Plant Archives Vol. 20, Supplement 2, 2020 pp. 249-255 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EVALUATION OF THE EFFECTIVENESS OF TREATMENTS FOR SANITIZING AGENTS FOR REMOVAL OF *LISTERIA MONOCYTOGENES* BIOFILM

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Abstract

Obtained results at the first phase of the trial showed the ability of *Listeria monocytogenes* to adhere on the surfaces of stainless steel, forming a microbial biofilm, the data obtained in the latter stages of the experiment, showed that peracetic acid is a very effective sanitizer to disable the cells of *L. monocytogenes* adherent to the surface. An obtained data showed that peracetic acid is a very effective sanitizer to disable the cells of *L. monocytogenes* adherent to the surface. This compound was used to obtain the total elimination of biofilms, to a low-temperature treatment (25°C), using an active solution scarcely concentrated (40-250 ppm). Such intervention conditions also minimize any problems of corrosion of the surfaces; peracetic acid is, in fact, compatible with the stainless steel, provided that the temperature does not exceed 45 °C and the duration of application is limited. The phenomenon of biofilm formation remains however, a complex process regulated by many factors, the effects of which are still poorly understood, several aspects remain to be clarified and a practical application of this study it is necessary to validate the results obtained in real conditions.

Keywords **:** Biofilm, *Listeria monocytogenes*, peracetic acid, stainless steel.

Introduction

Disinfection in the food processing environment can be performed by chemical or physical methods. The chemical principle relying on disinfectants is the most widely used in the food industry? Their application includes disinfection of production plant and food containers, the control of microbial growth in food and drinks and the decontamination of carcasses. *L. monocytogenes* is a ubiquitous bacterium were isolated from raw materials of food production, this bacteria have a remarkable ability to persist for months or years in a food processing plant (Chasseignaux *et al*., 2001; Giovannacci *et al*., 1999; Lundén *et al*., 2003; Norton *et al*., 2001; Wulff *et al.*, 2006). There are several hypotheses have been proposed to explain the persistence of *L. monocytogenes* (Borucki *et al*., 2003; Holah *et al.,* 2002; Lundén *et al*., 2000, Norwood and Gilmour, 1999; Wulff *et al.,* 200 are 6) including one stating that persistence could be due to an increased resistance or tolerance to the chemical disinfectants used. *L. monocytogenes* bacteria are a source of contamination of the cheese factories environment where they are found in places that are difficult to disinfect and where moisture and food debris are present (Tompkin, 2002) *L. monocytogenes* bacteria have the ability to adhere and form biofilms on inert surfaces and are considered food-borne pathogens.

(Shi, Zhu, 2009; Takahashi *et al.,* 2011). Due to the low level of bacterial sterilization, it has led to permanent contamination in food processing plants (Belessi *et al.,* 2011) It is a common disinfectant used in dairy plants where it is used in food factories in Brazil with a concentration of 300 – 700 mg/L (Ceragioli *et al*., 2010; Quarentei *et al.,* 2011) PAA has a broad spectrum and is a powerful oxidizing agent that disintegration into safe waste products (Van der Veen, Abee, 2011). It also has the ability to remove *Listeria monocytogenes* adhesion (Belessi *et al.,* 2011; Ibusquiza, Herrera and Cabo, 2011; Stoporth *et al.,* 2002). The objective of the experiments was to detection a suitable medium for biofilm formatting by an *L. monocytogenes* strain in comparison with an industrial isolate and to test the efficiency of the general used antiseptic against biofilm

Materials and Methods

Operating Conditions

In this phase of the study, 16 of sanitizing treatments have been tested for the elimination of biofilms formed by *L. monocytogenes* on stainless steel surfaces. As sanitizing agents were chosen an alkaline disinfectant (caustic soda solution diluted to 2%) and a disinfectant acid (peracetic acid solution to 40%). The treatments are listed in Table .1 have been tested on biofilms of 7 days. For the formation of biofilms, aliquots of 20 ml of BP (1 g/l, pH 6.5) were distributed in Coplin containers every containing 5 tiles in a vertical place so as to have the whole surface exposed to the medium

The tiles were sterilized by autoclaving at 121 °C for 15 minutes. All samples were then inoculated with *L. monocytogenes (*initial inoculum ~103 CFU/ml) and incubated without agitation at 30 °C. After 7 days, the tiles were removed In a sterile manner, To remove non-adherent cells, rinse with sterile distilled water and transported to disposable tubes each containing 40 ml of sterile the sanitizing solution to the expected concentration (or sterile saline in the case of the control). The contact time and temperature of the treating solution have been modulated in relation to what is reported in the table. After the application of the treatments, the tiles were removed and rinsed with sterile distilled water. The concentration of cells in the form sessile "survived" the action of the sanitizer was determined as previously described.

Statistical Analysis

The tests were conducted in duplicate. The microbial counts were transformed into logarithms before calculating the averages and expressed as log CFU/cm^2. Whereas the

controls were not all at the same level of growth, to allow a comparison between the experiments tested, the results were the results were a determination as to the percentage of cell mortality compared to its control**.**

Determination of the influence of the concentration of the sanitizing agent, the contact time and the age of the biofilm on the effectiveness of the treatment sanitizing.

Operating Conditions

In order to assess the individual and interactive effects of the concentration of sanitizing agent, the contact time and the age of the biofilm on the removal of biofilms formed by *L. monocytogenes* on stainless steel surfaces, was set up a Central Composite Design (Box *et al.,* 1978) in three variables and five levels. The coded levels of the independent variables are shown in Table 2. The 17 combinations obtained are shown in Table 3. How sanitizing agent has been chosen the solution of peracetic acid to 40%.

Table (4) shows the amount in ppm of peracetic acid corresponding to each pH value tested. Solutions used were prepared in distilled water at pH of the plan experimental and sterilized. The sanitizing treatments were tested on biofilms of 6, 9, 12, 15 and 18 days, as required by the CCD. For the formation of biofilms was followed the method described above. All samples, after inoculation of *L. monocytogenes* (~103 CFU/ml), were incubated, without shaking, at 30 °C. After the planned days of incubation, the pieces were removed aseptically, rinsed with sterile distilled water to remove non-adherent cells and transferred into disposable tubes each containing 40 ml of sterile sanitizing solution at the concentration expected from the experimental plan (or sterile saline in the case of control). The contact time was modulated in relation to what is reported in Table 3, the temperature of the treating solution was maintained at 25 °C. After application of the treatment, the tiles were removed and rinsed with sterile distilled water. The concentration of cells in the form sessile "survived" the action of the sanitizer was determined as previously described.

Regression equations and statistical analysis

The tests were conducted in duplicate. The microbial counts were transformed into logarithms before calculating the averages and expressed as log CFU/cm^2. Whereas the controls were not all at the same level of growth, to allow a comparison between the experiments tested, the results were expressed as the percentage of cell mortality compared to its control. The data relating to the variation of the percentage of mortality as a function of the variables of the CCD (sanitizer concentration, contact time and age of the biofilm) were analyzed using the procedure "Multiple Regression"**.** It was obtained as a quadratic polynomial equation, Whose characterize effects of the independent variables on the average of mortality, in individual terms, quadratic and interactive as in the equation below

$$Y = B_0 + \Sigma B_i * x_i + \Sigma B_{ii} * x_i^2 + \Sigma B_{ij} * x_i * x_j$$

That B_0 is a constant, B_i, B_{ii} and B_{ij} are the regression coefficients, $_{xi}$ and $_{xj}$ are The importance of the model obtained was estimated by the regression coefficient (R), the value of the test of Fisher (F), the p-level and the corresponding standard error (SE). that illustrate the individual and reactive effects of the independent variables on the rate of mortality, have been built "contour plot", obtained by ordering one variable at a time to a constant value (the central point of the CCD).

Results and Discussion

16 treatments of sanitization were tested for the removal of biofilm 7 days of *L. monocytogenes*, made form BP (1 g/l; pH 6.5) proved in Phase 1 of the experiment, the substrate that promoted greater adherence.

As sanitizing agents were chosen an alkaline disinfectant (sodium hydroxide) and as an acid disinfectant (peracetic acid). The alkaline solutions such as NaOH are generally used in detergents to remove carbonaceous sediments, oils and grease (Vasseur *et al*., 1999) by virtue of this capacity to promote protein denaturation and the saponification of fats. Furthermore, the bactericidal action is attributed to them because they are able to damage the cytoplasmic membrane, ribosomes, DNA and proteins (Rowbury *et al.,* 1996). Typically soda is very corrosive to aluminium and brass, and stainless steel is corroded only by boiling solutions of soda. The risk of corrosion, which generally increases with increasing temperature and duration of application, can undoubtedly be considered negligible in the experimental conditions which we adopted.

Peracetic acid is used in the food industry as a cleaner and as a disinfectant. Since the beginning of the fifties, has been used for the removal of bacteria and fungi from fruits and vegetables, today, peracetic acid is used for the disinfection of medical supplies and to prevent biofilm formation in the industries of pulp. It is much used in systems C.I.P. (Cleaning In Place: ie cleaning and disinfecting of the internal parts of the equipment, containers, tanks, piping performed by pumping solutions for). The peracetic acid produces irreversible oxidative action that alters the cellular components. It is compatible with stainless steel, aluminium, Teflon, polystyrene and polyethene, but not with copper, zinc, bronze, cement and lime plasters. It is active at low temperatures (maximum operating temperatures = 40-45 °C beyond which the molecule breaks down releasing toxic).

The 16 treatments tested for the removal of biofilms of *L. monocytogenes* varied, in addition to the type and amount of active ingredient used, even for the contact time (5 or 25 minutes) and for the temperature treatment (25 or 35 °C).

The results in Table 5 showed the effectiveness of each treatment is expressed in terms of percentage of mortality of adherent cells, calculated through the following formula:

$$P\% = 100 - (\log CFU * cm^{-2} \text{ treated sample} / \log CFU * cm^{-2} \text{ control}) * 100$$

The total removing of biofilms (P% = 100%) was obtained only in treatments 9, 10, 13 and 14 wherein the pH of the active solution, based on peracetic acid, was equal to 4 (800 ppm of sanitizing agent). The effectiveness of the treatments is halved (P =% ~ 50%) in the treatments 11, 12, 15 and 16, where the active solution of acid was not concentrated (pH 5.5). Modest was the influence of contact time and temperature of treatment on the values of P% in treatments based on acid.

The treatments with NaOH have shown all less effective than those based on peracetic acid, with values of P% around 50% obtained only in the most extreme conditions (maximum concentration, higher temperature and longer operation time contact). The treatment with this

compound, therefore, at least under the conditions tested, they were never able to completely eliminate biofilms formed by *L. monocytogenes* on stainless steel.

In the last phase of the experiment was studied the influence of the concentration of the sanitizing agent, the contact time and the age of the biofilm on the effectiveness of the treatment, using the methodology of the Central Composite Design (Box *et al*., 1978). The sanitizing treatments were tested on facts to form biofilms in BP (1 g/l, pH 6.5) was used as a sanitizing agent is peracetic acid, which proved more effective than NaOH in Phase 2 of the experiment.

After the application of the treatments, the data for the percentage of mortality, with an elaborate procedure of multiple regression, allowed us to obtain a "best fit equation " that describes in individual terms, quadratic and interactive influence of the concentration of peracetic acid [pH], the contact time [t], and the age of the biofilm [E] on the rate of mortality (P %) of *L. monocytogenes* in sessile form (Table 6). Were included in the equation only the terms with a high significance ($p < 0.05$). The R-value indicates that the proposed model is well suited to describe the experimental data, and the Fisher exact test also emphasizes the high significance polynomial equation obtained.

Table 7 "Best fit equation" for individual effects, quadratic and interactive of the concentration of peracetic acid [PH], the contact time[t], and the age of the biofilm[E] on the rate mortality (P%) of *L. monocytogenes* in the form sessile.

According to the equation, P % was positively influenced by [t] and adversely from [E] (both present as quadratic terms) to indicate, as expected, a greater inactivation of the cells with increasing duration of treatment and with decreasing age of the biofilm. The major acquired resistance of biofilms older could be explained by assuming that they have already developed a three-dimensional structure of biofilms unlike younger. Cell layers more interior, being constituted by cells metabolically less active due to the reduced presence of oxygen and nutrients, are generally more resistant to sanitizers also due to mutations in the gene level (Tremolo *et al.*, 2002; Prigent - Combaret *et al*., 1999). The formation of a mature biofilm can lead, therefore, to an increase in the resistance of the cells to the sessile sanitizers, especially if the phenomenon took place for several days. this underlines how important it is to interfere in a protective route to avoid or reducing the formation the biofilm on surfaces in touch with food during their transformation.

The concentration of peracetic acid (expressed in terms of pH) intervened as individual term positive, quadratic negative and positive interaction with the age of the biofilm.

The peracetic acid-based disinfectants are commercially available solutions in suitably stabilized at 40%. Peracetic acid breaks down in the environment, in a short time, in acetic acid, oxygen and hydrogen peroxide. Of these molecules, in itself already active individual antimicrobial agents, peracetic acid is the most active, as other organic peracids, it produces irreversible oxidative action that alters the cellular components. Unlike what happens with the disinfectant containing caustic soda, the activation energy is low and the bactericidal effect is significant even at temperatures slightly higher: just a modest increase in temperature to increase the inactivating effect, even if you recommend that you never exceed 40-45 °C, beyond which the molecule is degraded releasing toxic substances.

The figure1 represents the "contour plot" related to the effects of the interaction contact time (min) / Age of biofilm (days) on the percentage of death rate (P %) of *L. monocytogenes* in the form sessile, using a 40 ppm solution (pH 5.5) of peracetic acid. The analysis of the graph confirms the possibility to completely remove the biofilm from steel, using a solution of 40 ppm peracetic acid, only prolonging the contact time up to 25 minutes, regardless of the age of the biofilm. With regard to the latter, you may notice a bell-shaped effect (dashed line corresponding to P % = 80%) that emphasizes more effective treatments for biofilms of 12-15 days. This result is not confirmed in the literature and highlights the need for further research in this direction.

Figure 2 Shows the Pareto chart relative to individual and reactive effects of the independent variables on the change in the percentage mortality of *L. monocytogenes* in sessile form. This diagram shows the effect of different parameters on the normalized dependent variable, calculated as the proportion of the regression coefficients and standard errors (Bi / ES). In particular were considered significant effects that exceed the vertical lines, corresponding to the critical value of the t-student ($p = 0.05$). Can be observed as the concentration of active ingredient, either as an individual term, the positive quadratic negative was the variable that most significantly influenced the efficacy of treatments. Less significant influence of the other variables studied: contact time > age of the biofilm.

Conclusions

The results showed the ability of Listeria bacteria to adhere to stainless steel surfaces and the formation of biofilm. We also note that the components of the medium significantly affect the ability of bacteria to adherence. Bacteria are more adherent when food is available compared to lower adhesion when food is reduced in the medium.

The conditions are similar to the industrial hardly laboratory culture conditions (Leriche and Carpentier, 2000), however, he greater ease with which *L. monocytogenes* biofilms form when nutrient availability is weak(recurrent condition in a plant) probably of particular benefit to the food industry, stressing the need to interfere in a protective route to avoid or at least limit, the formation of biofilms on surfaces in touch with the food through their processing**.**

With regard to the latter, the data obtained in the latter stages of the experiment exhibit that peracetic acid is a very efficient sanitizer to disable the cells of *L. monocytogenes* adherent to the surface. This compound was used to obtain the total elimination of biofilms, to a low-temperature treatment (25°C), using an active solution scarcely concentrated (40-250 ppm). Such intervention conditions also minimize any problems of corrosion of the surfaces; peracetic acid is, in fact, compatible with the stainless steel, provided that the temperature does not exceed 45 °C and the duration of application is limited. An obtained results in this study placements are a useful approach to solve the problem, but the trial cannot be said to be far from over, in fact, the response of adherent cells of *L. monocytogenes* to disinfection treatments tested, it may not be the same as other

microorganisms. Because of the great number of factors involved in the formation of a biofilm (surface kind, availability of food and oxygen, microbial species, etc Each biofilm is different from the other. It is, therefore, a clear need for further research in this direction, in order to identify methods of sanitation standards that are effective in removing any type of biofilm.

The phenomenon of biofilm formation remains, not well understood there are many factors involved in the formation of the biofilm in a complex way, several aspects remain to be clarified, and above all, before you can think of a practical application of these studies, it is necessary to validate the results obtained in real conditions.

Table 1: Treatments sanitizing tested

The temperature of Treatment (C°)	Contact time (min)	Concentration (PH of solution)	Sanitizing agent	Treatment
25	5	10	NaOH	1
25	25	10	NaOH	2
25	5	12	NaOH	3
25	25	12	NaOH	4
35	5	10	NaOH	5
35	25	10	NaOH	6
35	5	12	NaOH	7
35	25	12	NaOH	8
25	5	4	Peracetic acid	9
25	25	4	Peracetic acid	10
25	5	5,5	Peracetic acid	11
25	25	5,5	Peracetic acid	12
35	5	4	Peracetic acid	13
35	25	4	Peracetic acid	14
35	5	5,5	Peracetic acid	15
35	25	5,5	Peracetic acid	16

Table 2 : Levels of the three independent variables coded

Age of biofilm (days)	Contact time (min)	Concentration of sanitizing	Levels
6	5	4,5	α(-2)-
9	10	5,0	-1
12	15	5,5	0
15	20	6,0	+1
18	25	6,5	+ α(+2)

Table 3 : Combinations of the three independent variables

Age of biofilm (days)	Contact time (min)	Concentration of sanitizing	Combination
9	10	5	1
15	10	5	2
9	20	5	3
15	20	5	4
9	10	6	5
15	10	6	6
9	20	6	7
15	20	6	8
12	15	5,5	9
6	15	5,5	10
18	15	5,5	11
12	5	5,5	12
12	25	5,5	13
12	15	4,5	14
12	15	6,5	15
12	15	5,5	16
12	15	5,5	17

Table 4 : Concentrations of peracetic acid (ppm) corresponding to the values of pH tested

Peracetic acid concentration	pH
250 ppm	4,5
200 ppm	5,0
40 ppm	5,5
5 ppm	6,0
0 ppm	6,5

Table 5 : The effectiveness of sanitizing treatments tested

(p%)	the treatment temperature (◦C)	Time of contact (min)	Concentration (pH of solution)	Sanitizing agent	Treatment
18.67	25	5	10	NaOH	1
32.08	25	25	10	NaOH	2
33.61	25	5	12	NaOH	3
34.23	25	25	12	NaOH	4
34.06	35	5	10	NaOH	5
39.20	35	25	10	NaOH	6
49.85	35	5	12	NaOH	7
53.80	35	25	12	NaOH	8
100	25	5	4	Peracitic acid	9
100	25	25	4	Peracitic acid	10
44.98	25	5	5.5	Peracitic acid	11
49.02	25	25	5.5	Peracitic acid	12
100	35	5	4	Peracitic acid	13
100	35	25	4	Peracitic acid	14
51.65	35	5	5.5	Peracitic acid	15
50.8	35	25	5.5	Peracitic acid	16

Table 6 : Best fit equation

ES [e]	R[d]	F[c]	Best fit equation
20.99	0.96	62.43	$P\% = 57.0604\ [pH] - 13.0040\ [pH]^2 + 0.0927\ [t]^2 - 0.7136\ [E]^2 + 3.4510\ [pH]\ [E]$

- concentration of Peracitic acid is expressed in terms of pH
- P% = 100 – (Log CFU / cm2 treated sample / Log CFU / cm2 control)*100
- Value of Fisher's exact test
- Regression coefficient
- Standard Error

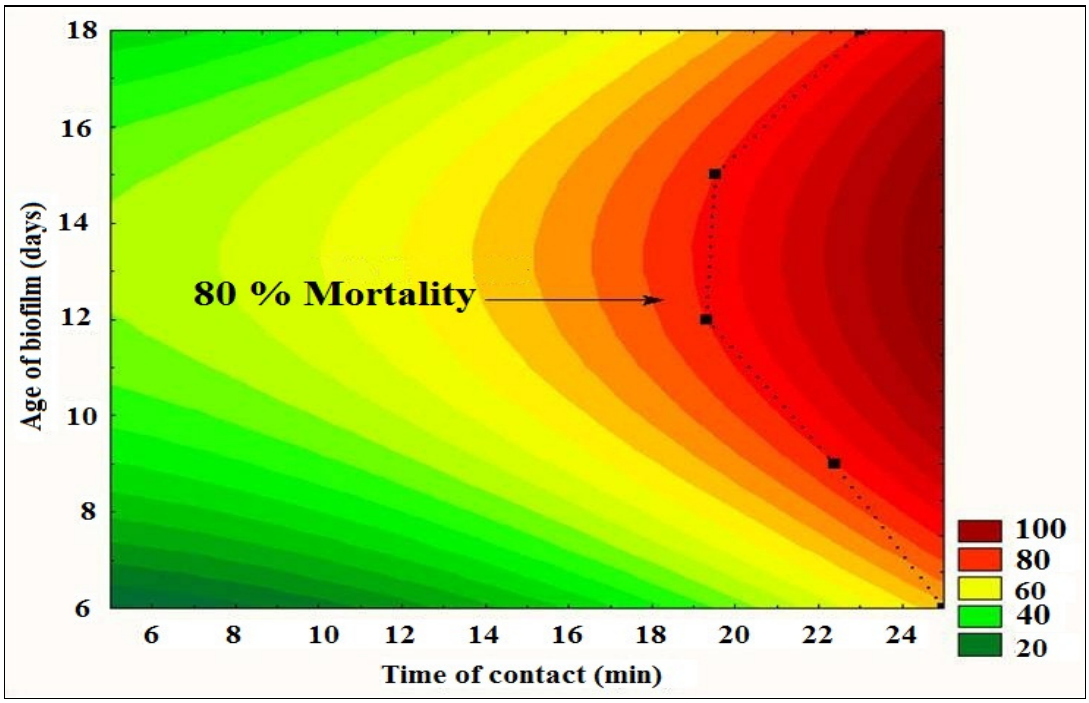

Fig. 1 : Represents the "contour plot" related to the effects of the interaction contact time (min) / Age of biofilm (days) on the percentage of mortality (P %) of L. monocytogenes in the form sessile, using a 40 ppm solution (pH 5.5) of peracetic acid.

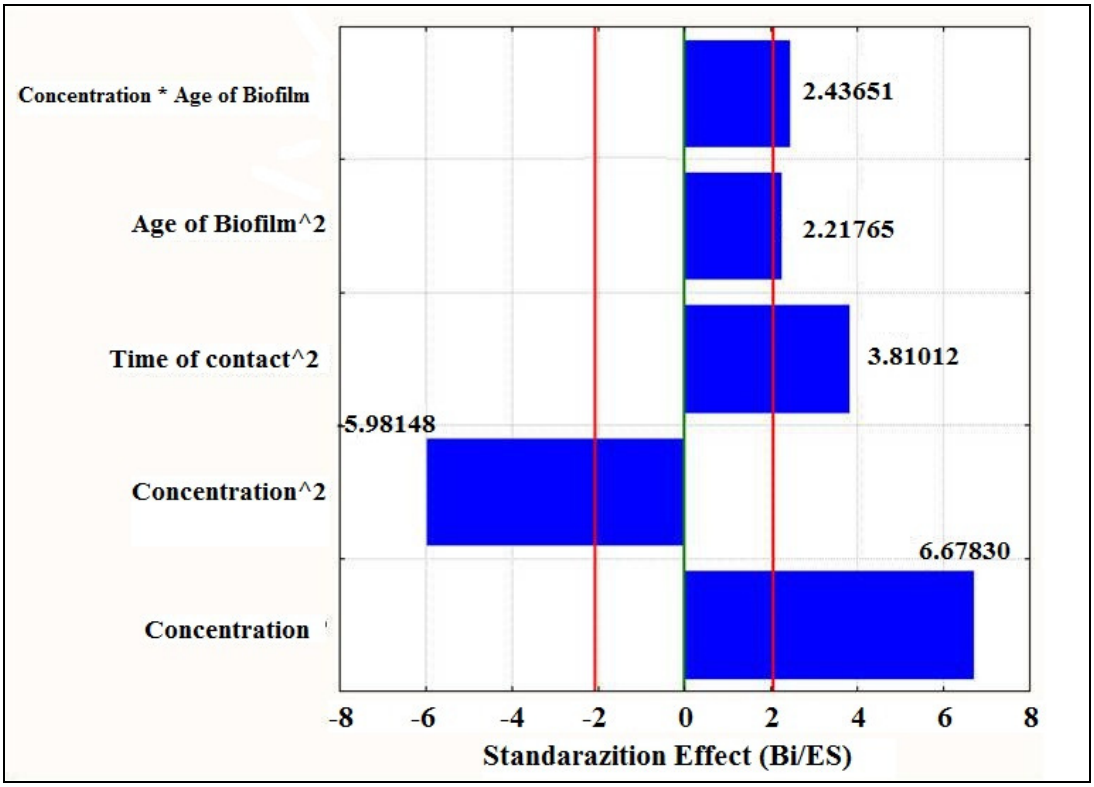

Fig. 2 : Pareto Diagram relating to the individual and interactive effects of the concentration of peracetic acid, the contact time and the age of the biofilm on the percentage of mortality (P%) of *L. monocytogenes* in the form sessile

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