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

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

12

13 **Research note**

14

15 **Characterization and growth under different storage temperatures of ropy slime-producing**

16 ***Leuconostoc mesenteroides* isolated from cooked meat products**

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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 Two strains of LAB were isolated from vacuum packed, cooked hams, which had been withdrawn from the market for the so-called «ropy slime» defect and identified as *Leuconostoc mesenteroides*.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<sup>5</sup> cfu ml<sup>-1</sup> 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

44 Meat spoilage is one of the most important causes of food waste and market rejection of meat  
45 products (12, 38). The shelf-life of meat depends on the type of bacteria initially present and their  
46 ability to grow on food. Favourable growth conditions during storage, such as storage temperature,  
47 should be determined for each bacterial genus involved (16, 22). Knowledge of bacterial growth  
48 kinetics is, therefore, considered the first step to prevent the development of spoilage microbiota on  
49 meat products (8, 37). Although a large variety of species can be isolated from meat and meat  
50 products, the majority of attention has focused on lactic acid bacteria (LAB), widely found in nature  
51 and in the processing plants (30, 34). They are «Generally Regarded As Safe» (GRAS) micro-  
52 organisms (18, 20, 30, 32) and are not only traditionally applied to the manufacture of fermented  
53 meat products (4, 5, 7, 9, 48), such as salami, but are also used as biopreservatives (9, 43, 44, 46).  
54 However, some strains of LAB are defined as specific spoilage organisms (SSO) and represent the  
55 major cause of spoilage of vacuum and modified-atmosphere packed, cooked meat products (25,  
56 30, 31, 35). Typical, detectable, organoleptic alterations are: off-flavours, discoloration, gas  
57 production, an excessive decrease in pH and slime formation, resulting in the reduction of the  
58 product's shelf-life (2). Spoilage can occur within the shelf-life period and this requires producers to  
59 make withdrawals (17). Even though these bacteria are usually below detection level ( $<10 \text{ cfu g}^{-1}$ )  
60 after packaging, the products may spoil quickly at a later stage (13, 27, 39). Among the sensory  
61 changes, the accumulation of ropy slime on the surface of cooked meat products is probably one of  
62 the most important and least tackled defects (28, 34). Ropy slime-producing LAB are able to grow  
63 and survive at refrigeration temperatures, competing with other bacteria in meat products and meat  
64 processing plants (24, 41). Consequently, the use of low temperatures in the preparation and storage  
65 of meat products may not prevent the formation of ropy-slime, even though refrigeration storage  
66 temperatures determine a longer shelf-life of the product (19). The constant bacterial growth is  
67 favoured by the slime formation, which creates a barrier between the surrounding environment and  
68 competitors (45). The slime is due to long-chain, high-molecular-mass, viscosifying or gelling,  
69 exocellular polysaccharides (10).

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

## 77 MATERIALS AND METHODS

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

85 **Isolation and identification of *L. mesenteroides*.** Approximately 25 cm<sup>2</sup> of ham were  
86 aseptically swabbed and transferred to 225 ml of sterile, buffered, peptone water (PW, Oxoid,  
87 Milan, Italy, CM1049), and homogenized at room temperature in a stomacher (PBI International)  
88 for 1 min at low speed and 1 min at high speed. Serial decimal dilutions in buffered PW were  
89 prepared and triplicate 1 ml or 0.1 ml samples of appropriate dilutions were poured or spread on de  
90 Man, Rogosa, Sharpe (MRS) Agar (Oxoid, CM0361), added with Vancomycin (20 µg/ml) to create  
91 a selective media for vancomycin-resistant bacteria (29, 40) and incubated for 48 hours at 30°C in  
92 microaerophilic conditions and further purified by four steps of sequential streaking on MRS-Va  
93 agar. Circular shaped colonies, with a convex elevation, 3-4 mm high, 4-6 mm in diameter, and a  
94 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<sub>2</sub>O<sub>2</sub>) 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<sup>8</sup> cfu ml<sup>-1</sup>. A dilution series to get a final concentration of 10<sup>4</sup> cfu

120 ml<sup>-1</sup> 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<sup>-1</sup>). 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<sup>2</sup> cfu ml<sup>-1</sup> and of the pour plate was 10 cfu ml<sup>-1</sup>. 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<sup>-1</sup>.

168 The highest population level ( $3.58 \log$  cfu ml<sup>-1</sup> and  $3.94 \log$  cfu ml<sup>-1</sup> for *L. mesenteroides* 649 and  
169 650, respectively) was reached after 4 hours at 44°C. The bacterial concentration was below the

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

174 At 30°C, the highest population level (9.22 log cfu ml<sup>-1</sup> and 9.40 log cfu ml<sup>-1</sup> for *L. mesenteroides*  
175 649 and 650) occurred after 48 hours. Bacterial concentration was below the detection limit after 8  
176 days and 13 days for *L. mesenteroides* 649 and 650, respectively.

177 At 20°C, the maximum population (8.53 log cfu ml<sup>-1</sup> and 8.56 log cfu ml<sup>-1</sup> for *L. mesenteroides* 649  
178 and 650 respectively) was reached after 8,8 days and 7,7 days for *L. mesenteroides* 649 and 650. At  
179 12°C, the maximum population (8,1 log cfu ml<sup>-1</sup> and 8.16 log cfu ml<sup>-1</sup> for *L. mesenteroides* 649 and  
180 650, respectively) was reached after 15.4 days and 13.7 days for *L. mesenteroides* 649 and 650; the  
181 stationary phase was maintained for two weeks. Then, bacterial concentration was below the  
182 detection limit after 120 days of incubation for both strains. At 4°C, the maximum population (8,9  
183 log cfu ml<sup>-1</sup> and 8.97 log cfu ml<sup>-1</sup> for *L. mesenteroides* 649 and 650, respectively) was reached after  
184 42.8 days and 44.7 days for *L. mesenteroides* 649 and 650. Bacterial concentration was maintained  
185 over 5 log cfu ml<sup>-1</sup> after 120 days of incubation for both strains.

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

188 **Canonical Discriminant Analysis.** Table 2 indicates the estimated days of the growth peak  
189 ( $D_{max}$ ), and log ( $Y_{max}$ ) at peak and the growth rate ( $b$ ) to reach the maximum viable cell  
190 concentration. With the exception of the abuse temperature of 44°C with an immediate decrease in  
191 the population, the maximum growth rate detected was at 30°C. The maximum length of complete  
192 inhibition was described during each temporal slot. However, viable cells were still detectable at  
193 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 (12°C-20°C-30°C; 37°C; 4°C and 44°C) 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  $1.98 \log \text{cfu g}^{-1}$ , and after 45 days of storage raised  
205 values of  $7.59 \log \text{cfu g}^{-1}$  at 4 °C and  $8.25 \log \text{cfu g}^{-1}$  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 et 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  $2.9 \log \text{cfu g}^{-1}$  on  
210 average, whereas the amount increased to  $7.7 \log \text{cfu g}^{-1}$  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 ( $9.22 \log \text{cfu ml}^{-1}$ ) on day 2.2 and 650 ( $9.40 \log \text{cfu ml}^{-1}$ ) 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  $8.9 \log \text{cfu ml}^{-1}$ )

219 were reached (strain 649 on day 42.8 and 650 on day 44.7 at 4°C). These findings show that starting  
220 from an initial concentration of 4 log cfu ml<sup>-1</sup>, the maximum population concentration reached over  
221 8 log cfu ml<sup>-1</sup> for all storage temperature considered (except for 44°C). However, the length of the  
222 lag phase stretched to a greater or lesser extent. In addition, it demonstrated that viable cells are still  
223 detectable after several days of incubation at refrigeration temperature (>5 log cfu ml<sup>-1</sup> on day 120)  
224 and this supports the hypothesis of the abundant bacterial growth and subsequent accumulation of  
225 slime on the surface of meat products, even though the cold chain is maintained throughout product  
226 shelf life. CDA (Figure 2) showed that four groups (12°C-20°C-30°C; 37°C; 4°C and 44°C) are  
227 clearly identified and that the growth dynamics of *L. mesenteroides* at 4°C and 12°C are more  
228 similar to the growth dynamics at 20°C and 30°C (fastest growth rate and highest bacterial  
229 concentration in cfu ml<sup>-1</sup>) than the abuse at 40°C. From the bacterial growth perspective, the  
230 refrigeration at 4°C or at a minor temperature abuse of 12°C is worse than a mismanagement at  
231 37°C or at 44°C.

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

240

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377

## FIGURE LEGEND

378 FIGURE 1. Growth curves of strains 649 (\_\_\_) and 650 (---) at 4-12-20-30-37-44°C (log cfu ml<sup>-1</sup>,  
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 CCCCTGCTGCCTCCCGTAGGAGT	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)
<i>Leuconostoc</i> spp. 16S rRNA	Lu1r Lu2	CCACAGCGAAAGGTGCTTGAC 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)
<i>L. mesenteroides</i> 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 <sup>2</sup>	Kinetic parameters			
			D <sub>max</sub> (days)	Y <sub>max</sub> (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

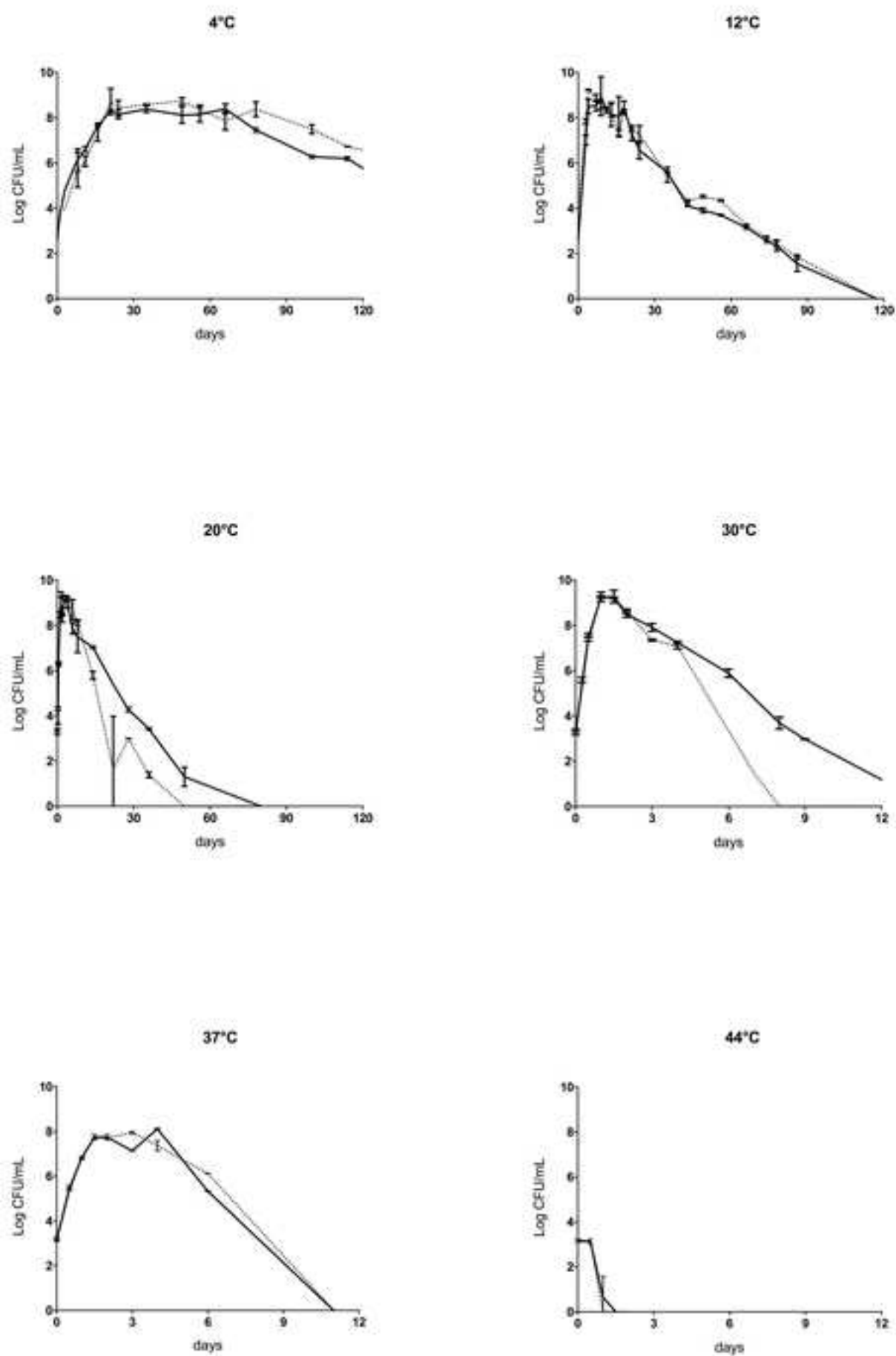
D<sub>max</sub>: days; Y<sub>max</sub>: log cfu ml<sup>-1</sup>; b: growth rate; Dtot: length of the experiment in days

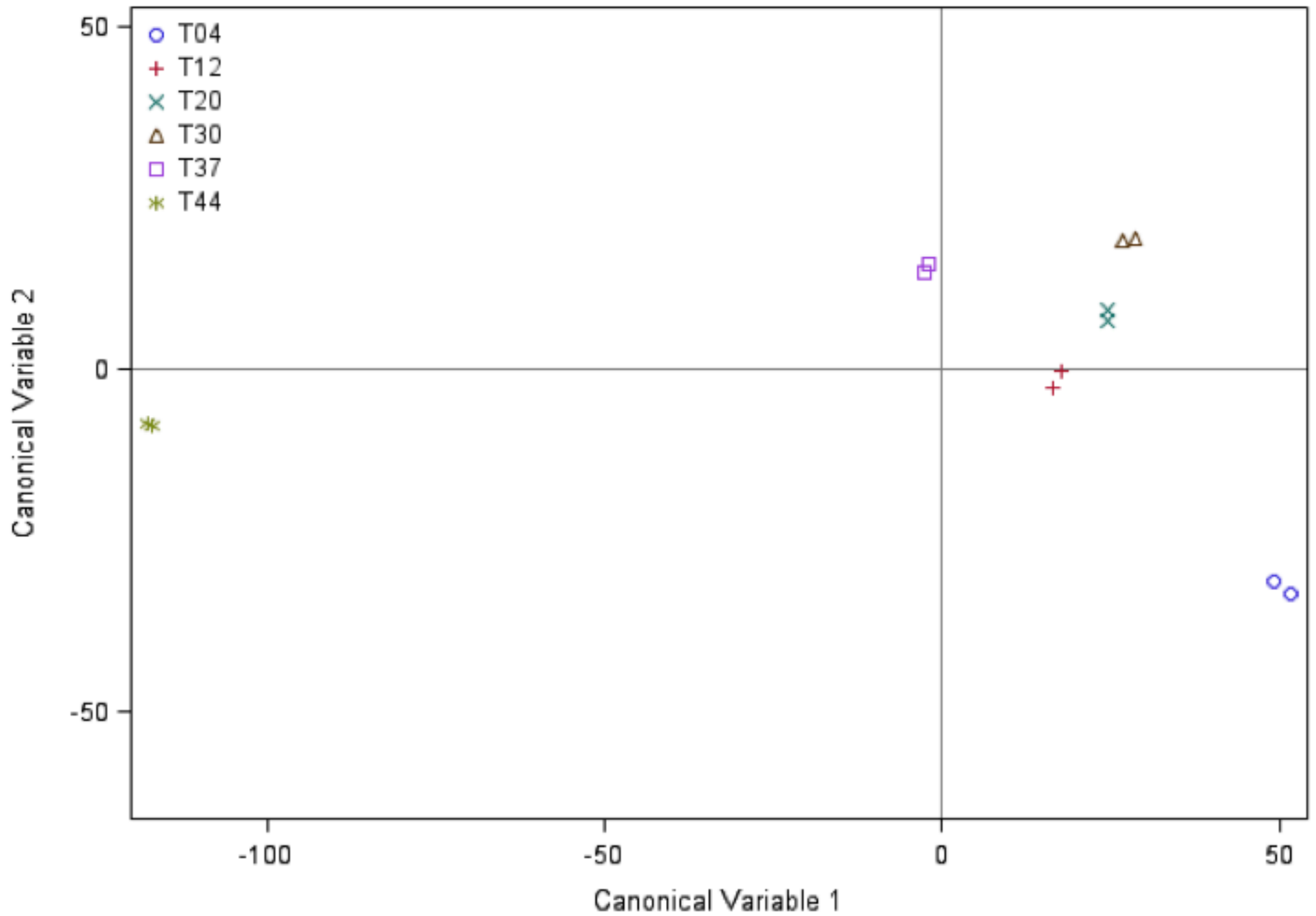
TABLE 3. Polynomial coefficients.

Strain	Temperature °C	R <sup>2</sup>	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







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