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Effect of Acid Adaptation of *Listeria monocytogenes* on Its Mild Thermal Inactivation in a Simulated Fruit Juice Supplemented with Carvacrol

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AT, JJEN, SSK and FXE designed the study. Author AT wrote the protocol and managed the analyses of the study with author MJ. Author SSK performed the statistical analysis with author FXE. Authors AT and JJEN wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The use of mild heat treatment in combination with carvacrol has been reported as a possible way to quickly inactivate *L. monocytogenes* in fruit juices. This study aimed at assessing the possible effect of a prior adaptation of this pathogen to fruit juice acidity on its inactivation under the effect of such combined process.
Place and Duration of Study: Department of Microbiology of the University of Yaoundé I between November 2014 and April 2015.
Methodology: Citric acid-adapted and non-adapted cells were first produced. Their inactivation were then followed at 60℃, with and without carvacro l supplementation (30 µL/L), in simulated fruit juices adjusted to pH 4.5 and Brix 12, pH being adjusted in one case with hydrochloric acid, in another with citric and lastly with malic acid. Inactivation curves were fitted to the Weibull inactivation model.

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Results: Acid adaptation of cells greatly increased their tolerance to the mild heat treatment. In fact, inactivation scales of acid-adapted cells were lower than those of non-adapted cells in all the tested conditions. The acidification of the model juice with citric or malic acid enhanced non-adapted cells inactivation. In contrast, with acid-adapted cells, only malic acid had a positive impact on inactivation with time. In presence of citric acid, this inactivation was even lower than in model juice acidified with hydrochloric acid. A slight positive impact of carvacrol supplementation on non-adapted cells inactivation was noticed either comparing inactivation scale or inactivation with time in the tested conditions. This effect of carvacrol was minor with acid-adapted cells.
Conclusion: Microorganism acid adaptation phenomenon should be taken into consideration while studying the antimicrobial efficiency of low thermal process treatment of fruit juices.

Keywords: Fruit juice; L. monocytogenes; acid adaptation; thermal tolerance; carvacrol.

1. INTRODUCTION

Thermal treatment is generally the method used to ensure the safety and stability of fruit juices. However, the severity of the heat treatment generally applied for conventional pasteurization has been reported to lead to the reduction of their nutritional quality, and changes in their physicochemical, rheological and sensorial properties [1-4]. This negative impact and the increasing consumers demand for high quality, minimally processed and microbiologically safe foods, have encouraged the research and development of low thermal processes among which irradiation, high hydrostatic pressure, pulsed electric fields ultraviolet [5,6]. Unfortunately, the widespread use of these cutting edge technologies has been limited due to the high investment required to acquire the relevant equipment.

Mild thermal treatment in combination with natural antimicrobial supplementation have been intensively studied during the last decade and proposed as durable alternative [7-12]. However, these researches related to the study of the antimicrobial efficiency of this combined process have not taken into account the possibility of a prior acid adaptation of microorganisms before the treatment. This acid adaptation phenomenon observed with some microorganisms has been reported to increase their mild thermal tolerance [13,14]. Citric acid is one of the predominant acids found in many fruits [15]. Isolation of microorganisms from fruit juices suggests they could have already adapted to the acidic nature of these juices, and then to this acid.

Many natural antimicrobials have been tested in association to mild heat treatment for low thermal pasteurisation of fruit juices, among which carvacrol. This latter is a Generally Recognised As Safe (GRAS) food additive with a wide and strong antimicrobial potential, and is the main component of *Origanum* spp. essential oil [16,17]. Sado et al. [12] observed that its supplementation in a carrot juice at 30 mg/L increased mild thermal inactivation of *Listeria monocytogenes* 56 LY inoculated inside. This study was then carried out in order to assess how an acid adaptation of this pathogen which has been associated to some outbreaks linked to fruit juices consumption [18], could affect its inactivation under the effect of that combined treatment.

2. MATERIALS AND METHODS

2.1 Microorganism

Listeria monocytogenes 56 LY was used in this study as target microorganism. It was kindly provided by the Food Microbiology Laboratory of the University of Bologna, Cesena, Italy and stored at -80℃ until use. Before the experiment, the strain was sub-cultured in nutrient broth at 37℃ for 24 h thrice.

2.2 Preparation of Simulated Fruit Juices

Three simulated (or model) fruit juices adjusted to pH 4.5 and Brix 12 were prepared, the difference between them being the acid used to adjust their pH (hydrochloric, citric or malic acid). These pH and Brix values were chosen to represent the mean values generally reported in the literature for fruit juices. Each model juice was prepared as described by [19]. Sucrose was first dissolved in distilled water at an amount corresponding to the Brix value. The mixture was heated for total dissolution, cooled and the pH adjusted with the corresponding acid and a 0.1N sodium hydroxide solution when needed. A portable pH meter (pH Meter HI 98103, Hanna instruments, Inc., USA) and an ATAGO refractometer (model 2313; ATAGO Co. Ltd., Tokyo, Japan) were used for these preparations.

2.3 Acid Adaptation of the Strain

Citric acid-adapted cells were obtained by culturing the strain at 37℃ for 24 h in a moderate acidic growth condition as suggested by [20]. Nutrient broth adjusted to pH 6.5 with citric acid was used. This specific pH was chosen so that citric acid does not exhibit an inhibitory effect on the strain growth which could have been a stress before thermal treatment. Besides, cells were also grown in nutrient broth pH 7 to serve as control (non acid-adapted cells).

2.4 Thermal Treatment

Inactivation kinetics at 60℃ of both acid-adapted and control cells were each followed in the different prepared model juices supplemented or not with carvacrol (natural, 99%, Food Grade, Sigma-Aldrich, St. Louis, USA) at 30 µL/L. The thermal treatment was conducted in a thermostatically controlled water bath as described in a previous study [11].

2.5 Inactivation Kinetics and Resistance Distributions

The inactivation kinetics obtained in the different tested conditions were fitted to the Weibull equation [21]:

$$\log S(t) = \log(N_t/N_0) = -bt^n \quad (1)$$

Where Nt/N0 are the survival ratio S(t) after the treatment time t. The parameters “b” and “n” are the inactivation scale and shape, respectively. Depending on the “n” parameter value, three shapes are possible: n<1, upward concavity; n>1, downward concavity; and n=1, first-order kinetic (linear).

The values of b’ and n’ obtained from the exponential form of the Weibull model, were used to generate the distribution of resistance during the treatment (Eq2):

$$dS(t)/dt = b'n't^{n'-1}\exp(-b't^{n'}) \quad (2)$$

Statistical parameters describing these distributions of resistance were calculated as indicated by Peleg & Cole [21]. In particular, the distribution mode, which represents the treatment time at which the majority of the population is inactivated; the mean (average inactivation time) and its variance; and the skewness (skew of the distribution) were assessed.

3. RESULTS

Fig. 1 presents the inactivation kinetics of acid-adapted and non-adapted *L. monocytogenes* cells in the different prepared model juices. The use of organic acids to acidify the model juice greatly increased the inactivation of control cells with time. In contrast, with acid-adapted cells, in comparison to the inactivation kinetic observed in model juice acidified with hydrochloric acid, the inactivation was higher in the one acidified with malic acid, but lower with citric acid.

The inactivation kinetics of acid-adapted and non-adapted cells in each model juice supplemented or not with carvacrol are presented in Fig. 2. In all the tested conditions, inactivation of acid-adapted cells was clearly lower than those of control cells. This negative impact of a previous acid adaptation of cells on their mild heat inactivation was more pronounced in the model juice acidified with organic acids. Supplementation of carvacrol improved the heat inactivation of control cells in the model juice acidified with hydrochloric acid. This was not the case in the model juices acidified with citric and malic acids where the presence of carvacrol did not affect the inactivation kinetics of those cells. With acid-adapted cells, no important change of inactivation kinetics was observed in presence of carvacrol.

The values of the inactivation parameters “b” and “n” obtained by fitting the experimental inactivation data to the Weibull inactivation model in the different tested conditions are presented in Table 1. The sum of squared errors (SSE) and determination coefficient (R^2) values demonstrate that the Weibull model could fit well the experimental inactivation data. All the values of the shape parameter “n” were lower than 1. This is in accordance with the upward concave shape of the inactivation curves observed. A comparison of the inactivation scales obtained (“b” values) clearly showed that a previous acid adaptation of cells before the thermal treatment increased their resistance. Indeed, an important reduction of “b” values was observed with acid-adapted cells compared to control cells. The acid used to acidify model juice accounted on the sensitivity of control cells to heat treatment. The “b” values were higher when using organic acids (citric and malic acids) rather than hydrochloric acid. This was not the case with acid-adapted cells where the “b” values were similar no matter the acid used. The supplementation of the model

juice with carvacrol slightly increased "b" values of non-adapted cells. But with acid-adapted cells this slight increase was only observed in the model juice acidified with hydrochloric acid.

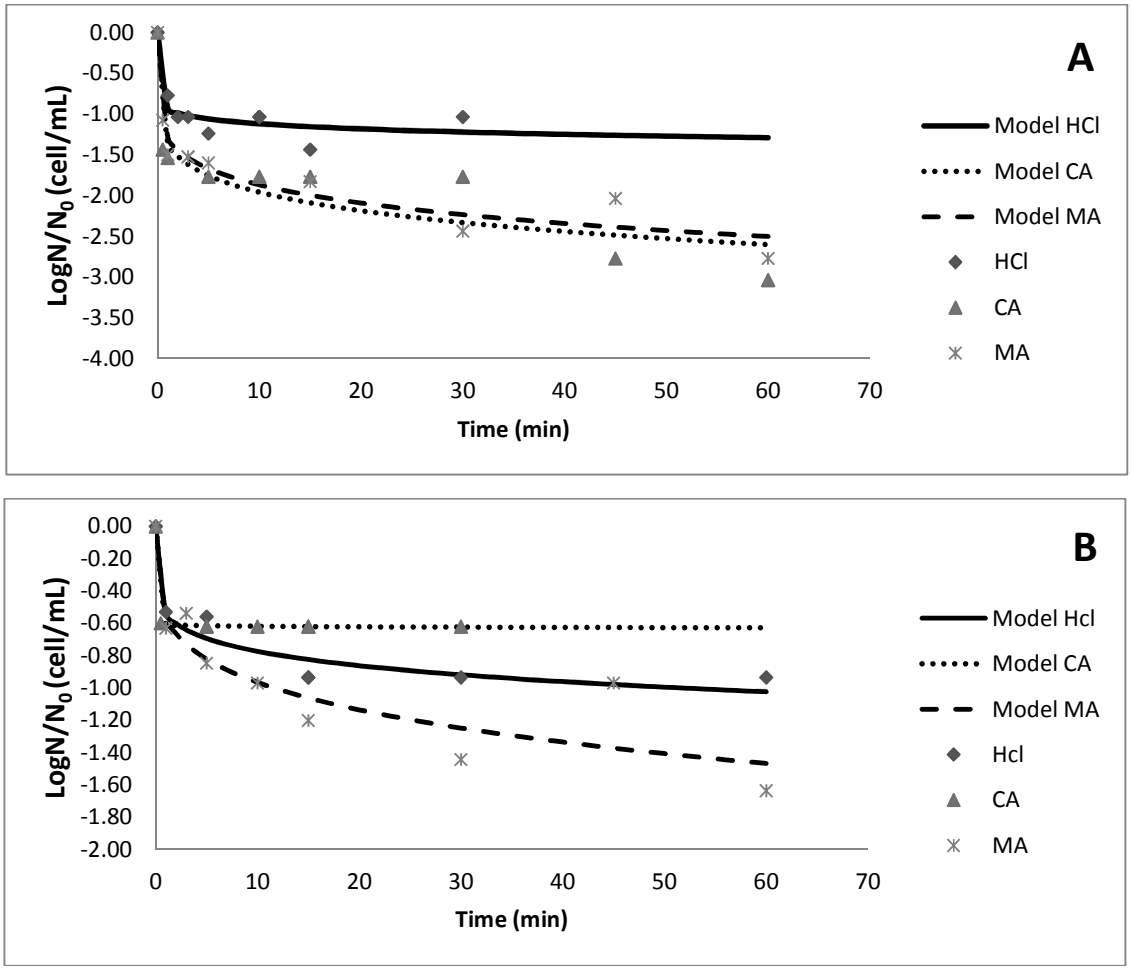

Fig. 1. Inactivation kinetics at 60℃ (experimental data and model) of non acid-adapted (A) and acid-adapted (B) cells in simulated juice with pH adjusted with hydrochloric (Hcl), citric (CA) and malic (MA) acids

Table 1. Weibull distribution parameters obtained in the different tested conditions*

Treatment medium			Cells treated	b			n			Model	
pH-Brix	pH adjusted with	Carvacrol (µL/L)		Value	SE	p	Value	SE	p	SSE	R^2
4.5-12	HCl	0	Control	0.94	0.11	0.00	0.08	0.05	0.17	0.19	0.85
		30	Control	1.08	0.22	0.00	0.21	0.08	0.03	0.76	0.83
		0	Acid-adapted	0.54	0.08	0.00	0.16	0.05	0.02	0.05	0.93
		30	Acid-adapted	0.78	0.13	0.00	0.09	0.06	0.22	0.18	0.82
	CA	0	Control	1.36	0.19	0.00	0.16	0.04	0.01	0.81	0.86
		30	Control	1.44	0.14	0.00	0.15	0.03	0.00	0.28	0.95
		0	Acid-adapted	0.61	0	0.00	0.01	0	0.00	0	0.99
		30	Acid-adapted	0.6	0.01	0.00	0.01	0	0.07	0	0.99
	MA	0	Control	1.28	0.11	0.00	0.16	0.03	0.00	0.32	0.94
		30	Control	1.4	0.13	0.00	0.14	0.03	0.00	0.54	0.9
		0	Acid-adapted	0.56	0.11	0.00	0.23	0.06	0.01	0.29	0.85
		30	Acid-adapted	0.61	0.05	0.00	0.12	0.03	0.01	0.02	0.97

**HCl, hydrochloric acid; CA, citric acid; MA, malic acid; SE, standard error; SSE, sum of squared errors; R^2, coefficient of determination; P, P value (Parameter with P<0.05 are significant)*

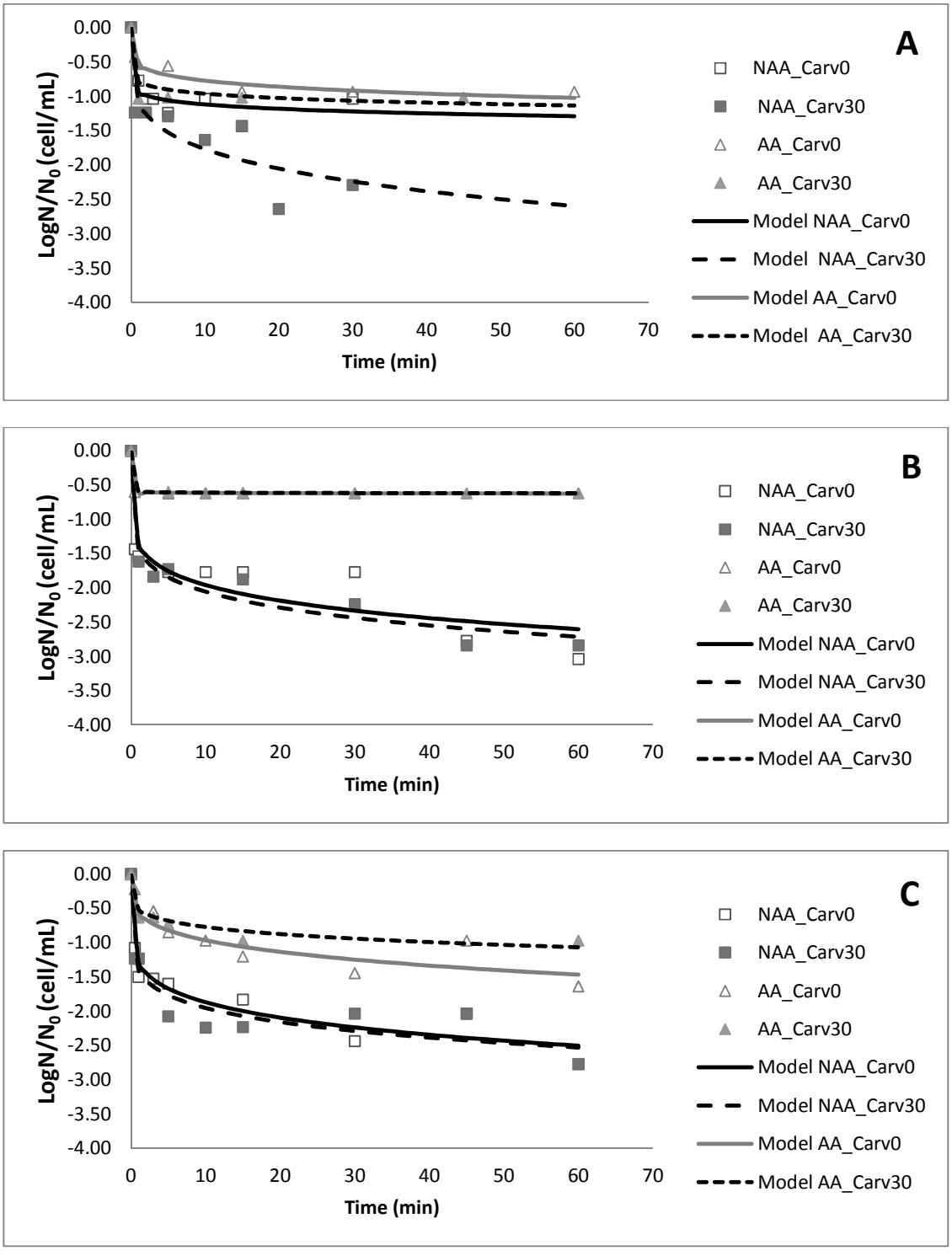

Fig. 2. Inactivation kinetics at 60℃ (experimental data and model) of acid-adapted (AA) and non acid-adapted (NAA) cells in simulated juice with pH adjusted with hydrochloric (A), citric (B) and malic (C) acids, supplemented (carv30) or not (carv0) with carvacrol

The frequency distributions of resistance of the cell population during the treatment in the different tested conditions are presented in Fig. 3. The difference between these distributions could be more appreciable comparing the associated calculated statistical parameters using their respective b' and n' (Table 2). No mode was obtained for these distributions. In fact, when n<1 the mode tends to be at infinitive.

Comparing conditions without carvacrol supplementation, we could observed that acid adaptation of cells before treatment led to an increase in their average inactivation time (mean), but this was not observed in model juice with pH adjusted with hydrochloric acid. The lowest variances were noticed in model juice with pH adjusted with malic acid. Regarding the effect of the acid used to acidify the model juice, the average inactivation time of control cells decreased in presence of organic acids (especially with malic acid), but the distributions were more skewed to the right. For acid-adapted cells, a decrease was also observed but only in model juice with pH adjusted with malic acid. A strong increase of this time was rather observed in medium with pH adjusted with citric acid, as well as a lacked of skew. Supplementation of model juices with carvacrol led to a decrease in the average inactivation time of control cells but an increase in the skew was observed. A decrease of the average inactivation time of acid-adapted cells was also noticed when supplementing carvacrol in model juice acidified with hydrochloric acid and citric acid. In contrast, an increase was rather observed in model juice acidified with malic acid.

4. DISCUSSION

The inactivation kinetics of *L. monocytogenes* at mild heat had already been reported to have an upward concave shape (n<1) [11,12]. A similar observation was made in our tested conditions. Coupled to the lack of mode observed with the frequency distributions of resistance, this shows that a majority of the cell population was quickly destroyed under the effect of the process, leaving behind the most resistant cell fraction [21].

Citric and malic acids are the predominant organic acids naturally present in fruits, and then in fruit juices [15]. As observed in this study, their presence plays a key role in thermal inactivation of *L. monocytogenes*. In fact, we could observe that they greatly increased the sensitivity of non acid-adapted cells to the heat treatment applied. This is certainly due to their antimicrobial properties, which has been linked to their capacity to reduce the pH of the medium, to decrease the internal pH of microorganism cells and to alter their membrane permeability [22]. Supplementation of fruit juices with natural antimicrobials has been described by many authors as a possible way to increase sensitivity to mild heat treatment of microorganisms present inside. Supplementation of our model juices with carvacrol effectively led to an increase of inactivation scales of *L. monocytogenes* non acid-adapted cells. This effect of carvacrol was also observed by Sado et al. [12] on the same strain and has also been related to its antimicrobial potential. This compound has as first target cell membrane where it increases fluidity and permeability.

Considering the possibility of the presence in juice of *L. monocytogenes* cells which have grown in the presence of citric acid, we clearly observed that this previous acid adaptation of cells may greatly improve their mild thermal tolerance. This phenomenon had already been observed with microorganisms like *Enterococcus faecium* [20], *Escherichia coli* O157:H7 [23] and *Salmonella typhimurium* [13]. In fact, acid adaptation has been reported to induce a change in membrane fatty acid composition, especially a decrease in the unsaturated to saturated ratio (UFA/SFA) leading to a decrease in membrane fluidity and permeability [13,14]. A possible decrease in membrane permeability when cells were acid-adapted could have therefore limited the entrance of organic acids and carvacrol into cells and then their antimicrobial action.

Organic acids with smaller molecular weight have been reported by Eswaranandam et al. [24] to have higher antimicrobial activity. These authors suggest that smaller organic acids like malic acid (HOOC-CH2-CH(OH)-COOH, 134.09 Dalton) might easily enter bacteria cells and change their internal pH than larger acids like citric acid (HOOC-CH2-C(OH)(COOH)-CH2-COOH, 192.13 Dalton). This statement was confirmed in our study where the inactivation of acid-adapted cells during time was higher in model juices acidified with malic and hydrochloric acids than with citric acid. It was also observed by analysing the frequency distribution of resistance of the cell populations. In fact, a lower average inactivation time of cell and variance was observed when model juice was acidified with malic acid compared to citric acid.

Furthermore once cells were acid-adapted, the presence of citric acid didn't affect anymore cells sensitivity, an increase in the average inactivation time was rather noticed.

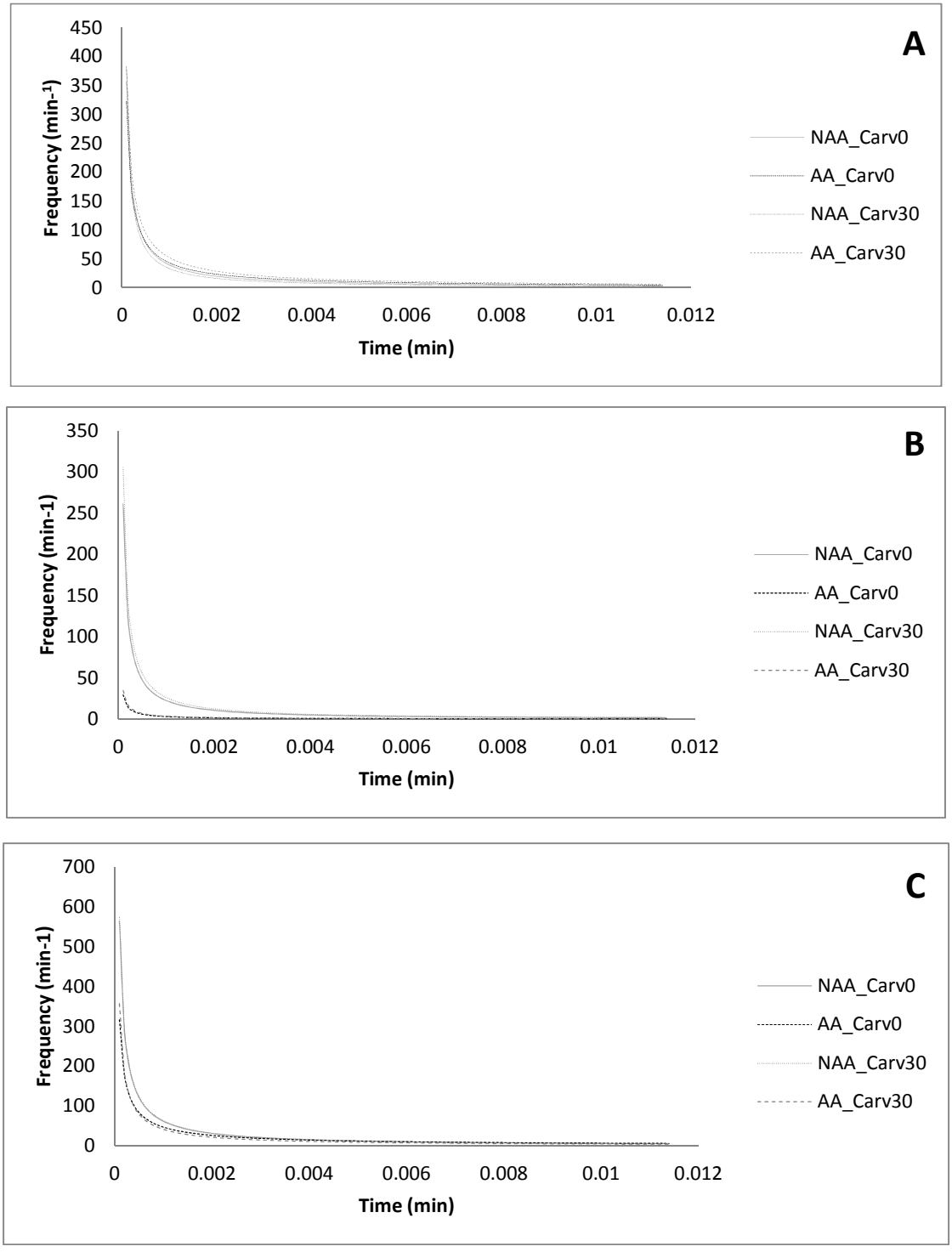

Fig. 3. Frequency distribution of resistance of acid-adapted (AA) and non acid-adapted cells (NAA) during the thermal treatment in the simulated juice with pH adjusted with hydrochloric (A), citric (B) and malic (C) acids, supplemented (carv30) or not (carv0) with carvacrol

Table 2. Statistical parameters of frequency distribution of resistance in the different tested conditions

Treatment medium			Cells treated	b'	n'	Mode	Mean	Variance	Skewness
pH-Brix	pH* adjusted with	Carvacrol (µL/L)							
4.5-12	HCl	0	Control	2.01	0.11	-	1114.33	9.28E+10	0.53
		30	Control	2.87	0.10	-	123.78	3.75E+09	7742.25
		0	Acid-adapted	1.17	0.18	-	156.77	1.43E+07	1.20E-03
		30	Acid-adapted	1.53	0.20	-	16.86	82900.25	0.14
	CA	0	Control	3.50	0.08	-	173.44	1.0169E+11	8.45E+06
		30	Control	3.68	0.09	-	17.80	1.57E+08	1.57E+07
		0	Acid-adapted	1.40	0.01	-	5.221E+182	-	-
		30	Acid-adapted	1.38	0.01	-	1.28E+149	-	-
	MA	0	Control	2.96	0.17	-	1.02	900.41	2635.63
		30	Control	3.02	0.17	-	0.82	485.83	3184.61
		0	Acid-adapted	1.29	0.22	-	18.47	49193.25	0.03
		30	Acid-adapted	1.39	0.13	-	2002.63	3.86E+10	4.99E-04

**HCl, hydrochloric acid; CA, citric acid; MA, malic acid*

This suggests that citric acid was no more able to enter in the acid-adapted cells membrane. This absence of activity of citric acid on acid-adapted cells could also be appreciated through the skewness observed. Indeed, the treatment of control cells in model juice acidified with citric acid showed a high skew, which demonstrates a high heterogeneity of the thermal tolerance of the population [21]. In contrast, a lack of skew was noticed with acid-adapted cells in this citric acidified model juice, which rather suggests homogeneity of the thermal tolerance of cell population which had greatly increased as shown by the average inactivation time.

The antimicrobial activity of carvacrol helped in decreasing the average inactivation times of control and acid-adapted cells and their variances. The particularity observed with acid-adapted cells, in model juice acidified with malic acid where the presence of carvacrol rather increased those parameters, suggests a possible antagonism between malic acid and carvacrol. Indeed, alone, both these antimicrobials rather decreased average inactivation time of acid-adapted cells and its variance. However, this statistical antagonism could be minimised since in that condition, the inactivation kinetics and inactivation scales in presence and absence of carvacrol were almost similar. Moreover, no information on this possible antagonism has been found in literature. Further researches need to be done before confirming it.

5. CONCLUSION

A previous acid adaptation of a pathogen like *L. monocytogenes* could deeply limit its inactivation at mild temperature, even when a natural antimicrobial is supplemented. This parameter should therefore be taken into consideration while studying the antimicrobial efficiency of such combined low thermal processing of fruit juice.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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