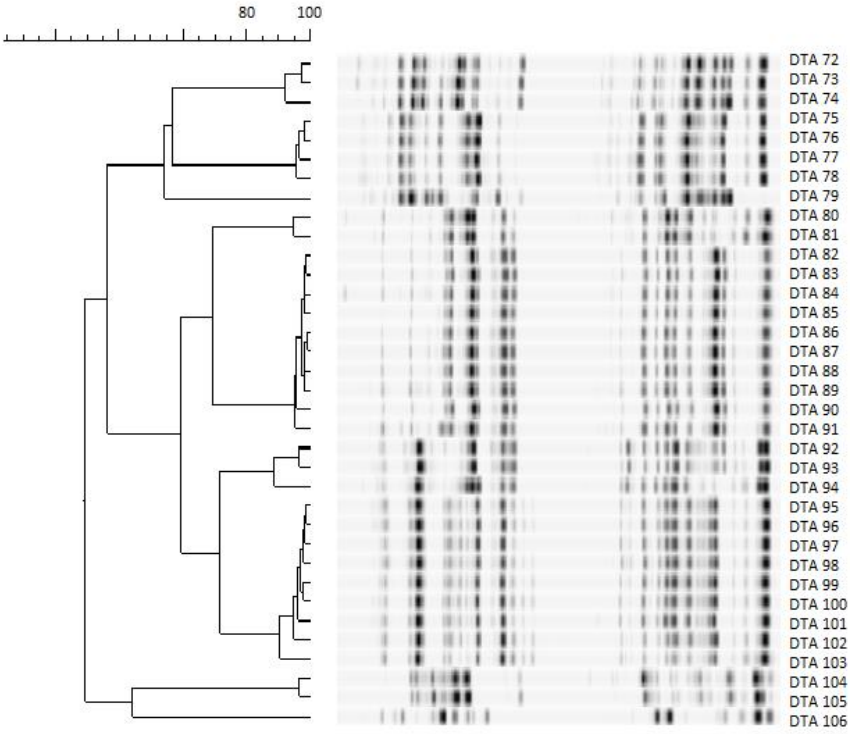
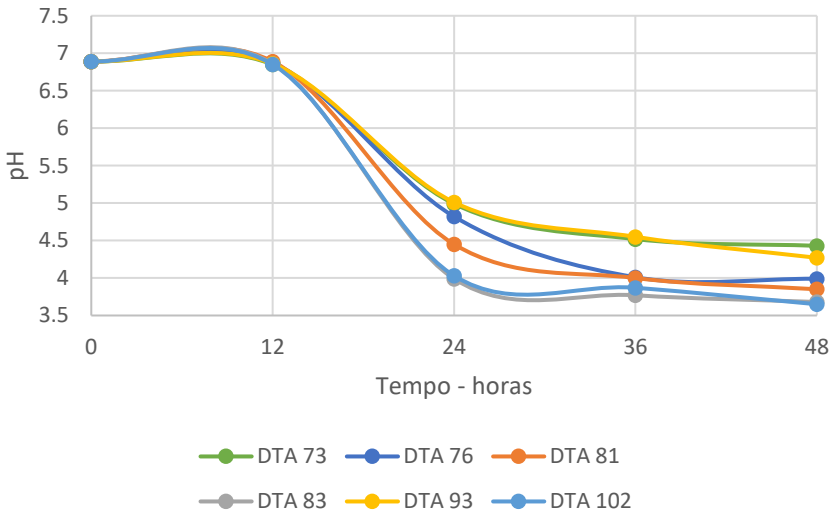
Supplementary Material

A predictive method to carry out a durability study to validate the shelf-life of vacuum packaged cooked sausages

1. *Acidification potential of Lacticaseibacillus*



(a)



(b)

Figure S1. (a) Cluster analysis of RADP-PCR profiles obtained of 35 *Lacticaseibacillus* isolates from stool samples of infants aged between 7 to 21 days. The amplification patterns were analyzed using the software Gel Compar 4.1 (Applied Maths) [1], and **(b)** potential of *Lacticaseibacillus* to acidify the pasteurized deMan, Rogosa and Sharp broth medium.

2. *Characterization of sausage spoilage*

2.1. Microbial Community Identification

High-throughput sequencing was performed for sequencing multiple microbial DNA molecules extracted from sausages. The total microbial DNA of sample groups (N = 3) was evaluated to determine the microbial agents responsible for sausages spoilage. The DNA of the samples was extracted with the Stool PSP Spin DNA kit (STRATEC Biomedical AG, Germany). The V3-V4 primers with the following conditions were employed: the first polymerase chain reaction (PCR) primer with the Illumina sequences based on TruSeq structure adapter (Illumina, San Diego, CA) and the second PCR with indexing sequences. The first PCR reaction was carried out using Platinum Taq (Invitrogen, USA) with 95 °C for 5 min, 95 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s (25 cycles), and a final extension of 72 °C for 2 min. The second PCR with 95 °C for 5 min, 95 °C for 45 s, 66 °C for 30 s and 72 °C for 45 s (10 cycles), and a final extension of 72 °C for 2 min. For comparison, Illumina 16S protocol was used (Illumina Technical Note 15044223 Rev. B). The final PCR reaction was cleaned up using AMPureXP beads (Beckman Coulter, Brea, CA) and samples were pooled in the sequencing libraries for quantification. The pool amplicon estimations were performed with Picogreen dsDNA assays (Invitrogen, USA) and the pooled libraries were diluted for accurate qPCR quantification using KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems, Woburn, MA). The libraries were sequenced in a MiSeq system using the standard Illumina primers provided in the kit. After sequencing, bioinformatics sequence demultiplexing, adaptor, and primer trimming were performed (Figure S1).

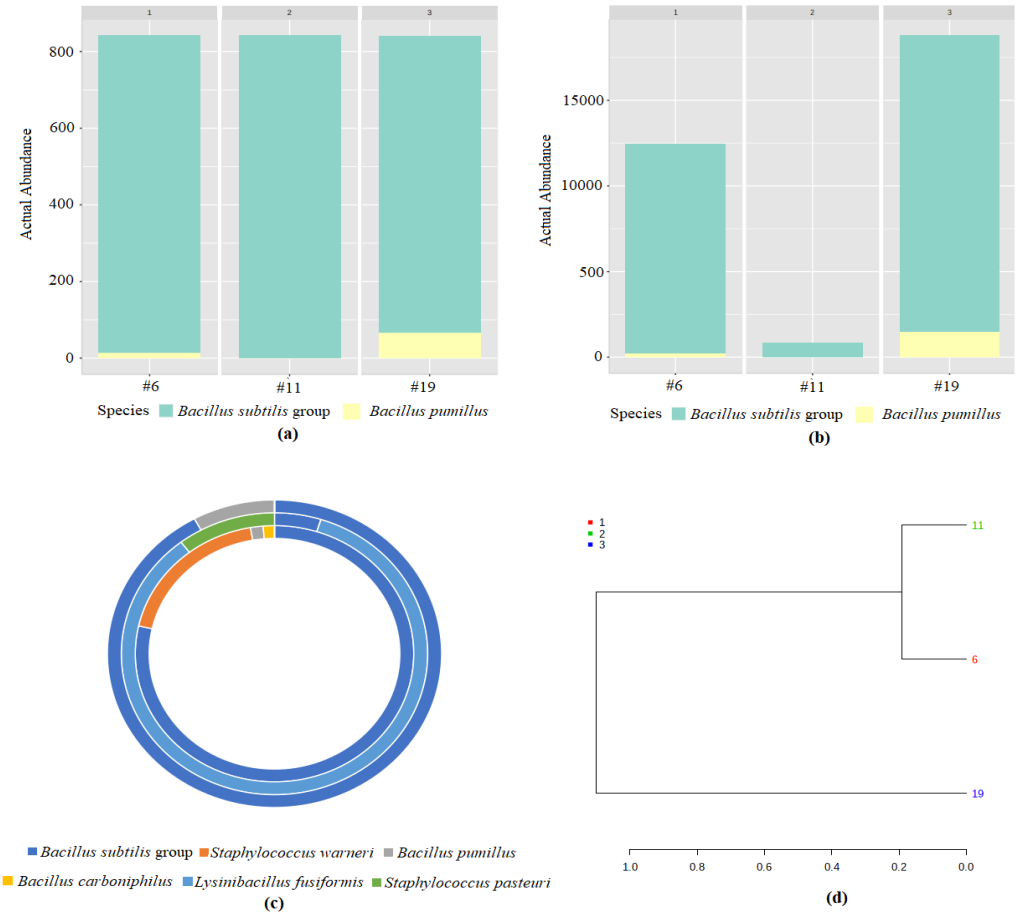


Figure S2. The relative abundance (**a** and **b**), Krona plot **(c)**, and dendrogram of similarities and discrepancies of high-throughput sequencing of bacterial phyla of vacuum-packaged cooked sausages.

2.2. Microbial growth curve during sausage spoilage

Total bacteria growth was monitored during the stimulation of natural microbiota growth by incubation at 4, 12, 24, and 36 °C. The initial decimal suspension (1/10) was prepared by weighing and homogenizing the sausage in its package. This step was conducted to count the microorganisms in the sausage, as well as the ones accumulated in the liquid inside the package after syneresis. The method ISO 4833 (2013) was used for enumeration of microorganisms in samples. Colonies were counted using a colony counter (Interscience, Saint Nom, France), and the results were calculated using at least two successive dilution levels, according to Equation S1 [2].

$$N = \frac{\sum C}{V\,[n1+0.1n2)\,d} \qquad \text{(Equation S1)}$$

Where, ∑c - sum of the colonies counted on the two plates retained from two successive dilutions (at least one of which contains a minimum of 10 colonies), V - volume of inoculum placed in each well (mL), n1 and n2 - number of wells selected in the first dilution and number of wells selected in the second dilution, respectively, and d - level of the first dilution retained.

Xy-scatter charts relating the microbial number (log cfu/g) and time (hour) for each temperature were plotted to profile the microbial population growth. The angular coefficient was used to determine specific growth rates after normalizing the phases (Figure S2).

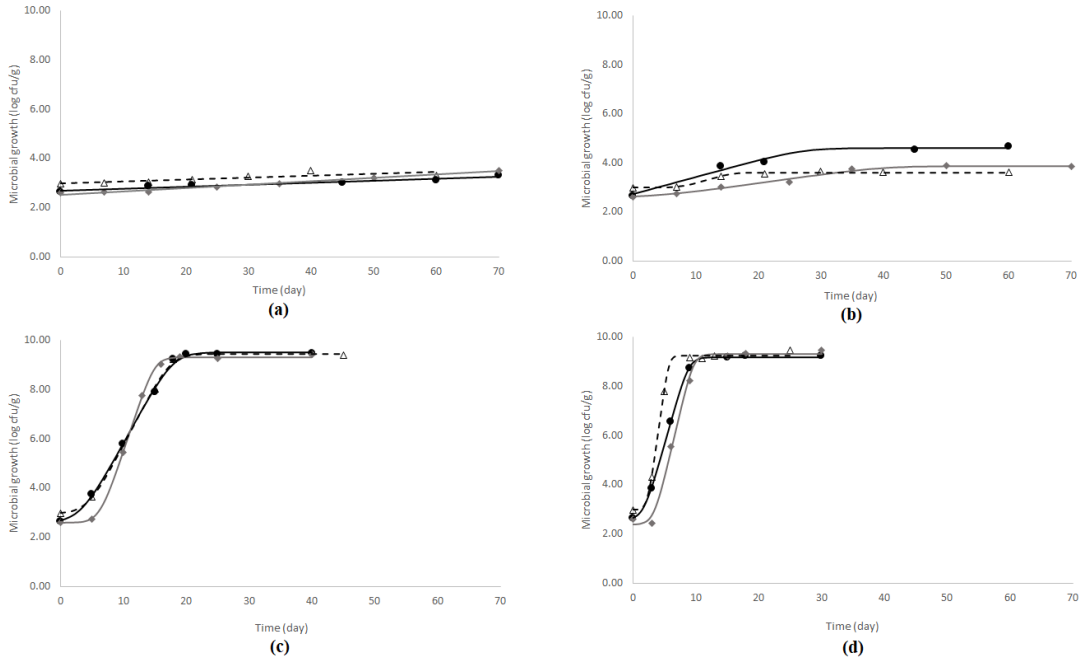


Figure S3. Microbial growth curves at 4 °C **(a)**, 12 °C **(b)**, 24 °C **(c)**, and 36 °C **(d)**. They were plotted regarding the natural microbiota of vacuum-packaged cooked sausages (sample #1; sample #2\; sample #3). Drop-plate technique was used to count total bacteria. Baranyi's mathematical model was applied to model the microbial growth at each temperature. The initial population was ca. 2.8 log cfu/g. The growth (log) phase started suddenly after incubation at 24 and 36 °C, and extended up to 8.2 log cfu/g. Stationary phase started after the population had reached ca. 9.3 log cfu/g. The period between the log and the stationary phases was considered the deceleration phase.

2.2.1. Instrumental color measurement and detection of slime formation

Simultaneously to the microbial counting, consumer appraisal of vacuum-packaged cooked sausages was investigated by measuring the color and detecting the formation of slime. For this purpose, the growth curves were evaluated to determine when changes in sensory attributes related to the appearance occur.

The color was analyzed in two ways: on the packaged sausage surface and after withdrawing the pieces from the packages and cleaning up their surface with an absorbent tower paper. The method reported in the Meat Color Measurement Guidelines was used for measurements [3]. A colorimeter device (450 G, Delta Color, Brazil) with an 8-mm diameter measuring area and a 50-mm diameter illumination area was used. Illuminant A (average incandescent, tungsten-filament lighting, 2857 K) and 10º standard observer were used to place more emphasis on the proportion of red wavelengths and capture a larger portion of the sample scanned. The instrument was standardized based on scans of black and white standardized tiles. Color values were expressed by L* (100 = white, 0 = black), a* (positive values = redness, negative values = greenness), and b* (positive values = yellowness, negative values = blueness). Each piece was scanned at five different positions to obtain a representative area by calculating the average. The total color differences (ΔE) between the control samples and the other treatments, chroma (C*) (color intensity, also known as saturation index), and the parameter to indicate shifts in color over time towards discoloration (H°) were calculated according to Equations 3 to 5, respectively. All measurements were carried out at room temperature (23 ± 1°C).

$$\Delta E = \sqrt{(L_0^* - L_T^*)^2 + (a_0^* - a_T^*)^2 + (b_0^* - b_T^*)^2} \qquad \text{(Equation 3)}$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad \text{(Equation 4)}$$

$$H^\circ = arctagent\ (b^*/a^*) \qquad \text{(Equation 5)}$$

The formation of ropy slime on the surface of the sausages during the incubation period was collected by rinsing the surfaces (package and sausage) with 50 mL of buffer phosphate pH 7.2 added with 0.8 % of polysorbate 80 (Tween 80) (Dinâmica, Jaraguá do Sul, Santa Catarina, Brazil). After homogenization, the absorbance was measured on a spectrophotometer device (Biospectro, Spectrum SP-2000UV, Brazil) at 538 nm. A quartz standard cuvette (10 mm2, 3.5 cm), wiped with soft cotton, was used for all readings. The results were expressed by subtracting the values obtained during the incubation and the zero time to the same sample group. The same phosphate buffer used for rinsing was used as the blank of all measurements. Samples incubated at different temperatures were treated separately (Table S1).

Table S1. Instrumental color measurement (on the unopened packaged sausages and after withdrawing the sausages from the packages and cleaning up their surfaces) and slime formation detection.

Incubation conditions		Color of sausages																						Absorbance (538 nm)
		Unopened package										Opened package												
Temperature (°C)	Time (days)	L*		a*		b*		C*		H°		ΔE		L*		a*		b*		C*		H°		ΔE		
4	7	40.89	± 1.04	37.36	± 1.64	11.67	± 0.17	39.63	± 1.59	19.47	± 0.64	0.58	± 0.16 [a]	21.26	± 0.99	31.15	± 1.34	11.67	± 0.58	33.28	± 1.07	20.58	± 1.71	0.73	± 0.50	0.216	± 0.013
	14	40.50	± 0.77	37.04	± 1.22	11.61	± 0.11	39.33	± 1.17	19.63	± 0.53	0.27	± 0.16 [a]	21.87	± 1.39	31.08	± 1.37	11.61	± 0.42	33.18	± 1.13	20.52	± 1.53	0.94	± 0.33	0.215	± 0.012
	25	40.66	± 1.03	37.36	± 1.36	11.47	± 0.18	39.57	± 1.32	19.26	± 0.56	0.37	± 0.13 [a]	21.79	± 1.20	31.17	± 1.51	11.47	± 0.37	33.23	± 1.29	20.25	± 1.52	0.99	± 0.37	0.220	± 0.005
	35	40.30	± 1.10	37.14	± 1.48	11.60	± 0.08	39.38	± 1.41	19.42	± 0.67	0.50	± 0.11 [a]	20.72	± 1.12	31.34	± 0.95	11.60	± 0.39	33.42	± 0.77	20.32	± 1.18	1.23	± 0.73	0.233	± 0.009
	50	40.34	± 0.91	37.05	± 1.19	11.60	± 0.14	39.30	± 1.17	19.46	± 0.40	0.62	± 0.30 [a]	21.25	± 0.80	31.29	± 1.23	11.60	± 0.42	33.38	± 1.01	20.38	± 1.40	0.90	± 0.14	0.239	± 0.046
	70	40.40	± 0.57	37.03	± 1.00	11.65	± 0.12	39.29	± 0.97	19.53	± 0.38	0.42	± 0.30 [a]	20.99	± 0.92	30.75	± 1.09	11.65	± 0.63	32.89	± 0.86	21.18	± 1.81	1.10	± 0.44	0.238	± 0.045
12	7	40.61	± 1.03	37.33	± 1.26	11.54	± 0.04	39.60	± 1.20	19.50	± 0.56	0.29	± 0.13 [a]	22.40	± 1.34	31.04	± 1.19	11.54	± 0.27	33.12	± 1.09	20.42	± 0.95	1.29	± 0.25	0.212	± 0.014
	14	40.05	± 0.99	36.89	± 1.33	11.56	± 0.21	39.22	± 1.31	19.84	± 0.41	0.55	± 0.19 [a]	22.50	± 1.61	31.14	± 1.12	11.56	± 0.29	33.22	± 0.96	20.39	± 1.12	1.02	± 0.45	0.215	± 0.026
	25	40.37	± 0.95	36.94	± 1.40	11.58	± 0.10	39.20	± 1.29	19.58	± 0.83	0.34	± 0.10 [a]	21.77	± 1.21	31.30	± 1.14	11.58	± 0.53	33.38	± 0.91	20.32	± 1.49	0.75	± 0.09	0.247	± 0.045
	35	40.30	± 0.81	37.08	± 0.89	11.62	± 0.04	39.35	± 0.84	19.33	± 0.22	0.49	± 0.07 [a]	21.40	± 1.19	31.44	± 1.21	11.62	± 0.36	33.53	± 1.03	19.45	± 0.74	0.80	± 0.50	0.226	± 0.047
	50	39.86	± 0.36	37.09	± 0.99	11.56	± 0.02	39.30	± 0.94	19.31	± 0.46	0.74	± 0.65 [a]	21.36	± 0.99	31.23	± 1.06	11.56	± 0.23	33.30	± 0.92	20.34	± 0.98	0.79	± 0.24	0.202	± 0.004
	70	39.86	± 0.58	37.15	± 1.67	11.64	± 0.03	39.36	± 1.58	19.28	± 0.76	0.81	± 0.60 [a]	21.60	± 1.01	31.11	± 1.68	11.64	± 0.38	33.23	± 1.44	20.56	± 1.62	0.81	± 0.42	0.216	± 0.014
24	5	40.36	± 0.85	37.50	± 1.45	11.60	± 0.15	39.75	± 1.35	19.36	± 0.79	0.35	± 0.32 [a]	21.47	± 0.89	31.43	± 1.36	11.60	± 0.32	33.51	± 1.20	20.28	± 1.21	0.66	± 0.45	0.217	± 0.022
	10	40.05	± 0.36	36.96	± 1.13	11.54	± 0.09	39.19	± 1.04	19.41	± 0.68	0.61	± 0.53 [a]	21.28	± 1.00	31.49	± 1.14	11.54	± 0.38	33.54	± 0.94	20.15	± 1.28	0.67	± 0.66	0.223	± 0.028
	15	40.01	± 0.47	34.65	± 2.96	11.43	± 0.18	37.02	± 2.81	20.69	± 1.49	2.68	± 3.88 [a]	21.41	± 0.76	31.33	± 1.35	11.43	± 0.53	33.36	± 1.08	20.08	± 1.67	0.92	± 0.31	0.238	± 0.027
	18	39.26	± 0.79	30.04	± 0.13*	11.57	± 0.59	32.62	± 0.21*	22.92	± 1.00*	7.39	± 1.42 [b]	21.65	± 1.15	31.22	± 1.07	11.57	± 0.60	33.30	± 0.86	20.36	± 1.51	1.04	± 0.19	0.361	± 0.055*
	20	30.26	± 0.86*	24.78	± 1.06*	11.52	± 0.64	27.16	± 0.97*	23.75	± 1.59*	16.23	± 2.05 [c]	21.55	± 1.13	31.74	± 1.23	11.52	± 0.48	33.77	± 1.22	20.04	± 0.97	1.04	± 0.59	0.458	± 0.017*
	25	19.62	± 0.61*	17.33	± 2.54*	11.56	± 0.30	19.86	± 2.21*	29.35	± 3.64*	29.06	± 1.65 [d]	21.26	± 1.19	31.19	± 1.12	11.56	± 0.57	33.26	± 1.07	20.34	± 1.12	1.20	± 0.14	0.521	± 0.069*
	40	17.99	± 0.58*	11.46	± 0.94*	11.56	± 0.61	14.69	± 0.37*	38.65	± 4.07*	34.42	± 1.43 [e]	21.22	± 0.79	30.81	± 0.82	11.56	± 0.47	32.91	± 0.64	20.57	± 1.20	0.97	± 0.79	0.641	± 0.072*
36	3	40.36	± 1.13	37.05	± 1.08	11.48	± 0.21	39.41	± 1.08	19.94	± 0.26	0.37	± 0.16 [a]	21.37	± 1.80	30.56	± 0.56	11.48	± 0.18	32.65	± 0.48	20.59	± 0.61	1.48	± 0.50	0.209	± 0.013
	6	40.33	± 0.90	37.22	± 1.02	11.45	± 0.34	39.55	± 1.06	19.76	± 0.27	0.32	± 0.31 [a]	21.66	± 1.73	30.75	± 1.20	11.45	± 0.22	32.82	± 1.06	20.44	± 1.05	1.16	± 0.10	0.233	± 0.032
	9	35.00	± 4.42	33.78	± 4.36*	11.59	± 1.56	35.68	± 4.53	18.78	± 1.50*	7.20	± 6.42 [b]	21.30	± 1.27	30.56	± 1.31	11.59	± 0.24	32.69	± 1.14	20.80	± 1.19	1.07	± 0.59	0.385	± 0.067*
	15	17.99	± 0.58*	20.37	± 0.74*	11.52	± 0.13	22.47	± 0.70*	24.94	± 0.71*	28.33	± 1.24 [d]	21.45	± 1.18	31.00	± 1.16	11.52	± 0.49	33.07	± 0.93	20.41	± 1.46	0.81	± 0.48	0.491	± 0.127*
	18	16.33	± 1.00*	18.33	± 0.88*	11.51	± 0.27	20.53	± 0.77*	26.73	± 1.39*	30.90	± 0.18 [d]	21.26	± 0.97	31.03	± 0.72	11.51	± 0.28	33.10	± 0.65	20.36	± 0.71	0.92	± 0.09	0.624	± 0.063*
	30	15.65	± 1.53*	11.59	± 1.18*	11.20	± 0.31	14.92	± 1.07*	39.09	± 2.32*	35.87	± 0.30 [e]	21.76	± 0.84	30.46	± 0.50	11.20	± 0.31	32.45	± 0.43	20.19	± 0.71	1.20	± 0.22	0.769	± 0.024*
Control	zero time	40.45	± 0.95	37.22	± 1.25	11.66	± 0.02	39.50	± 1.18	19.58	± 0.62			21.77	± 0.81	31.48	± 0.85	11.66	± 0.36	33.57	± 0.68	20.33	± 1.07			0.229	± 0.006

Color values were expressed by L* (100 = white, 0 = black), a* (positive values = redness, negative values = greenness), and b* (positive values = yellowness, negative values = blueness). L - White standard plate. Different letters and asterisks in the same column indicate significant differences by Fisher's (LSD) or by Dunnett's test ($P < 0.05$), respectively. Sausage in the time zero was included as the control in the Dunnett's test.

3. Microbial Growth Predictor - MicroLab_ShelfLife

3.1 Sample incubation design

Packages (n = 5) from the same batch and manufactured under the same conditions are used to estimate 'use-by' or 'best-before' date using the natural microbiota of the meat products. A borderline limit can be entered in the computational package, based on preliminary studies for the matrix, in order to preview the 'use-by' or 'best-before' date of the test. The method ISO 4833 (2013) may be used for enumeration of microorganisms in samples [4]. Following the procedure, a package (n = 1) must be analyzed as soon as the product arrives in the laboratory (time zero). Microbial population in the remained packages (n = 4) are stimulated to grow by pair incubation at a lower and a higher temperature. Laboratories can determine the incubation temperatures; however, lower and higher temperatures with a value comprehended from 4 to 20 °C and 25 to 36 °C must be used, respectively. Except for the time zero, there is no pre-defined time for microbial counting once the computational predictive modeling package can process any time; however, microbial growth (log) phase must be included at least in one of the counts (Figure S3). Results related to the colony counting must be entered in the computational predictive modeling package to obtain information about the parameters of the method and the microbial growth curve at a chosen dynamic temperature profile.

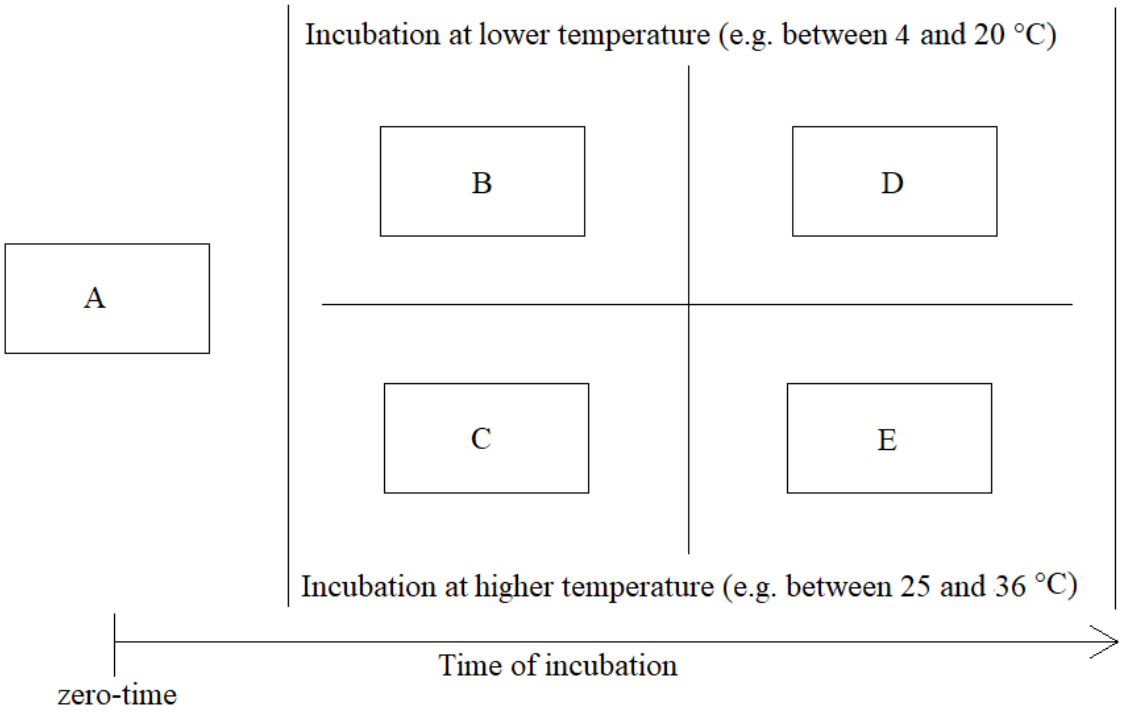


Figure S4. Sample incubation design. Microbial count at time zero must be below 8.2 log cfu/g to validate the test. Besides the time zero, there is no pre-defined time for microbial counting once the computational predictive modeling can process any time; however, microbial growth (log) phase must be included at least in one of the counts. Laboratories can determine the incubation temperatures; however, lower and higher temperatures between 4 and 20 °C, 25 and 36 °C, respectively, must be used.

3.2. Growth phase (log) modeling

Specific growth rates per hour (log cfu/g/h) at lower and higher temperatures were obtained by determining the angular coefficient of the growth (log) phase (L) in each growth curve. They were calculated to one unit of degree Celsius (log cfu/g/h/°C) by dividing the mean value of the angular coefficient by the difference between the higher and the lower temperature (Equation S2). This parameter was used to calculate the microbial growth per hour at each the temperature profile. Hourly microbial growth was obtained by multiplying the specific growth rate (log cfu/g/h/°C) by the temperature value during 1 hour. Daily growth was obtained by the sum of all hourly growth (Equation S3).

$$N(Tgrowth)\ (log\ cfu/g/h/°C) = \left(\left(\frac{\alpha(HT) - \alpha(LT)}{2}\right).\left(\frac{1}{HT-LT}\right)\right) / 24 \quad \text{(Equation S2)}$$

$$Ngrowth\ (log\ cfu/g) = \sum_{k=1}^{24} n.(N(Tgrowth)) \quad \text{(Equation S3)}$$

Where, *N(Tgrowth)* - rate of microbial growth per degree Celsius (log cfu/g/day/°C) in the growth (log) phase, α(HT) and α(LT) - angular coefficients at the higher (HT) and lower (LT) temperatures (°C), respectively, n - hourly temperature ranging from 4 to 36 °C, *Ngrowth* - daily microbial growth (log cfu/g) in the L phase, and k - time (hour).

3.2.1 Deceleration phase modeling

Correlation variable factor FT(n) (Equation S4) was created and inserted into Equations S2 and S3 to model the microbial growth in the deceleration phase (D) based on the value of the L phase (Equations S5 and S6. The ratio of the L and D phases period was determined at four different temperatures (4, 12, 24, and 36 °C), and xy-scatter charts were plotted with L/D values (coordinate abscissa) and incubation temperature (axial abscissa). Linear regression was used for mathematical modeling of values. The first-degree equation was considered to determine the variable factor FT(n) value for any temperature profile related to storage in the market. By the estimation of the daily population growth at the L and D phases, it is possible to preview when the population will achieve the stationary (S) phase.

$$FT(n) = L/D \quad \text{(Equation S4)}$$

$$N(Tdeceleration)\ (log\ cfu/g/h/°C) = N(Tgrowth) / FT(n) \quad \text{(Equation S5)}$$

$$Ndeceleration\ (log\ cfu/g/dia) = \sum_{k=1}^{24} n.\left(N(Tdecedeleration)\right) / FT(n) \quad \text{(Equation S6)}$$

Where, *N(Tdeceleration)* - microbial growth rate per degree Celsius (log cfu/g/day/°C) in the D phase, *N(Tgrowth)* - microbial growth rate per degree Celsius (log cfu/g/day/°C) in the L phase, *FT(n)* - correlation variable factor to describe specific growth rate between L and D phases per degree Celsius, n - hourly temperature ranging from 4 to 36 °C, Ndeceleration - daily microbial growth (log cfu/g) in the D phase, and k - time (hour).

3.3. Computational predictive modeling package

3.3.1. Calculation method

The computational predictive modeling package was compiled using the Visual Basic Application – Excel 2016 (MicroSoft, Washington, USA) to serve as an auxiliary tool for laboratory routine to estimate the 'use-by' or 'best-before' dates of vacuum-packaged cooked sausage or

similar meat products. The computational predictive modeling tool was inputted with colony counting, at the time zero and after pair incubation of samples at two different temperatures. It was designed to calculate the results (log cfu/g) using at least two successive dilution levels, according to the ISO 7218 (2007), either by pour-plate or spread-plate or drop plate-techniques.

The 'Temperature (°C) for Shelving Period' platform was created to enter the temperature profile mimicking the sausage disposal in the market, with hourly variation during one day. The computational predictive modeling provides information regarding the 'use-by or 'best-before' dates according to the information on changes in the product appearance due to the formation of ropy slime on the surface of the sausages.

3.3.2. Predictive microbial growth curve

The computational predictive modeling was designed to create predictive microbial growth curves based on the results from the experimental design described in section 2.2. The correlation between L and D phases was performed based on the value of the FT(n), which considers the temperature profile during sausage storage and disposal for sale in the market. Microbial growth curves started with the result (log cfu/g) obtained at time zero. L and D phases were modeled based on Equations S.2.4 and S2.5, respectively (Figure S4).

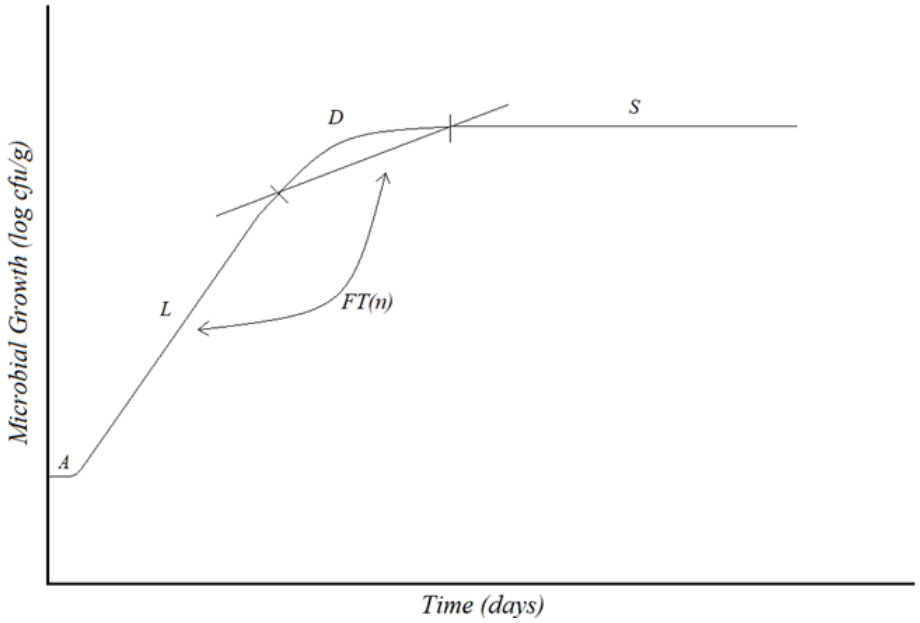


Figure S5. Illustration of the biological growth curve by predictive modeling. A – adaptation and acceleration growth phase; L – microbial growth (log) phase; D – deceleration phase; S – stationary phase. Correlations between specific growth rate in L and D phases were performed based on the correlation factor FT(n) value, according to the chosen temperature profile of the test.

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