*
Bacterial 16S rRNA	Y1 Y2	TGGCTCAGAACGAACGCTGGCCCG CCCACTGCTGCCTCCCGTAGGAGT	350	1 cycle at 94 °C for 3' followed by 30 cycles at 94 °C for 45'', 55°C for 45'' and 72°C for 1 min and final extension at 72 °C for 10'	*(47)*
Leuconostoc spp. 16S rRNA	Lu1r Lu2	CCACAGCGAAAGGTGCTTGCAC GATCCATCTCTAGGTGACGCCG	175	1 cycle at 94 °C for 3' followed by 30 cycles at 94 °C for 45'', 55°C for 45'' and 72°C for 1 min and final extension at 72 °C for 10'	*(47)*
L. mesenteroides 16S rRNA	L.mes-f L.mes-r	AACTTAGTGTCGCATGAC AGTCGAGTTACAGACTACAA	110	1 cycle at 94 °C for 5', followed by 30 cycles at 94°C for 1', 60°C for 1', 72°C for 2'' and final extension of 72 °C for 10'	*(3, 26)*

TABLE 2. Kinetic parameters.

Strain	Temperature °C	R^2	Kinetic parameters			
			D_{max} (days)	Y_{max} (log)	b (Δlog/day)	Dtot (days)
L. m. 649	4	0.9392	42.8	8.901	0.123	179
	12	0.8752	15.4	8.099	0.177	117
	20	0.7712	8.8	8.533	0.260	80
	30	0.9308	2.2	9.220	2.157	14
	37	0.9779	2.6	8.039	1.804	11
	44	1.0000	0.2	3.587	1.855	2
L. m. 650	4	0.9504	44.7	8.966	0.117	179
	12	0.8977	13.7	8.160	0.122	117
	20	0.8094	7.7	8.563	0.310	80
	30	0.9602	1.9	9.401	2.662	14
	37	0.9950	2.5	8.112	1.886	11
	44	1.0000	0.2	3.939	3.534	1

D_{max}: days; Y_{max}: log cfu ml^{-1}; b: growth rate; Dtot: length of the experiment in days

TABLE 3. Polynomial coefficients.

Strain	Temperature °C	R^2	a0	a1	a2	a3	a4
L. m. 649	4	0.9392	3.6375	0.2966	-0.0054	0.00003	-75 E-9
	12	0.8752	5.3856	0.4009	-0.0179	0.00023	-95 E-8
	20	0.7712	6.2350	0.5802	-0.0435	0.00087	-539 E-8
	30	0.9308	4.4547	5.0347	-1.6578	0.17387	-0.0059
	37	0.9779	3.3841	4.3766	-1.3325	0.14459	-0.0057
	44	1.0000	3.1761	3.9834	-10.8990	6.02439	-0.9839
L. m. 650	4	0.9504	3.7246	0.2924	-0.0054	0.00004	-88 E-9
	12	0.8977	6.4905	0.2724	-0.0132	0.00017	-697 E-9
	20	0.8094	6.1725	0.6857	-0.0577	0.00122	-778 E-8
	30	0.9602	4.2432	6.0930	-2.1975	0.23564	-0.0080
	37	0.9950	3.3326	4.6729	-1.5040	0.17864	-0.0075
	44	1.0000	3.1661	7.6398	-21.4817	12.9166	-2.2407

a0, a1, a2, a3, a4: coefficients of polynomial determined by the function «PROC REG» regression procedure in SAS 9.4

Figure 1

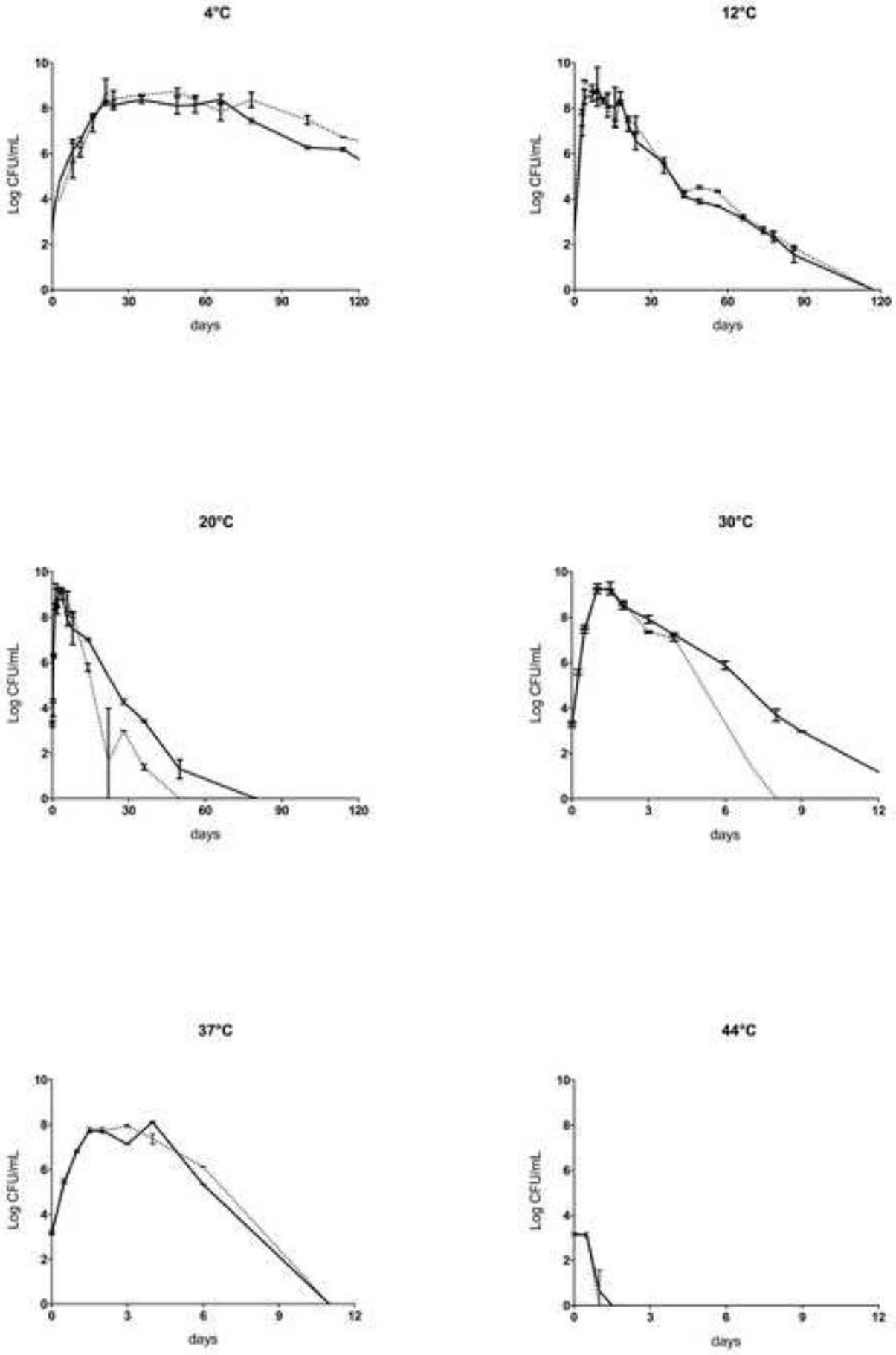

Figure 2