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HEAT RESISTANCE OF HUMAN PATHOGENS IN SOUS-VIDE PRODUCTS STUDIED IN MODEL NUTRITION MEDIA

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Sous-vide (French for ʻunder vacuumʼ) is a professional cooking method, by which, under oxygen-free conditions and precise temperature control, not only cooking but preservation is achieved. During the process the food matrix is vacuum-packed and undergoes a mild heat treatment, thus achieving an enhanced nutrition value and a better organoleptic character. Due to the mild heat treatment (55 to 90 °C), the high water activity, and the slight acidity of raw materials, the microbial quality assurance is a great challenge even for professionals. The heat treatment does not assure the inactivation of pathogen spores. In our experiments we used *Clostridium perfringens* representing the spore-forming pathogens, and *Salmonella* Enteritidis as a the food-borne infection bacterium. Effects of various temperatures were measured in normal and sous-vide type vacuum packaging. Higher thermal death rate in vacuum packaging was demonstrated for *Salmonella* Enteritidis and *Clostridium perfringens*.

Keywords: *Clostridium perfringens*, *Salmonella* Enteritidis, heat treatment, sous-vide, mild technologies

Prognosticating the proliferation of spoilage-causing pathogen microbes in food is fundamental for food processing, distribution, and consumption. This is especially important in mild technologies producing minimally processed product. Mild means preservation of food without altering its nutritional and organoleptic value, thus reducing the side-effects of heating, the main preservation treatment (Fellows, 2000). An emerging mild technology is sous-vide (French for ʻunder vacuumʼ), defined as ʻraw materials or raw materials with intermediate foods that are cooked under controlled conditions of temperature and time inside heat-stable vacuumized pouchesʼ (Schellekens, 1996). Typically, sous-vide cooking uses temperature lower than 100 °C, a kind of pasteurization, while "cooking in heat-stable, vacuumized pouches improves shelf-life and can enhance taste and nutrition" (Baldwin, 2012).

However, the low pasteurization temperature endangers the microbial safety and stability of sous-vide products, as it was already reported by Mossel and Struijk (1991). Heat treatment protocols for the microbial safety of traditionally pasteurized food specifies a minimum temperature value of 65 °C. Sous-vide technology, considering the sensory parameters of food, suggests not to exceed 56 °C core temperature for certain meat and fish products. The critical point of the technology is the sizing of temperature treatment. The European Chilled Food Federation (ECFF, 2006) directives specify the minimum heat treatment at 70 °C for 2 min or equivalent heat treatment for pasteurized, high water activity, refrigerated stored products with a pH value higher than 4.5 (ʻcook-chill productsʼ or ʻchilled pasteurized foodsʼ), if elimination of vegetative pathogens is the aim and other antimicrobial

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barrier is missing. However, such heat treatment does not inactivate bacterial spores and microbial toxins. For the 6 order of magnitude endospore reduction of psychrophylic (growing at and above +3.0 °C), non-proteolytic *Clostridium botulinum* serotypes 2' at 90 °C or equivalent heat treatment is necessary (HARMONY PROJECT, 1999). *Clostridium botulinum* thermal deactivation in sous-vide products was already studied by BETTS and GAZE (1995).

Professionals of the technology agreed in a 6D principle as the basis for the microbial safety of sous-vide products, thus using paired parameters of temperature-time, which serve as effective heat treatment for 6 order of magnitude reduction in the number of vegetative cells of psychrophylic *Clostridium botulinum* strains. Predictive models can help in the estimation of the target microbe's survival in a given range in certain food products (WHITING, 1995). These models, established upon studies on real foods, provide relevant data for food processers in order to size the heat treatment in the production, thus helping to produce safe food for consumers (ROSS & MCMEEKIN, 1994). Salmonellosis was the second among the most common reported food infections in 2009 by an EU survey, responsible for 108 614 human cases. *Salmonella* has remained the most common cause of foodborne outbreaks, mostly originating from chicken and turkey meat or pork (EFSA, 2011). *Salmonella* survival can be reduced by proper heat treatment, antimicrobial agents like carvacrol or cinnamaldehyde (JUNEJA et al., 2013). *Salmonella* thermal control in food is reviewed by BERMÚDEZ-AGUIRRE and CORRADINI (2012). The risk of *Clostridium perfringens* survival and toxin production in sous-vide products was already investigated by JUNEJA and MARMER (1996), JUNEJA (2006), and NOVAK and JUNEJA (2002). Effect of packaging is an important factor in bacterial survival and product safety, so it was also studied in this work.

1. Materials and methods

These experiments were performed in the accredited (NAT-1-1674/2012) Food and Water Test Laboratory of the Institute of Food Sciences at the University of West Hungary, Faculty of Agricultural and Food Sciences. NCAIM B 01417^T strain of *Clostridium perfringens* and ATCC-13076 of *Salmonella* Enteritidis were used to determine the heat resistance under normal (atmospheric conditions) and sous-vide type vacuum packing. For both pathogens the heat destruction parameters were determined at 55, 60, and 65 °C in order to compare the effects of packaging on the heat inactivation.

1.1. Study of Clostridium perfringens *vegetative cells heat resistance in model media*

NCAIM B 01417^T *Clostridium perfringens* strain was obtained as lyophilised preparate in vacuum packed double vial from the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Hungary. Based on protocol, sterile physiologic saline solution was pipetted after opening, and after a 20 min rehydration time it was inoculated into the advised RCM (Reinforced Clostridial Medium) broth followed by anaerobic incubation at 37 °C for 24–72 h. For the reproducibility of the experiment, the strain was preserved in Microbank® system as well. From the inoculated and translucent broth, TSA (Tripton-Soya Agar) plates were streaked and incubated at 37 °C for 72 h. The pure culture was processed to a 0.5 McFarland (1.5×10^8 ml^{-1}) suspension, adjusted for inoculating the study suspension.

To prepare the experimental samples, 50 ml RCM broth was pipetted to vacuumable polyethylene bag under sterile conditions. The same way samples were prepared under 99% vacuum in a sous-vide vacuum equipment. For both sample series heat treatment was

performed in sous-vide circulation thermostated water-bath with core thermometer at 55 °C, 60 °C, and 65 °C (accuracy: 0.1°C). Treatment time varied from 0.5 to 80 min. Sampling frequency was dependent on the temperature; it was shorter, 0.5 min, at higher temperatures and longer, 10 min, at lower temperatures. For sampling, inoculating aliquots of the same way treated sample units were taken one by one.

1.2. Study of Salmonella *Enteritidis heat resistance in model media*

After opening the double plastic vial, the lyophilised *Salmonella* Enteritidis ATCC (American Type Culture Collection)-13076 strain culture was transferred into the diluting solution of the kit following the protocol, and spread on TSA plates. After 24–48 h of incubation at 37 °C, 0.5 McFarland (1.5×10^8, determined by Densimat®, BioMerieux) suspension was prepared from the pure culture, and in sterile buffered peptone water the final concentration (10^8 ml^{-1}) was adjusted.

From the suspension, 50 ml units were filled in sterile heat stable plastic bags suitable for the number of samples. Half of them were vacuum-packed modelling sous-vide conditions (99% vacuum). The samples were heat treated in a circulating water bath. During sampling, bags were removed from the water bath and the content was used for the determination of viable count. This assured continuous and quick sampling, which is necessary for the immediate processing and reduces inaccuracy derived from the time factor in heat resistance investigations. Investigations were conducted on samples packed under atmospheric pressure and vacuum parallelly at 55 °C, 60 °C, and 65 °C with the same treatment times and sampling frequency.

1.3. Determination of viable count

The whole heat treatment investigation was performed in 3–3 independent experiments. Samples (packed under vacuum and atmospheric pressure) from each experiment, taken during the heat treatment in model media, were similarly processed. Decimal dilution series were prepared to 10^8, of which 1–1 ml aliquots were pipetted into sterile Petri dishes, into which RCM or TSA agar was poured. After solidification, they were incubated for 72 h at 37 °C under anaerobic conditions (for *Clostridium perfringens*), and for 24 h at 37 °C under aerobic conditions (for *Salmonella* Enteritidis). From each dilution 2 parallel plates were poured. Plates with colony counts between 10 and 300 were used for the enumeration. Viable counts on the enumerable plates are given as weighted average of the colony numbers concerning the dilution level.

1.4. Mathematical evaluation

Viable counts after heat treatment were obtained as weighted average considering dilution factor, as

$$\overline{c} = \frac{\sum c}{(n_1+0.1n_2)Vd} \tag{1}$$

where: $\overline{c}$ =weighted average of viable counts, Σc=sum of the calculated viable count, n_1=number of plates for the first evaluable dilution factor, n_2=number of plates for the second evaluable dilution factor, d=dilution factor of the first evaluable dilution, V=amount of plated culture (inoculum).

Decimal reduction time (D value): the time required at a certain temperature to inactivate 90% of the microorganisms being studied (one log unit reduction in $\log_{10}$ N value). It means the negative reciprocal of the slope of the survival curve ($\log_{10}$ N in CFU ml^{-1} values versus time). Relation between decimal reduction time and temperature is given by z value: the temperature required for one $\log_{10}$ unit reduction in the D value. It is the negative reciprocal of the slope of the heat resistance curve (log D versus temperature). The equations of survival curves were calculated by linear regression analysis. The results and statistical parameters are summarized in Tables 1–2.

Average and standard deviation of the viable count were calculated from n=6 values (obtained from 2 parallel plating × 3 independent experiments). Atmospheric and vacuum packed treatment datasets were compared by F- and two sample *t*-tests.

2. Results and discussion

The survival curves (log10 CFU ml^{-1} in function of time) of *Clostridium perfringens* are shown in Figures 1–2. Error bars represent standard deviation (n=6) values (obtained from 2 parallel plating × 3 independent experiments).

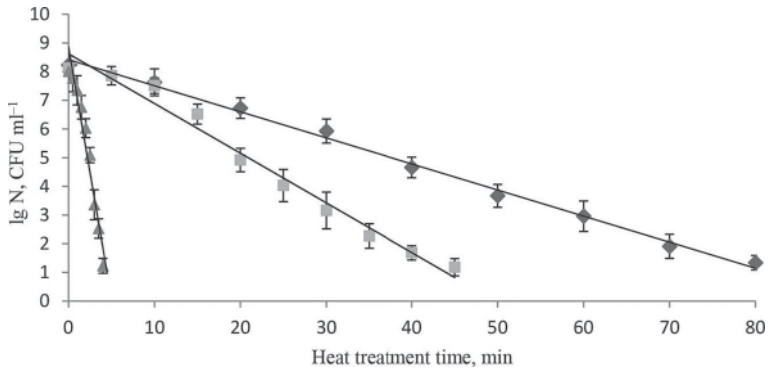

Fig. 1. Survival curves of *Clostridium perfringens* vegetative cells at 55 (◆), 60 (■), and 65 °C (▲) in RCM medium, atmospheric packaging

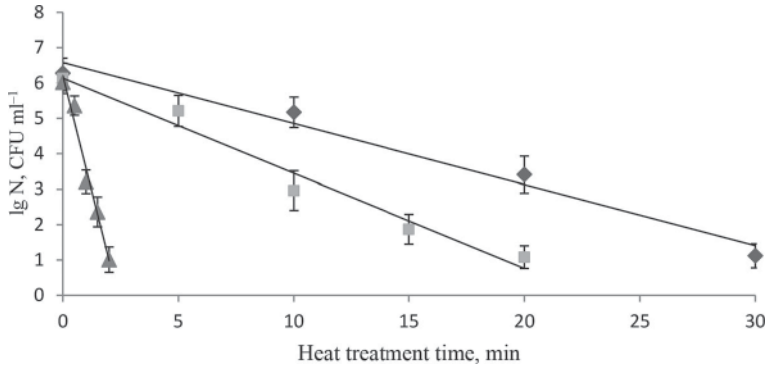

Fig. 2. Survival curves of *Clostridium perfringens* vegetative cells at 55 (◆), 60 (■), and 65 °C (▲) in RCM medium, under vacuum

Based on the survival curve of *Clostridium perfringens* NCAIM B 01417^T strain, compared to the starting viable count at 55 °C in the samples packed at atmospheric pressure (Fig. 1), in 80 min 7 orders of magnitude, while in the vacuum packed samples (Fig. 2), in 30 min 5 orders of magnitude reductions were detected. The treatment decreased the 10^6 initial viable counts below the detection limit of 10 CFU ml^{-1} after 35 min in the vacuum packed samples, and after 85 min in the control samples. At 60 °C, the viable counts of the atmospheric pressure samples declined 7 orders of magnitude for the 45th min of the heat treatment. In the vacuum packed samples, 5 orders of magnitude reduction was measured in 20 min. Survival curves of the 65 °C heat treatment demonstrate that from the starting 8.11 log CFU ml^{-1} viable counts of vegetative cells decreased to 1.23 log CFU ml^{-1} at the 4th min of heat treatment, which means a 7 orders of magnitude reduction. Five orders of magnitude reduction was achieved in the 2nd min in case of the vacuum packed samples, where viable counts decreased from 6.32 log CFU ml^{-1} to 1.01 log CFU ml^{-1}.

Changes in viable counts of samples inoculated with *Salmonella* Enteritidis ATCC-13076 in effect of heat treatment are shown in Figures 3–4. Error bars represent standard deviation (n=6) values.

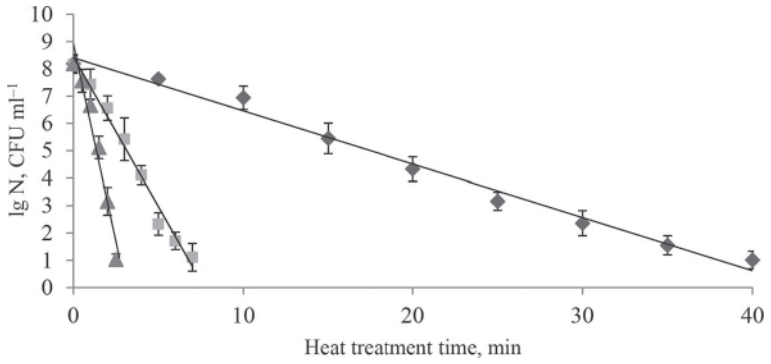

Fig. 3. Survival curves of *Salmonella* Enteritidis at 55 (◆), 60 (■), and 65 °C (▲) in buffered peptone water, atmospheric packaging

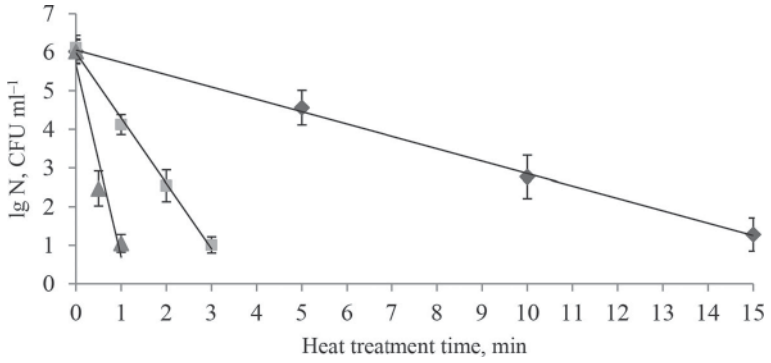

Fig. 4. Survival curves of *Salmonella* Enteritidis at 55 (◆), 60 (■), and 65 °C (▲) in buffered peptone water, under vacuum

Based on the survival curves of *Salmonella* Enteritidis ATCC-13076 strain, at 55 °C in the samples packed at atmospheric pressure, 7 orders of magnitude reduction was achieved in the 40th min (Fig. 3). In the vacuum packed samples in 15 min 5 orders of magnitude decimal reduction was detected (Fig. 4). At 60 °C for atmospheric samples 7 orders of magnitude reduction in 7 min, while for vacuum-packed samples 5 orders of magnitude in 3 min was achieved in the number of survived cells. At 65 °C for atmospheric samples 7 orders of magnitude reduction in 2.5 min, while for vacuum-packed samples 5 orders of magnitude in 1 min was achieved in the number of survived cells, in the latter no viable cells were detected after 1.5 min.

The statistical analysis of the survival curves and the log D values belonging to the applied temperatures of the heat treatments are summarized in Tables 1 and 2.

Table 1. Heat treatment parameters of the survival curves of *Clostridium perfringens*

T (°C)	Airspace	Datapair	Survival curve		St. error	P value	D (min)	lgD
			intercept	slope	of slope			
55	atm.	9	8.4	0.0905	0.0024	0.000000003	11.69	1.068
	vacuum	4	6.58	0.1724	0.0018	0.011500000	5.81	0.764
60	atm.	10	8.62	0.1731	0.0089	0.000000051	6.49	0.812
	vacuum	5	6.14	0.2688	0.0275	0.002292147	3.96	0.598
65	atm.	9	8.88	1.763	0.1465	0.000006259	0.58	0.237
	vacuum	5	6.38	2.724	0.2097	0.000985196	0.3	0.523

Table 2. Heat treatment parameters of the survival curves of *Salmonella* Enteritidis

T (°C)	Airspace	Datapair	Survival curve		St. error	P value	D (min)	lgD
			intercept	slope	of slope			
55	atm.	9	8.4	¬0.195	0.00811	0.0000001	5.57	0.746
	vacuum	4	6.05	¬0.320	0.0089	0.00078209	3.16	0.500
60	atm.	8	8.43	¬1.095	0.0561	0.0000012	0.99	¬0.004
	vacuum	4	5.98	¬1.691	0.0787	0.0021636	0.58	¬0.237
65	atm.	6	8.89	¬2.888	0.3082	0.0007233	0.34	¬0.469
	vacuum	3	5.66	¬4.97	1.2297	0.1544209	0.2	¬0.699

Plotting the log D values in function of temperature, heat resistance curves were obtained. The z values had been calculated as negative reciprocal of the slope of the resistance curves.

Heat resistance curves for *Clostridium perfringens* and *Salmonella* Enteritidis are shown in Figures 5–6.

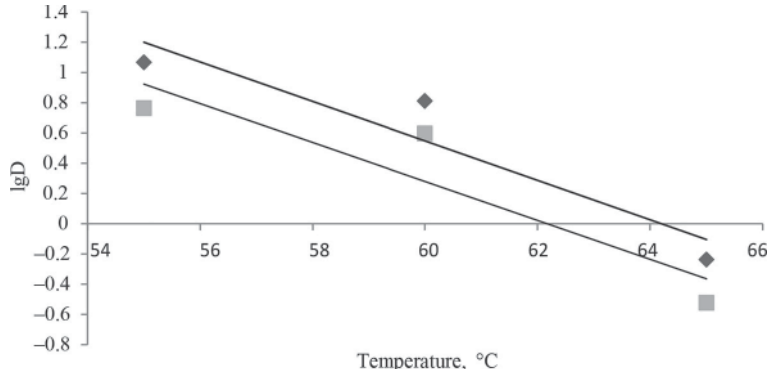

Fig. 5. Heat resistance curves and z values for *Clostridium perfringens*. Atmospheric samples (◆): logD= –01305T+8378, z=7.66 °C; vacuum packed samples (■): logD= –0.1287T+8.002, z=7.77 °C

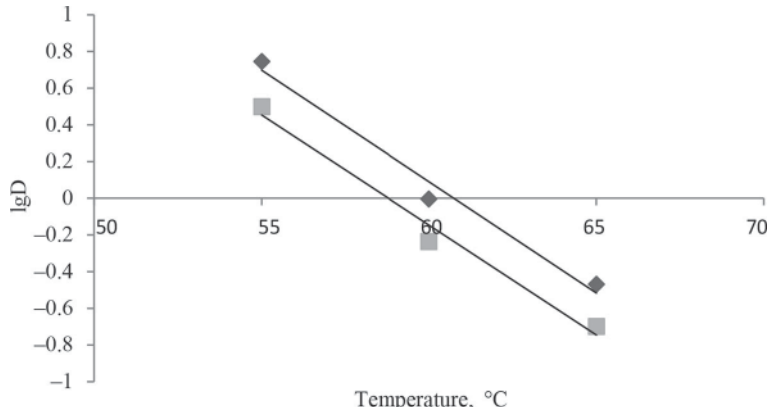

Fig. 6. Heat resistance curves for *Salmonella* Enteritidis. Atmospheric samples (◆): logD = –0.1215T+7.381, z=8.23 °C; vacuum packed samples (■): logD= –0.1199T+7.049, z=8.34 °C

For *Clostridium perfringens*, z value between 55 °C and 65 °C was 7.66 °C, this shows that 7.66 °C temperature increase would be necessary for one order of magnitude reduction of the decimal reduction time. Heat resistance curve for *Salmonella* Enteritidis is shown in Figure 6, intercept of the heat resistance curve is 7.3772, slope is –0.1214. For *Salmonella* Enteritidis, z value between 55 °C and 65 °C was 8.23 °C, this shows that 8.23 °C temperature increase would be necessary for one order of magnitude reduction of decimal reduction time.

Decimal reduction time (D) in case of *Clostridium perfringens* (NCAIM B 01417^T) in broth under atmospheric pressure and 55 °C is 11.69 min (D_{55}). Byrne and co-workers (2006) measured 16.3 min (55 °C) in pork meat for vegetative cells, and 2.2 min (100 °C) to 34.2 min (90 °C) for spores. At 60 °C it is 6.49 min in our present work (D_{60}), Byrne and co-workers measured 8.5 min. At 65 °C it was 0.58 min in our study (D_{65}), while Byrne and co-workers published 0.8 min (D_{65}). Z value between 55 °C and 60 °C is 7.6 °C, which approximately equals 7.7 °C published in the same paper. Our D-values are similar to those

(11.2–16.5 min range of D_{55}) reported by SARKER and co-workers (2000) for *Clostridium perfringens* strains that carry the enterotoxin gene on the chromosome, and differ from those (5.0–9.1 min range of D_{55}), that carry this gene on a plasmid. These suggest that our strain belongs to the first type of enterotoxin gene location, thus having higher heat resistance and an ability to cause food-borne infections.

Decimal reduction time (D) in case of *Salmonella* Enteritidis ATCC-13076 in broth was 5.57 min (D_{55}) under atmospheric pressure and 3.16 min (D_{55}) in vacuum packed samples.

JUNEJA and co-workers (2001) measured 5.74 min in chicken meat broth at 55 °C (D_{55}), while HUMPHREY and co-workers (1990) reported 7.8–8.5 min decimal reduction times (D_{55}) for whole eggs at 55 °C, both under atmospheric pressure. Decimal reduction time at 60 °C under atmospheric pressure was 0.99 min (D_{60}), in vacuum packed samples 0.58 min (D_{60}) in our study. JUNEJA and co-workers (2001) measured 0.89 (D_{60}) in chicken meat broth (atmospheric pressure), while for whole eggs 0.14–2.2 min (D_{60}) was reported by ANELLIS and co-workers (1954), 0.31–0.69 min by BAKER (1990), and 0.1–2 min by LACZAY (2008). At 65 °C, decimal reduction times were 0.34 min (D_{65}) under atmospheric pressure and 0.2 min for vacuum packed samples. JUNEJA and co-workers (2001) published 0.39 min (D_{62}) in chicken meat broth, while LACZAY (2008) for whole eggs reported 0.02–0.3 min.

Z value for *Salmonella* Enteritidis ATCC-13076 between 55 °C and 60 °C is 8.1 °C in chicken meat both under atmospheric pressure and in vacuum packed samples. This z value approximately equals to 8.83 °C published by JUNEJA and co-workers (2001), for the range between 58 °C and 65 °C in chicken meat.

3. Conclusions

Based on the survival curves of *Clostridium perfringens* NCAIM B 01417^T in model media (Figs 1 and 2), the following observations can be stated:

For vacuum packed samples applied in sous-vide technology, heat treatments of 55 °C for 35 min, 60 °C for 25 min, and 65 °C for 2.5 min reduced the initial cell number below the detection limit (10 CFU ml^{-1}) resulting in a 7 orders of magnitude destruction.

In vacuum packed samples the absolute values of the slopes of survival curves are higher, which proves a faster thermal death rate in vacuum packaging.

In vacuum packed samples the initial viable cell numbers were generally two orders of magnitude lower, which is partly the result of the death of cells sensitive to pressure changes before reaching the planned treatment temperature; and of the slightly different temperature reaching times.

Based on survival curves of *Salmonella* Enteritidis (ATCC-13076) strain in model media (Figs 3 and 4) the following observations can be stated:

For vacuum packed samples applied in sous-vide technology, heat treatments of 55 °C for 20 min, 60 °C for 4 min, and 65 °C for 1.5 min reduced the initial cell number below the detection limit (10 CFU ml^{-1}) resulting in a 6 orders of magnitude destruction.

In vacuum packed samples the absolute values of the slopes of survival curves are higher, which proves a faster thermal death rate in vacuum packaging.

In vacuum packed samples the initial viable cell numbers were approx. two orders of magnitude lower, which is partly the result of the death achieved before the planned treatment temperature, due to the higher death rate of cells sensitive to pressure changes.

The lower viable counts of vacuum packed samples was proven to be the effect of vacuum packaging due to the significant difference (*t*-probe $P<0.05$) in the viable counts for both strains at all temperatures. Thus, antibacterial effect of vacuum treatment in sous-vide products was demonstrated.

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