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EXTENDED VALIDATION OF DYNAMIC IRREVERSIBLE THERMOPORATION: A NOVEL THERMAL PROCESS FOR MICROBIAL INACTIVATION

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ABSTRACT

A novel thermal treatment for microorganism inactivation, characterized by a very rapid temperature increase (up to 30°C/s) and a low final temperature (up to 65°C) maintained for a relatively short holding time, has been recently presented and tested by the authors, showing microbial load reduction greater than 5 log units against several common bacteria and yeasts. With the aim of extending the possible use of the new thermal treatment to a wider microorganisms class, in this work the dynamic irreversible thermoporation (DIT) treatment was further tested on a well-known thermoresistant strain, the *Enterococcus hirae*: The results of these new experimental tests confirmed the reliability of the process, which allowed to reach the 5 log microbial reduction once the adequate holding time was employed.

The comparison with simple immersion in a thermostatic bath, where the very slow heating process with 0.3°C/s has been performed, confirmed the crucial role of the thermal shock for the success of the treatment. The inactivation kinetics of *E. hirae* in isothermal conditions immediately after the application of thermal shock has also been studied. Finally, the morphological analysis performed by using a scanning electron microscope clearly revealed the rupture of the cell membrane, leading to identification of the process called dynamic irreversible thermoporation (DIT).

PRACTICAL APPLICATIONS

The main features of the dynamic irreversible thermoporation (DIT) process produce two major advantages with respect to the traditional heat treatments commonly employed for beverages: one perfectly agrees with production economics principles, while the other concerns the quality of the product. First, the DIT process can be implemented directly on bottled products because its low maximum temperature of 65°C is compatible with the plastic materials usually employed for sealed beverages: Its online application in the filling process could hence be conveniently considered, thus avoiding both the use of an expensive aseptic environment and the risk of post-processing contamination. Second, by using such a low final temperature, the thermal degradation of the food components is avoided, with consequent preservation of the sensory and nutritional quality of the fresh-like food products.

INTRODUCTION

Nowadays, the most common methods for microorganism inactivation employed in the beverage industry rely on thermal treatments: to ensure safety and extend their shelf life, beverage products are usually subjected to pasteurization, a process that involves heat transfer at a prescribed time–temperature combination to kill pathogens. Although pasteurization is certainly effective for the inactivation of pathogenic microorganisms, the high temperatures reached may cause undesirable biochemical and nutritional changes, thus affecting the overall quality of the final product (Ortega-Rivas 2011).

With the increasing demand for processed food with unchanged nutritional attributes, food researchers focused on the development of improved preservation processes with a minimal impact on the fresh taste, texture and nutritional value of the food products. Besides a considerable number of nonthermal treatments of recent design and currently under development, such as high hydrostatic pressure and pulsed electric fields, the easiest way to preserve food quality while reducing its bacterial load is represented by low temperature treatments.

The dynamic irreversible thermoporation (DIT) process, a new thermal treatment not yet introduced in the industrial application, is characterized by high temperature gradients (up to 30°C/s) and relatively low final temperatures (up to 65C) maintained for a relatively short holding time, as shown in Fig. 2: The authors recently demonstrated its effectiveness in bacterial load reduction against several common food- and water-borne pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria innocua*, and spoilage microorganisms, such as *Candida albicans* (Cammalleri *et al*. 2015); thanks to its short duration and to the fairly low maximum temperature reached, the DIT process is compatible with already packaged products and conveniently applicable online with the filling process, making aseptic environments unnecessary and avoiding the risk of post-processing contamination in food.

With the aim of extending the use of the DIT treatment to a broader set of microorganisms, in the present work, the authors studied its effectiveness against a well-known thermal-resistant microorganism, the *Enterococcus hirae*: To this purpose, the authors assessed the bactericidal effectiveness of the DIT treatment on *E. hirae* aqueous suspensions, following the experimental procedures prescribed by the UNI EN 1276:2009 (UNI EN 2009), a widely employed standard for the evaluation of bactericidal activity in industrial and academic food research; in addition, the inactivation kinetics in isothermal conditions immediately after the application of thermal shock have also been investigated, and the morphological changes of bacterial cells due to treatment have been investigated by using a scanning electron microscope.

MATERIALS AND METHODS

Thermal Test Bench

The tests were carried out on a properly designed test bench based on the ohmic dissipation effect (see Fig. 1), whose detailed description is reported in the previous work by the same authors (Cammalleri *et al*. 2015).

Each test suspension was pumped by a Watson-Marlow peristaltic pump 120S/DV (Watson-Marlow Inc., Wilmington, MA, USA), equipped with a marprene tube, inside an AISI 316 stainless steel tube (outer diameter and thickness of 1.59 and 0.36 mm, respectively) which was subjected to a voltage drop across its terminals by means of a laboratory-grade direct current power supply.

In this way, the inner tube surface, heated by the ohmic dissipation, transfers heat to the flowing suspension, whose

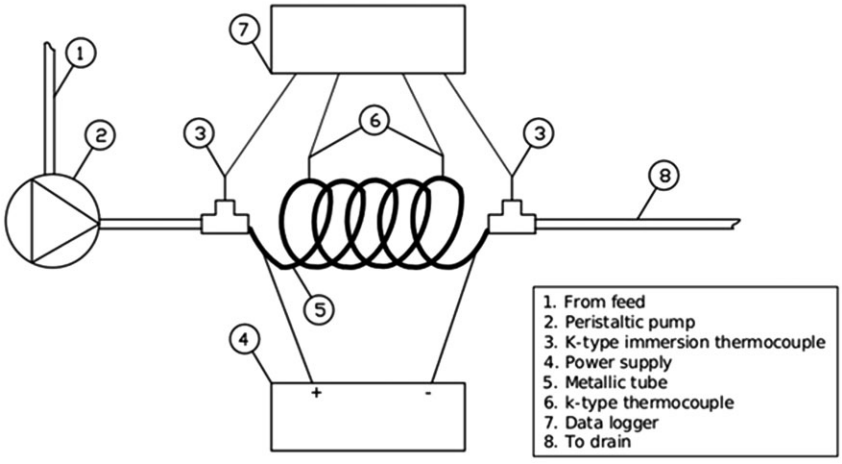

FIG. 1. SCHEMATIC REPRESENTATION OF THE TEST BENCH

temperature increment (ΔT) can be related to the electric power dissipation (W_{el}) through the following relation:

$$\eta W_{el} = \eta i^2 R = c_p G \Delta T \tag{1}$$

where i represents the electric current flowing in the metallic tube, R the tube electrical resistance, G the suspension mass flow, c_p the suspension specific heat at constant pressure, while η represents the heat transfer efficiency of the system.

Assuming the suspension as an incompressible fluid, both its density (δ) and volumetric flow (Q) can be considered constant along the whole tube:

$$Q = \frac{G}{\delta} = \text{constant} \tag{2}$$

Given also the constant tube diameter, the flow section area (A) does not change along the tube; it results then to

$$\frac{Q}{A} = v = \frac{l}{\Delta \tau} \tag{3}$$

where v represents the mean flow velocity and $\Delta\tau$ the duration of the rapid heating, i.e., the time required for the suspension to flow through the whole tube length (l).

From Eqs. (2) and (3), it follows that

$$G = \frac{lA\delta}{\Delta \tau} \tag{4}$$

and Eq. (1) becomes

$$\eta i^2 R = c_p \frac{lA\delta}{\Delta \tau} \Delta T \tag{5}$$

The rate of temperature rise (ϑ) of the solution can be expressed as

$$\vartheta = \frac{\Delta T}{\Delta \tau} \tag{6}$$

Hence, from Eq. (3), the volumetric flow (Q) required to obtain each fixed ϑ and ΔT is

$$Q = \frac{lA\vartheta}{\Delta T} \tag{7}$$

The relationship between the electrical current (i) and the process parameters can hence be derived from Eqs. (5) and (6):

$$i^2 = \frac{\vartheta c_p lA\delta}{\eta R} \tag{8}$$

Being the metallic tube resistance (R) related to the metal resistivity (ρ), to the tube length l and to the tube annular section (S) according to the relation:

$$R = \frac{\rho l}{S} \tag{9}$$

it follows that, given a certain rate of temperature rise (ϑ), the necessary electrical current (i) can be obtained as

$$i = \sqrt{\frac{\vartheta c_p \delta AS}{\eta \rho}} \tag{10}$$

This implies that, once both tube dimensions and fluid properties are determined, the electrical current (i) depends only on the rate of temperature rise (ϑ) and on the thermal efficiency (η), which, in the test performed, varied from 0.62 to 0.80.

The fluid temperature was measured both at the inlet (initial temperature T_1) and at the outlet (final temperature T_2) of the heated tube by means of K-type thermocouples connected to a USB TC-08 Pico Tech Data Logger (Pico Technology, Cambridgeshire, PE, UK). A personal computer was used to control the whole system, monitoring the sensors output signals and performing data acquisition by means of an expressly designed software developed by the authors in LabVIEW environment: Once the values of the treatment parameters ϑ and ΔT are fixed, the software sets the pump volumetric flow (Q) and, using the measured temperatures as feedback, controls the electric current (i) provided by the power supply with the aim of reaching the desired value of ΔT.

The heat losses from the tube to the environment have been minimized by using a proper insulating tape applied to the whole process line.

A tube length of 1.50 m was used to perform most of the experimental investigation; in some cases, however, in order to evaluate the influence of the flow conditions on the effectiveness of DIT treatments (as explained in the Kinetic analysis of reduction curves section), a tube length of 0.75 m has been employed.

The flow conditions have been identified by the Reynolds number,

$$Re = \frac{\delta v d}{\mu} \tag{11}$$

where d is the inner tube diameter and μ is the fluid viscosity, which, referring to pure water, has been assumed to be 1 mPa·s.

Table 1 summarizes the operative parameters of the thermal treatment system employed for the tests.

Microorganism and Test Suspension

As already mentioned, in this study a single bacterial strain has been employed, i.e., the *E. hirae* (ATCC 10541). *Enterococcus* species are gram-positive bacteria, facultative

A (mm^2)	l (m)	T_1 (°C)	T_2 (°C)	ϑ (°C/s)	i (A)	Q (mL/min)	Re	η
0.594	1.50	30	65	30	13.3	46.5	1,119	0.797
				20	11.5	31.0	746	0.711
				10	8.70	15.5	373	0.621
	0.750			20	10.8	15.5		0.806

TABLE 1. OPERATIVE PARAMETERS OF THE THERMAL TREATMENT SYSTEM

A = flow section area, l = tube length, T_1 = fluid inlet temperature, T_2 = fluid outlet temperature, ϑ = rate of temperature rise, i = electrical current, Q = volumetric flow rate, Re = Reynolds number.

anaerobic organism, catalase negative and non-spore-forming. They are able to survive stressful and hostile environments, with temperatures in the range of 5–65°C, slightly acidic or basic (pH = 4.5–10.0) and with high NaCl concentration, enabling them to colonize a wide range of niches (Fisher and Phillips 2009).

Enterococci inhabiting the gastrointestinal tract of humans and other animals have emerged as a major cause of nosocomial infections in recent decades; as regular inhabitants of the intestine, enterococci may serve as indicators of fecal contamination and are therefore of particular importance in food and public health microbiology (Franz *et al.* 1999).

The bacteria used in the test were cultured on Vancomycin Resistant Enterococci (VRE) Agar Base (Oxoid, Ltd., Hampshire, U.K.); according to the mentioned standard procedure UNI EN 1276:2009 (UNI EN 2009), on the basis of the fresh culture, an initial suspension was prepared in buffered peptone water, with a concentration of 10^8 cfu/mL; successively, test suspensions were prepared starting from the initial suspension, by adding hard water (19.84 g/L $MgCl_2$, 46.24 g/L $CaCl_2$, 35.02 g/L $NaHCO_3$ in sterile distilled water) and bovine albumin solution or sucrose solution (0.3 and 10 g/L, respectively) as interfering substance, thus creating a substrate characterized by ion content and organic matter similar to beverages such as aromatized water and fruit juices. The volumetric composition of each test suspension realized was 10% of the initial suspension, 80% of hard water, 10% of interfering substance, thus obtaining a concentration of 10^7 cfu/mL.

Test Procedure and Microbial Count

The test suspensions were submitted to a panel of DIT protocols with varying values of rate of temperature rise (ϑ) and holding time (HT) (represented in Fig. 2), with final temperature (T_2) = 65°C.

For fast referencing purpose, each protocol has been labeled using the acronym DIT followed by the values of the three main process parameters, i.e., DIT ϑ-T_2-HT; moreover, bovine albumin or sucrose has been employed as an interfering substance.

Before starting the rapid heating, each test suspension has been preheated in a thermostatic bath for 30 min at 30°C; at the end of the whole thermal process, instead, the collected samples were stored in an ambient at 4°C for 2 h.

According to the UNI EN 1276:2009 (UNI EN 2009), bacterial count was performed using the spread plate technique: 1 mL of sample of diluted test suspension was divided and spread onto three separate VRE agar plates, which were incubated for 48 h at 37°C. The number (N) of cells per milliliter of the seeded suspension was calculated as follows:

$$N = \frac{c}{n \times 10^{DF}} \tag{12}$$

where c is the sum of the cfu counted on the three plates considered, n is the volume of sample seeded in the plates (mL) and DF is the exponent of the dilution factor.

According to the mentioned UNI EN standards for microbial count on agar plates, the final concentration can be considered measurable if within the limits prescribed: the lower limit is represented by 14 cfu in the whole solution sample, while the upper limit is reached counting 330 cfu on a single plate.

With the aim of obtaining measurable bacterial loads for each DIT protocol tested, several serial 10-fold dilutions of each treated suspension have been seeded.

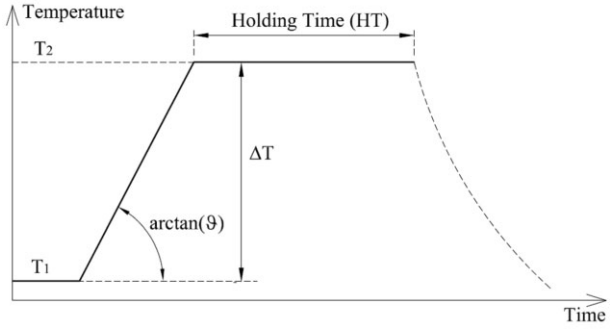

FIG. 2. SCHEMATIC REPRESENTATION OF A TEMPERATURE–TIME PROFILE OF DYNAMIC IRREVERSIBLE THERMOPORATION THERMAL PROCESS

ΔT = temperature increment of the suspension, ϑ = rate of temperature rise.

TABLE 2. EFFECTIVE TESTS OVER TOTAL TESTS OF DIT PROTOCOLS WITH HT 120 s AND 240 s AND MINIMUM AND MAXIMUM REDUCTION FACTOR LOG *R* OBTAINED

	DIT 20-65-120		DIT 30-65-120		DIT 20-65-240		DIT 30-65-240	
	Albumin	Sucrose	Albumin	Sucrose	Albumin	Sucrose	Albumin	Sucrose
Effectiveness	0/4	0/4	0/4	0/4	0/4	3/4	2/4	4/4
log R_{min}	2.0	3.7	1.1	2.6	4.0	4.4	3.7	5.0
log R_{max}	3.3	4.1	2.9	4.0	4.7	5.6	5.3	5.6

Bacterial count was performed twice by using two samples for every test and the average number of colonies was then recorded.

After each treatment, the DIT test bench was completely sanitized using 0.5% sodium hypochlorite and subsequently rinsed with sterile distilled water. Several validation procedures and controls were also performed for both interfering substance, according to the standard UNI EN 1276: 2009 (UNI EN 2009) adopted, as already described in Cammalleri *et al.* (2015).

The reduction of microbial concentration in the suspension submitted to DIT thermal treatment was evaluated as

$$R = N_i/N_f \tag{13}$$

where N_i and N_f represent the initial and the final bacterial concentrations, calculated by means of Eq. (12) before and after the DIT treatment, respectively: The treatment was considered effective when the reduction of counts gave as a result $\log R \geq 5$.

RESULTS AND DISCUSSION

DIT Treatment Effectiveness

As a first step for the evaluation of the effectiveness of the DIT treatment on the *E. hirae* suspensions, the authors adopted the same process parameters which proved effective in previous experimental tests carried out on different strains (Cammalleri *et al.* 2015): rates of temperature rise (ϑ) of 20 and 30°C/s, final temperature (T_2) = 65°C and holding times (*HT*) of 0 and 60 s.

On the contrary of the results obtained on the previously treated strains, this new set of tests revealed to be totally ineffective: The residual bacterial load ranged between 2.7×10^7 and 3.2×10^6 cfu/mL, corresponding to log *R* of 0.5 and 1.1, in the presence of albumin, while residual load in the range of 7.7×10^6–1.6×10^5 cfu/mL with log *R* of 0.5–2.2 was experimented in the presence of sucrose. The authors hence focused on the individuation and adaptation of the process parameters which may allow obtaining with the *E. hirae* suspensions the same good results already obtained with different strains. Indeed, the final treatment temperature (T_2) can be considered already at its maximum value if the application on already bottled plastic products is to be considered (Liga *et al.* 2015); on the other hand, the rate of temperature rise (ϑ) has already reached such a high value that a further increase would mean power levels too high for a feasible transfer of the DIT process to the industrial scale. The only parameter which could be then subjected to substantial variation is the duration of the final temperature holding phase *HT* (shown in Fig. 2), above all if it is considered that in the previous experience (Cammalleri *et al.* 2015), the increase of the *HT* allowed to significantly boost the effectiveness of DIT treatments: According to these observations, then *E. hirae* suspensions were subjected to DIT protocol variants with unchanged values of ϑ and T_2, and with increased holding times (*HT*) of 120 and 240 s. Separate tests were performed for both the interfering substances employed and the results are summarized in Table 2.

After the treatments with *HT* = 120 s, *E. hirae* residual load ranged between 1.9×10^6 and 4.4×10^4 cfu/mL with log *R* 1.1–3.3 in the presence of albumin, while residual loads between 6.6×10^4 and 1.5×10^2 cfu/mL with log *R* 2.6–4.1 were obtained using sucrose as an interfering substance. The extension of the holding time up to 240 s produced a further improvement of the bacterial load reduction: In more detail, in the presence of albumin, the residual load ranged between 9.8×10^3 and 152 cfu/mL with log *R* 3.7–5.3, while on sucrose suspensions, the residual bacterial load ranged between 969 and 261 cfu/mL, with log *R* ranging from 4.4 to 5.6. As an overall result, increasing both the rate of temperature rise and the holding time improved the effectiveness of the DIT thermal treatment.

Since an increment in the holding time is technologically easier to implement than the increase of the rate of temperature rise, the authors decided to carry out further investigations on the effect of prolonged holding times at the lower rate of temperature rise (i.e., ϑ = 20°C/s), aiming to obtain bacterial load reductions with log *R* = 5. Moreover, in this further experimentation, only bovine albumin has been employed as interfering substance, since, according to the results in Table 2, this revealed to be the worst condition.

Kinetic Analysis of Bacterial Load Reduction Curves

This further experimentation allowed investigating the thermal inactivation kinetic of *E. hirae* in isothermal

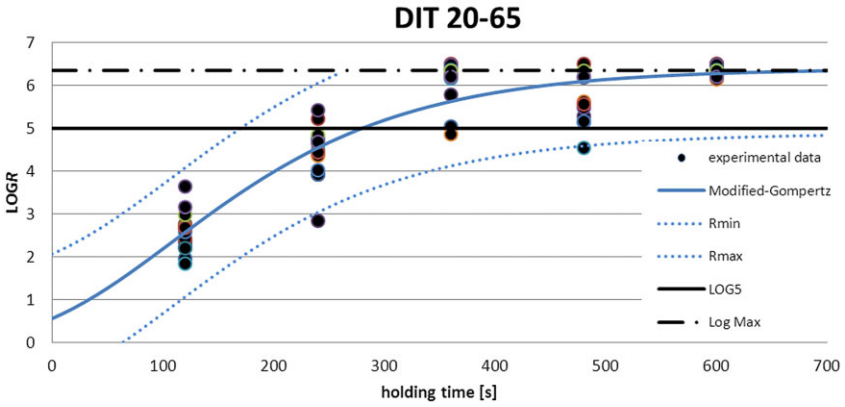

FIG. 3. BACTERIAL LOAD REDUCTION FACTOR OBTAINED WITH *ENTEROCOCCUS HIRAE* UNDER DIT 20-65 TREATMENT
Dots represent experimental data; solid line represents the best fit curve; dotted lines represent 90% confidence interval; log 5 is the threshold value according to the UNI EN 1276 standard; log Max is the asymptote of maximum average reduction measurable.

conditions immediately after the application of thermal shock imposed by the test bench. The experimental data were fit by the modified Gompertz model (Huang 2003), a nonlinear model commonly employed to describe the isothermal inactivation kinetic of heated microorganisms (Huang 2009),

$$\log R(HT) = \log N_i - \log N_f(HT) = \left[\log \bar{N}_i - \log L\right]\exp\{-\exp[-\alpha(HT - M)]\} \quad (14)$$

where N_i and N_f represent the already mentioned initial and final bacterial concentrations (cfu/mL), $\bar{N}_i$ is the mean value of N_i evaluated over the total amount of tests performed for each DIT protocol, α is the relative inactivation rate (s^{-1}), M is the model time constant (s), while L is the lower limit for bacterial counts on agar plates (i.e., 14 cfu/mL): The difference $\left[\log \bar{N}_i - \log L\right]$ is then the asymptotic value for time approaching infinity and represents the maximum average reduction achievable.

At the end of each rapid heating, the test tubes containing the treated bacterial suspension were placed into a thermostatic bath at temperature T_2 for varying holding times, with increments of 120 s up to a maximum HT of 10 min. These new experiments, labeled DIT 20-65, were all carried out in nine replicates.

The experimental results obtained have been fitted by the modified Gompertz model of Eq. (14) using the Least Square Error procedure performed by the software Curve Expert Professional, thus obtaining the best fit values of the two model parameters α and M; Fig. 3 shows the experimental bacterial load reduction as a function of the holding time, together with the best fit curve and the 90% confidence interval: This has been calculated, according to UNI EN 1276:2009 (UNI EN 2009) standard, using Student's t-distribution with p-k degrees of freedom, with p being the number of experimental points and k the number of parameters of the regression model.

Table 3 reports the best fit values of the model parameters α and M, together with some statistical index related to the goodness of fit.

The results clearly show an increase of the thermal treatment effectiveness with the holding time, until a saturation value is reached, which is close to the maximum reduction achievable; the modified Gompertz model employed for data fitting predicts that the 5 log reduction can be achieved using the DIT 20-65 treatment if a holding time of at least 300 s is adopted.

These results may lead to the conclusion that the effectiveness of the thermal treatment relies exclusively on the isothermal bath and not on the thermal shock. To remove this doubt, an aliquot of the *E. hirae* suspension was submitted to a conventional slow heating test (DIT 0-65 treatment) in a thermostatic bath. The experiments started with a warm-up phase, during which the test suspension is immersed in a 75°C thermostatic bath, where a temperature gradient as low as 0.3°C/s has been realized; once the temperature of 65°C has been reached, the test suspension was transferred in a second thermostatic bath at 65°C and maintained for the holding time. Figure 4 shows the resulting bacterial load reduction factors as a function of the holding times, which has been evaluated after the warm-up phase: The bacterial reduction of 5 log units was never obtained, even with very prolonged holding times (18 min).

TABLE 3. RESULTS OF THE FITTING PROCEDURE FOR THE *ENTEROCOCCUS HIRAE* REDUCTION FACTOR UNDER DIT 20-65 TREATMENT USING THE MODIFIED GOMPERTZ MODEL

DIT 20-65	
α (s^{-1})	0.0067
M (s)	93
Minimum HT for 5 log reduction (s)	300
Standard error	0.74
Correlation coefficient	0.86
Confidence interval (90%)	±1.2

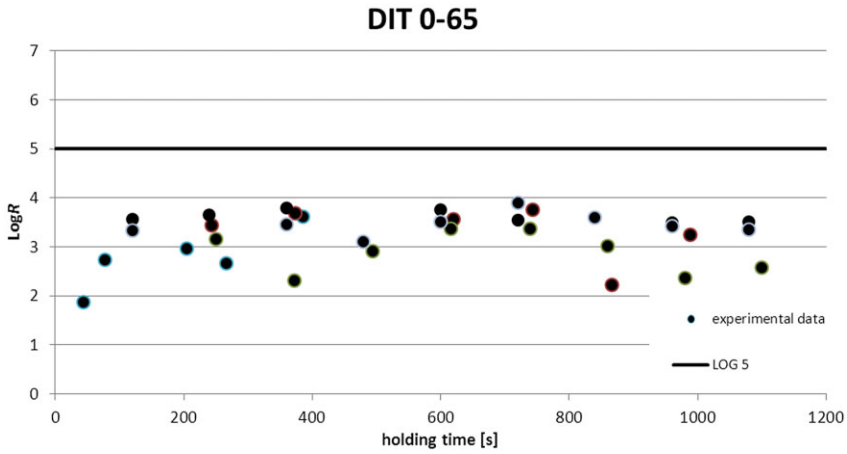

FIG. 4. *ENTEROCOCCUS HIRAE* REDUCTION FACTOR OBTAINED BY CONVENTIONAL SLOW HEATING THERMAL PROCESS IN THERMOSTATIC BATH

The result of this thermostatic bath test effectively proved the importance of the thermal shock performed with the DIT treatment, which, for a 5 log *E. hirae* load reduction, should be about two orders of magnitude higher than the thermostatic bath gradient (0.3°C/s), which, instead, revealed ineffective.

The application of lower temperature gradients would be however a considerable advantage in terms of technical requirements and lower power consumption, especially if large-scale application is considered. With the aim of assessing the possibility of a reduction of the rate of temperature rise (ϑ), the authors decided to test the effectiveness of the DIT protocol with a temperature gradient of 10°C/s, i.e., the DIT 10-65 protocol. The same test procedure and statistical analysis performed for the previous tests have been carried out. As a result, Fig. 5 reports the obtained bacterial reduction factor as a function of the duration of the holding time, together with the best fit curve and the 90% confidence interval; the best fit values of the model parameters α and M are instead reported in Table 4 together with the goodness of fit associated indices.

Also, in this case, the microbial reduction increased with the holding time duration, reaching a saturation value close to the measurable upper limit; as also shown, the best fit curve predicts that a holding time of about 240 s allows the DIT 10-65 treatment to reach the bacterial reduction of 5 log units.

A comparison based on the best fit curves resulting from the application of DIT 20-65 and DIT 10-65 protocols (see Figs. 3 and 5, respectively) points out that in the second case (i.e., with a slower temperature increase), the 5 log reduction factor can be pursued with shorter holding time (240 s instead of 300): This unexpected and apparently inconsistent result can be explained if the testing apparatus and procedure is considered. In effect, for the same tube and temperature increment ΔT, a higher temperature gradient (ϑ) means a higher flow rate (Q) (Eq. 7), or higher mean flow velocity (v) (Eq. 3) which is exactly the same thing. As

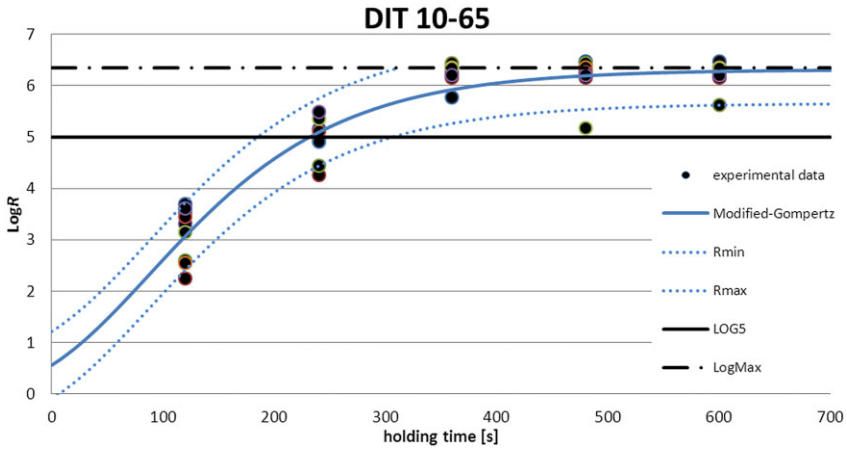

FIG. 5. BACTERIAL LOAD REDUCTION FACTOR OBTAINED WITH *ENTEROCOCCUS HIRAE* UNDER DIT 10-65 TREATMENT
Dots represent experimental data; solid line represents the best fit curve; dotted lines represent 90% confidence interval; log 5 is the threshold value according to the UNI EN 1276 standard; log Max is the asymptote of maximum average reduction measurable.

TABLE 4. RESULTS OF THE FITTING PROCEDURE FOR THE *ENTEROCOCCUS HIRAE* REDUCTION FACTOR UNDER DIT 10-65 TREATMENT USING THE MODIFIED GOMPERTZ MODEL

DIT 10-65	
α (s^{-1})	0.010
M (s)	88
Minimum *HT* for 5 log reduction (s)	240
Standard error	0.39
Correlation coefficient	0.95
Confidence interval (90%)	±0.66

TABLE 5. RESULTS OF THE FITTING PROCEDURE FOR THE *ENTEROCOCCUS HIRAE* REDUCTION FACTOR UNDER DIT 20-65 LR TREATMENT USING THE MODIFIED GOMPERTZ MODEL

DIT 20-65 LR	
α (s^{-1})	0.0078
M (s)	39
Minimum *HT* for 5 log reduction (s)	210
Standard error	0.59
Correlation coefficient	0.95
Confidence interval (90%)	±0.99

a higher mean flow velocity means a shorter time interval to flow across the whole tube length, a higher temperature gradient with same tube length may have caused an incomplete heat transfer to the inner part of the fluid core, thus giving rise to a lower bacterial reduction: This explains the longer holding time required by the DIT 20-65 protocol, together with its wider 90% confidence interval. All these considerations induced the authors to carry out an additional test, with the aim of assessing the effectiveness of DIT protocol with temperature gradient $\vartheta = 20$°C/s and the same mean flow velocity of the DIT 10-65 protocol, i.e., with the same Reynolds number $Re = 373$. To this purpose, the tube length has been halved (as reported in Table 1), hence allowing to perform a DIT 20-65 protocol using the same flow rate of the DIT 10-65. The results obtained by the new tests, labeled DIT 20-65 LR (i.e., low Reynolds), are shown in Fig. 6: Here, the bacterial reduction factor is represented as a function of the holding time duration, together with the best fit curve and the 90% confidence interval; as for the previous tests, the best fit values of the model parameters α and M are reported in Table 5 together with the goodness-of-fit indices. In these new conditions, the best fit curve predicts that an average reduction factor of 5 log units can be obtained by the DIT 20-65 LR if a holding time of at least 210 s is employed: It is worth noting that this holding time is slightly shorter than the one required by the DIT 10-65 treatment, which is 240 s (see Fig. 5). This result underlines a critical aspect of the DIT treatment, which must be adequately considered, above all if the industrial implementation of the process is considered: The necessary high temperature gradients, if not adequately realized, may compromise the uniformity of the thermal treatment, with a final detrimental effect on the whole microorganism inactivation effectiveness.

On the whole, the obtained results seem to suggest that a sufficiently high temperature gradient causes a substantial damage to the bacteria which become irreversible only with a proper holding time (DIT 20-65-210, DIT 10-65-240). Moreover, considering that the treatment with the very low temperature gradient of 0.3°C/s resulted ineffective, further research could be directed to the identification of the minimum temperature gradient ϑ, which, together with adequate *HT*, makes the microorganism inactivation process effective.

Nevertheless, the results illustrated in this paper definitely demonstrate the capability and reliability of the DIT process as thermal treatment for microbial inactivation in beverages, whenever a proper temperature increase rate and adequate holding time have been ensured.

Table 6 shows the mean reduction factor together with other statistical indices obtained considering HT ≥ 240 s for the two DIT protocols with equal Reynolds number, DIT

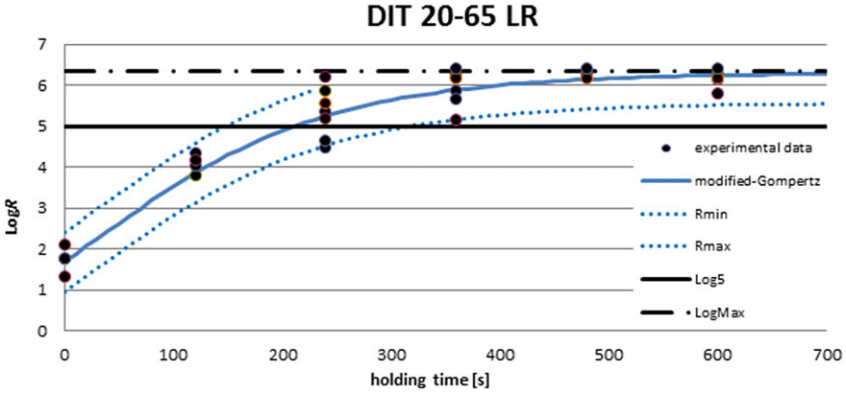

FIG. 6. BACTERIAL LOAD REDUCTION FACTOR OBTAINED WITH *ENTEROCOCCUS HIRAE* UNDER DIT 20-65 LR
Dots represent experimental data; solid line represents the best fit curve; dotted lines represent 90% confidence interval; log 5 is the threshold value according to the UNI EN 1276 standard; log Max is the asymptote of maximum average reduction measurable.

TABLE 6. RESULTS OF THE STATISTICAL ANALYSIS PERFORMED ON THE DATA OBTAINED FOR HT ≥ 240 s BY THE TWO DIT PROTOCOLS WITH EQUAL REYNOLDS NUMBER

	DIT 20-65 LR	DIT 10-65
Mean log *R*	5.9	5.9
Standard deviation	0.53	0.63
Standard error	0.10	0.11
90% confidence interval	0.17	0.18
99.7% confidence interval	0.34	0.34

20-65 LR and DIT 10-65. As shown, the microbial load reductions are extremely high and the confidence intervals are narrow; moreover, a very good agreement has been found between the results of the two DIT protocols.

Scanning Electron Microscopy Analysis

To better understand the biological mechanisms behind the effectiveness of the DIT protocols, the authors focused on assessing the morphological changes produced by the DIT treatment on the membrane surface of *E. hirae* cells.

Scanning electron microscopy (SEM) is a useful technique for investigating the surface structure of the biological samples and is a powerful technique for revealing the fine structure of the living systems; the main advantages of this technique consist of its higher spatial resolution compared with other imaging techniques together with the ability to measure and quantify topological data. The authors hence employed a scanning electron microscope to observe and compare the microstructural features of the bacterial cells of both treated and untreated *E. hirae* test suspensions. To this purpose, 5 mL of sample of the test suspension was centrifuged at 1,500 rpm for 20 min; the resulting pellet was resuspended in 5 mL of 10% formaldehyde, and after a fixing period of 10 min, centrifuged again at 1,500 rpm for 20 min; after the chemical fixation, the pellet was resuspended in 5 mL of phosphate buffered saline, centrifuged again at 1,500 rpm for 20 min and washed three times by resuspension in sterile bi-distilled water; a further centrifugation at 1,500 rpm for 20 min followed and, finally, the bacteria were resuspended in 0.5 mL of sterile bi-distilled water. At this point, the sample was then drop cast on an Al SEM stub and coated with a thin layer of Au by a sputter coater (Scancoat Six EDWARDS, HHV Ltd., Crawley, U.K.), operated for 1 min at 1 kV and 30 mA using Ar as carrier gas: The samples thus prepared were observed by using a FEI Quanta 200 FEG MK2 scanning electron microscope (FEI Company, Hillsboro, Oregon) in a high vacuum chamber. A critical observation of the images of the sample not subjected to the thermal treatment (see left image in Fig. 7) showed no alteration in the cell morphology: No defects, such as pinholes or cracks, were observed on the cell membrane; on the contrary, the images taken on the thermally treated sample revealed malformed and rough surfaces with well-rendered lacerations on the cell membrane (see right image in Fig. 7). As these membrane lacerations originate from a pure thermal treatment, i.e., without any chemical or electrical contribution, the authors considered this as a thermoporation, hence identifying the whole thermal process as dynamic irreversible thermoporation.

CONCLUSIONS

A new thermal treatment for microorganism inactivation in beverages, called DIT (dynamic irreversible thermoporation),

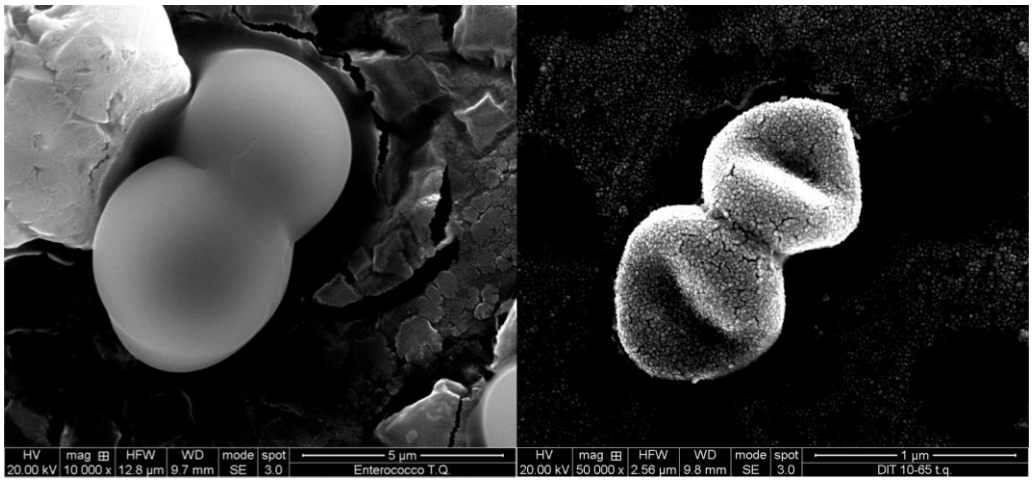

FIG. 7. SCANNING ELECTRON MICROSCOPE IMAGES OF *ENTEROCOCCUS HIRAE* BACTERIAL CELLS BEFORE (ON THE LEFT) AND AFTER THE DIT-10-65 TREATMENT (ON THE RIGHT): THE MODIFICATIONS OF CELL MEMBRANE IS EVIDENT

has been recently presented and successfully tested on several commons strains (*P. aeruginosa*, *E. coli*, *S. aureus*, *L. innocua* and *C. albicans*). This new thermal treatment, characterized by a rapid heating with a relatively low final temperature (65°C), has a great potential for the food industry as it can be applied on already bottled products, thus avoiding the use of expensive aseptic environment. In the present paper, the authors aimed to further assess the effectiveness of the DIT treatment on a known thermal-resistant strain, i.e., the *E. hirae*. To this purpose, new series of experimental tests have been carried out, meticulously following the procedures prescribed by the UNI EN 1276:2009 (UNI EN 2009), on a properly designed test bench, which allowed treating several *E. hirae* aqueous suspensions with different DIT protocol variants, obtained by maintaining a fixed maximum temperature of 65°C and varying the other two main process parameters, i.e., the *temperature gradient* (ϑ) and the final temperature *holding time* (*HT*). The results of the experimental tests outlined that the required 5 log bacterial load reduction can be achieved by a proper combination of the process parameters ϑ and *HT*: In particular, the treatment revealed fully effective with a temperature gradient as low as 10°C/s and holding times of at least 240 s, which make the industrial application of this process less critical.

The thermal inactivation kinetics of *E. hirae* for varying *holding times* has also been studied and described by a nonlinear model, the modified Gompertz, which allows estimating the holding time required to obtain the average reduction factor prescribed by the UNI EN 1276 standard.

The tests carried out with different rates of temperature rise ϑ and same flow conditions (identified by the same Reynolds number) reported very similar results, thus pointing out the importance of the heating process uniformity on the treated suspension mass.

Finally, a morphological investigation has also been carried out by means of a scanning electron microscope, with the aim of observing alterations produced by the DIT thermal treatment on the microorganism cell morphology; the SEM analysis revealed well-rendered lacerations of the cell surface of the thermally treated suspensions, thus confirming the effectiveness of the process; being the pinholes and cracks produced only by a pure thermal treatment, the process has been definitely called DIT.

On behalf of this second experimental investigations, the authors consider the DIT treatment a valid alternative to common, and more expensive, microorganism inactivation process; as already mentioned, this new treatment could be easily applied to already bottled products if a more efficient and uniform heating system would be available, such as radio frequency–based system; moreover, further experimentation should be carried out to prove that the microorganism inactivation effectiveness of the DIT process applied to beverage products, such as sport drink, energy drink, fruit juice, whose physical, chemical and electric properties (Lombardo *et al.* 2015) is different from pure water.

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