Journal of Advances in Microbiology

11(3): 1-8, 2018; Article no.JAMB.42424
ISSN: 2456-7116

Antimicrobial Potential of Carvacrol and Its Effect at Sub-lethal Concentration during Low Thermal Pasteurization of Fruit Juices

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Authors' contributions

This work was carried out in collaboration between all authors. All members contributed in designing the study. Authors AT and CP wrote the protocol and managed the analyses of the study. Author SSK performed the statistical analysis together with author AT who wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2018/42424
Editor(s):
(1) Dr. Niranjala Perera, Department of Food Science & Technology, Wayamba University of Sri Lanka, Sri Lanka.
Reviewers:
(1) Mariana-Atena Poiana, Banat's University of Agricultural Sciences and Veterinary Medicine, Romania.
(2) Adriana Pavelková, Slovak University of Agriculture in Nitra, Slovakia.
(3) Mehtap Akın, Selçuk University, Turkey.
(4) Azza Anwar Abou-Arab, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/25584

Original Research Article

Received 29th April 2018
Accepted 9th July 2018
Published 17th July 2018

ABSTRACT

Aims: This study aimed at assessing the relationship between the initial antimicrobial activity of carvacrol and its effect at sub-lethal concentration when used for low thermal treatment of fruit juices.
Place and Duration of Study: Faculty of Food Science and Technology, University of Agricultural Science and Veterinary Medicine of Cluj-Napoca for a period of 5 months.
Methodology: The antimicrobial potential of carvacrol on *Escherichia coli* ATCC 25922; *Listeria monocytogenes* 56 LY and *Zygosaccharomyces bailii* was evaluated through the macrodilution

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method. The survival of these two latter strains grown in different acid conditions was also assessed after a treatment at 55°C in pineapple, orange and watermelon juices supplemented or not with carvacrol (30 µL/L) as performed in a previous study for *E. coli*. For each strain, the comparative length of treatment was the time required to reduce non-acid adapted cells in watermelon juice supplemented with carvacrol by 99.9%.

Results: Carvacrol exhibited a MBC value of 200 µL/L on *E.coli*; 1900 µL/L on *L. monocytogenes*, and a MFC value of 500 µL/L on *Z. bailii*. A prior growth of these microorganisms in acid conditions globally led to an enhancement of their survival ratio to heat treatment. *Z. bailii* inactivation was affected by the nature of the treated juice (higher in pineapple juice followed by orange juice). Supplementation of carvacrol at 30 µL/L had a positive impact on microorganisms thermal inactivation but with strength which appears to be inversely proportional to its MBC or MFC on the target strain (R^2=0.98).

Conclusion: The choice of the natural aroma compound to use for such combined treatment of fruit juices should be made based on its antimicrobial potential on the contaminant microorganisms.

Keywords: Carvacrol; antimicrobial activity; fruit juices; mild heat; combined treatment; safety.

1. INTRODUCTION

Heat treatment is the most frequently used method of ensuring the safety and stability of fruit juices. It is generally performed at temperatures equal to or higher than 80°C and often has a negative impact on the nutritional and sensorial properties of the fruit juices depending on the length of treatment. Furthermore, it requires high energy consumption, an energy which is not always available, especially in developing countries. Alternatives such as irradiation, high hydrostatic pressure, pulsed electric fields and ultraviolet treatment have been developed [1,2,3,4]. Unfortunately, the high initial investment required to acquire the technical equipment has limited their widespread use [5].

Mild thermal treatment of fruit juices in combination with natural aroma compounds has been intensively studied during the last decade as another potential alternative. For instance, Belletti et al. [6] described the use of mild thermal treatment (55°C) in combination with citral, (E)-2-hexenal and citron essential oil to inactivate *Saccharomyces cerevisiae* in soft drinks. Char et al. [7] found that supplementation of orange juice with vanillin greatly increased the inactivation of *Listeria innocua* at temperatures between 57 and 61°C. Belletti et al. [8] also described the use of mild heat treatment (55°C/15 min) in combination with low essential oils components for the inactivation of *S. cerevisiae* in citrus based beverages. Sado et al. [9] observed that the combination of carvacrol, (E)-2-hexenal and citral was a way of enhancing the mild heat inactivation of *L. monocytogenes*. As shown by Essia et al. [10], the use of *Eryngium foetidum* essential oil in combination with mild heat was also effective for the inactivation of *L. monocytogenes* in pineapple juice.

The possibility of a previous growth of microorganisms in an acidic environment was not taken into consideration in those studies. This acid adaptation phenomenon has been demonstrated yet to enhance microbial thermal tolerance [11,12,13,14]. In a previous work [15], we observed that supplementation of carvacrol (major compound of *Origanum* spp. essential oil) in fruit juices at a consumers' accepted concentration was helpful in reducing the survival ratio of acid-adapted cells of *E. coli*. This study aimed at assessing the antimicrobial efficiency of such combined process on other acid-adapted microorganisms using the same experimental approach, this in order to evaluate the relationship between the initial antimicrobial activity of carvacrol (Fig. 1) on the target strain and its efficiency at sub-lethal concentration during the treatment.

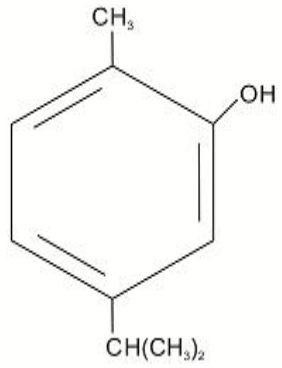

Fig. 1. Chemical structure of carvacrol (2-methyl-5-isopropylphenol)

2. MATERIALS AND METHODS

2.1 Microorganism Strains

Three microorganisms provided by the Food Microbiology Laboratory of the University of Bologna (Cesena, Italy) were used in this study: *Escherichia coli* ATCC 25922; *Listeria monocytogenes* 56 LY and *Zygosaccharomyces bailii*. They were sub-cultured thrice in Nutrient Broth (CM0001, Oxoid Ltd., Basingstoke, UK) at 37°C before being used.

2.2 Study of the Antimicrobial Activity of Carvacrol on the Studied Strains

The antimicrobial activity of carvacrol (natural, 99%, Food Grade, Sigma-Aldrich, St. Louis, USA) on each of the studied strains was determined using the macro-dilution method [16,17]. Tubes containing Brain Heart Infusion (BHI, Fluka 53286, Sigma-Aldrich, St. Louis, MO) at different concentrations of carvacrol (with a gap of 100 µL/L between the tubes) were inoculated with the tested microorganism for an initial concentration of 6 Log cell/mL. The final volume in the tubes was 10 mL (9.8 mL of BHI + 0.1 mL of carvacrol ethanolic solution + 0.1 mL microorganism culture dilution). After 24h of incubation at 37°C, the lowest concentration of carvacrol which prevented any visible growth was considered as the Minimal Inhibitory Concentration (MIC). One mL from tubes not showing growth was then sub-cultured in tubes containing 9 mL of BHI which were incubated at 37°C for 24 hours. The lowest concentration of carvacrol which prevented any visible growth was considered as the Minimal Bactericidal Concentration (MBC) for bacteria or Minimal Fungicidal Concentration (MFC) for the yeast.

2.3 Heat Treatment of Microorganisms in Fruit Juices Supplemented with Carvacrol at Consumers Tolerated Concentration

The responses of *L. monocytogenes* and *Z. bailii* cell cultures to a mild thermal treatment in autoclaved pineapple (pH 3.25; Brix 12), orange (pH 3.61; Brix 8.9) and watermelon (pH 5.4; Brix 6.8) juices supplemented or not with carvacrol were assessed using the same experimental approach and protocol as were used for *E. coli* in a previous study [15].

Microorganisms were first cultured at 37°C for 24h in nutrient broth adjusted at pH values, 6.5, 6, 5.5, 5, 4.5 and 4 with hydrochloric (35-38%, POCH SA, Gliwice, Poland), citric (≥99.5%, Food Grade, Sigma-Aldrich, St. Louis, USA) or malic (≥99.5% purity, MERCK, Darmstadt, Germany) acid. Besides, two additional growth media were prepared consisting of nutrient broth supplemented or not with glucose (1% W/V) and with a pH value adjusted to 7 using hydrochloric acid (0.1N) and sodium hydroxide (0.1N). The condition at pH7 without glucose supplementation was used as control to obtain non-acid-adapted cells.

Preliminary culture conditions in which the pH value did not exhibit a strong inhibitory effect on the growth of the strain were selected for subsequent thermal treatment. Inactivation levels were assessed after a heat treatment at 55°C for 12 minutes for *Z. bailii* or 30 minutes for *L. monocytogenes*, in triplicate samples of each fruit juice supplemented or not with carvacrol at a consumers' tolerated concentration of 30µL/L [15]. For each strain, the selected length of treatment was the time needed at 55°C to reduce non-acid-adapted cells in watermelon juice supplemented with carvacrol by 99.9% as estimated in a preliminary experiment.

2.4 Statistical Analysis

Statistical analyses were performed using Statistica.10 software of Statsoft. A one-way ANOVA (Fisher LSD Post hoc test) analysis was performed to study the significant differences between the mean values obtained. A covariance analysis (ANCOVA) was also performed to better appreciate the effect of carvacrol on microbial thermal inactivation.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Activity of Carvacrol

Carvacrol showed 100µL/L as MIC value on all the three studied strains. However, its microbicidal concentration varied from one microorganism to another. Indeed, its MBC value was 200 µL/L on *E.coli*, 1900 µL/L on *L. monocytogenes,* and its MFC 500 µL/L on *Z. bailii*. This compound is known for its strong and wide antimicrobial activity. It first targets the cell membrane, where it increases fluidity and permeability. The possibility of an interaction with membrane proteins and periplasmic enzymes has also been suggested [18,19]. The observed concentrations required for its antimicrobial activity on our *E. coli* and *Z. bailii* strains were

found to be near those reported by Xu et al. [20] and Rivera-Carriles et al. [21] who observed a MIC of 200 mg/L on *E. coli* and below 250 ppm on *Z. bailii*, respectively. In contrast, the MBC value obtained with *L. monocytogenes* (1900 µL/L) was far higher than that obtained by Sado et al. [9] (300 mg/L) on the same strain and with the same initial cell concentration. The difference in the subculture growth medium used (nutrient broth in our case and BHI in their case) might explain this disparity. Indeed, this microorganism produces biofilm during its growth, in quantities increasing with the nutrient content of the culture medium [22]. In comparison to BHI, nutrient broth is a less suitable growth media for *L. monocytogenes*. The growth rate inside is much slower [23]. This slow growth rate of biofilm cells has been reported to protect the cells from antimicrobial action [24].

3.2 Growth of the Microbial Strains in Acidic Environments

Table 1 presents *L. monocytogenes* cells concentrations obtained after incubation in the different growth media, as well as the change of the pH value induced by this growth. The highest cell concentration was observed in presence of malic acid at pH≥6. This cell growth was strongly inhibited in all the conditions with pH≤5. It also appeared that cell concentration in condition with glucose was lower than in its absence. This was probably due to the growth inhibition as soon as the pH decreased to 5 due to glucose fermentation.

Based on these results and those obtained with *Z. bailii* in a previous study [25], the selected conditions for subsequent thermal treatment were those with initial pH values of 6.5 and 5.5 for *L. monocytogenes*, and 6 and 5 for *Z. bailii*, this together with cells grown at pH 7.

3.3 Survival Ratio of Produced Cells to Combined Treatment in Fruit Juices

Table 2 presents the levels of inactivation of *L. monocytogenes* cells after the heat treatment. In conditions without carvacrol, a previous acid-adaptation of cells led to an inactivation which was statistically lower or similar to that of control cells. Indeed, acid adaptation has been reported as enhancing microorganisms' heat tolerance by decreasing the ratio of unsaturated fatty acids to saturated fatty acids (UFA/SFA) in the membrane [11,26]. In this study, an exception was noticed with cells grown in the presence of glucose which were observed to have a significantly higher inactivation. This may be the result of the stress observed during cell

Table 1. Change of pH and cell concentration of *L. monoctyogenes* after 24 hours of incubation at 37°C of 6 log cell/mL in the growth media

Initial growth conditions		pH after 24 hours	Cell concentration after 24 hours (Log cell/mL)
Medium	**Initial pH**		
Nutrient broth + Glucose + HCl	7	4.99 ± 0.15	7.1 ± 0.1 d
Nutrient broth + HCl	7	5.83 ± 0.03	7.6 ± 0.1 bc
Nutrient broth + HCl	6.5	5.69 ± 0.04	7.7 ± 0.1 abc
	6	5.55 ± 0.02	7.7 ± 0.1 ab
	5.5	5.35 ± 0.05	7.5 ± 0.0 bc
	5	5.04 ± 0.03	6.5 ± 0.1 e
	4.5	4.62 ± 0.02	6.2 ± 0.1 f
	4	4.12 ± 0.04	6.3 ± 0.1 f
Nutrient broth + Citric acid	6.5	5.91 ± 0.01	7.6 ± 0.1 abc
	6	5.81 ± 0.06	7.7 ± 0.0 abc
	5.5	5.43 ± 0.06	7.4 ± 0.1 c
	5	5.22 ± 0.04	6.2 ± 0.1 f
	4.5	4.57 ± 0.05	6.2 ± 0.1 f
	4	4.11 ± 0.02	6.2 ± 0.0 f
Nutrient broth + Malic acid	6.5	5.78 ± 0.04	7.9 ± 0.2 a
	6	5.81 ± 0.08	7.9 ± 0.1 a
	5.5	5.50 ± 0.07	7.5 ± 0.1 abc
	5	5.06 ± 0.02	6.2 ± 0.0 f
	4.5	4.59 ± 0.03	6.2 ± 0.0 f
	4	4.07 ± 0.05	6.2 ± 0.0 f

**Mean cell concentrations that are not followed by the same letter are significantly different (P ≤ 0.05)*

Table 2. Inactivation levels of *L. monocytogenes* cells after 30 minutes of treatment at 55°C in fruit juices supplemented or not with carvacrol*

Cells growth medium**		Inactivation level (Log N/N_0)					
		Pineapple juice	Orange juice	Watermelon juice	Pineapple juice + [carvacrol]	Orange juice +[carvacrol]	Watermelon juice + [carvacrol]
Control	NB_7_H	ab -3.23 ± 0.83 a	bc -3.03 ± 0.67 a	ab -3.09 ± 0.30 a	ab -3.40 ± 1.18 a	ab -3.56 ± 0.80 a	bc -3.58 ± 0.36 a
Acid-adapted	NB+Glucose_7_H	a -3.98 ± 0.80 a	a -4.18 ± 0.52 a	a -4.19 ± 0.21 a	ab -3.63 ± 0.20 a	ab -3.83 ± 0.81 a	ab -4.21 ± 0.51 a
cells	NB_6.5_H	ab -3.43 ± 0.65 a	ab -3.87 ± 0.32 a	abcd -3.22 ± 0.00 a	ab -3.33 ± 0.78 a	ab -3.32 ± 1.00 a	bc -2.71 ± 0.00 a
	NB_5.5_H	abc -2.91 ± 0.25 a	abc -3.20 ± 0.49 a	abc -2.87 ± 0.75 a	ab -3.37 ± 0.35 a	ab -3.46 ± 0.24 a	bc -3.50 ± 0.27 a
	NB_6.5_C	c -1.98 ± 0.62 a	bc -2.81 ± 0.32 a	d -1.41 ± 1.09 a	b -2.75 ± 1.34 a	b -2.95 ± 1.05 a	c -2.92 ± 0.53 a
	NB_5.5_C	bc -2.43 ± 0.96 a	c -2.43 ± 0.73 a	bcd -1.65 ± 1.20 a	b -2.79 ± 0.69 a	ab -3.04 ± 0.61 a	c -2.38 ± 1.54 a
	NB_6.5_M	ab -3.24 ± 0.62 bc	bc -2.94 ± 1.11 c	abcd -2.65 ± 0.91 c	a -4.52 ± 0.00 ab	a -4.53 ± 0.42 ab	a -4.84 ± 0.13 a
	NB_5.5_M	bc -2.59 ± 0.20 bc	c -2.37 ± 0.31 c	cd -1.61 ± 0.46 d	ab -3.10 ± 0.45 ab	ab -3.43 ± 0.07 a	bc -3.47 ± 0.57 a

**Within the same column, inactivation levels not preceded by the same letter are significantly different ($P \leq 0.05$). Within the same row, inactivation levels not followed by the same letter are significantly different ($P \leq 0.05$)*

***NB_X_Y where NB is Nutrient Broth; X, the pH of the medium and Y, the acid used to adjust the pH (H, C, M are Hydrochloric, Citric and Malic acids, respectively)*

Table 3. Inactivation levels of *Z. bailii* cells after 12 minutes of treatment at 55°C in fruit juices supplemented or not with carvacrol*

Cells growth medium**		Inactivation level (Log N/N_0)					
		Pineapple juice	Orange juice	Watermelon juice	Pineapple juice + [carvacrol]	Orange juice + [carvacrol]	Watermelon juice +[carvacrol]
Control	NB_7_H	a -5.06 ± 0.56 a	a -4.25 ± 2.08 a	a -3.19 ± 0.84 a	ab -4.90 ± 0.70 a	a -4.46 ± 1.71 a	a -3.47 ± 1.23 a
Acid-adapted cells	NB+Glucose_7_H	a -4.73 ± 1.53 ab	a -3.22 ± 0.64 bc	b -0.36 ± 0.29 d	a -5.87 ± 0.51 a	a -4.78 ± 0.91 a	a -2.22 ± 0.51 c
	NB_6_H	a -3.64 ± 2.77 a	a -2.94 ± 0.11 ab	b -0.11 ± 0.12 b	c -3.13 ± 1.71 ab	a -3.29 ± 1.98 ab	a -2.00 ± 1.01 ab
	NB_5_H	a -4.49 ± 1.14 a	a -4.06 ± 0.26 a	b -0.77 ± 0.15 c	ab -4.79 ± 0.00 a	a -4.27 ± 0.07 a	a -1.80 ± 0.14 b
	NB_6_C	a -4.44 ± 0.00 a	a -4.48 ± 0.79 a	b -0.75 ± 0.29 c	ab -4.57 ± 0.46 a	a -4.93 ± 0.05 a	a -2.20 ± 0.00 b
	NB_5_C	a -3.63 ± 1.00 ab	a -3.64 ± 1.21 ab	b -0.22 ± 0.22 c	bc -4.37 ± 0.16 a	a -3.68 ± 1.48 ab	a -1.64 ± 2.23 bc
	NB_6_M	a -3.81 ± 1.51 a	a -3.56 ± 2.83 ab	b -0.51 ± 0.18 b	abc -4.37 ± 0.30 a	a -3.59 ± 1.56 a	a -3.21 ± 0.67 ab
	NB_5_M	a -4.10 ± 0.53 ab	a -2.36 ± 1.31 abc	b -0.20 ± 0.14 c	b -4.49 ± 0.80 a	a -3.83 ± 1.69 ab	a -1.88 ± 2.06 bc

**Within the same column, inactivation levels not preceded by the same letter are significantly different ($P \leq 0.05$). Within the same row, inactivation levels not followed by the same letter are significantly different ($P \leq 0.05$).*

***NB_X_Y where NB is Nutrient Broth; X, the pH of the medium and Y, the acid used to adjust the pH (H, C, M are Hydrochloric, Citric and Malic acids, respectively)*

Table 4. Impact of carvacrol on the inactivation levels observed*

Strain	Log N/N_0 SS	F	P	R^2
L. monocytogenes	9.79	18.63	0.00	0.48
Z. bailii	19.15	14.46	0.00	0.63
*E. coli***	90.18	97.54	0.00	0.78

**Log N/N_0 SS (Sum of Squares) characterises the impact of carvacrol on strain inactivation; P, P value ($P \leq 0.05$ suggests a significant effect on the strain inactivation); F, Fisher; R^2, coefficient of determination.*
***Data from Tchuenchieu et al. (2018)*

culture in that condition as it is suggested that a combination of stresses reduces heat tolerance [27]. Comparing the survival ratios in the three fruit juices, it can be observed that they were globally the same. The higher acidity of pineapple and orange juices therefore did not have an impact on this strain inactivation. The supplementation of juices with carvacrol significantly enhanced the inactivation of only the cells which were previously cultured with malic acid. Cells grown in the presence of this acid at pH 6.5 were even the most inactivated cells in the presence of carvacrol. This contributes to the hypothesis developed above suggesting that this strain is most sensitive to carvacrol after growth in suitable condition. In fact, the growth of this microorganism was rather stimulated in presence of malic acid compared to the other acid culture conditions at the same pH values.

Table 3 presents the levels of inactivation of *Z. bailii* cells in the juices after 12 minutes of treatment at 55°C. In conditions without carvacrol, It was noted that a previous growth of this microorganism in acidic conditions significantly reduced its inactivation in watermelon juice. This was not the case in orange and pineapple juices where the survival ratio was not significantly affected by the growth condition of the strain. The higher content of pineapple and orange juices in organic acids like citric and malic acids may therefore have acted as another hurdle due to their antimicrobial properties [28,29], therefore making cells more sensitive to heat treatment. The levels of inactivation in the three juices were statistically similar for nonacid-adapted cells but different for those adapted. The inactivation of these latter tended to be higher in pineapple juice followed by orange juice. This observation had already been made with *E. coli* [15] and shows that juices with low pH values may be more suitable for this combined process. A positive effect of carvacrol supplementation on *Z. bailii* inactivation was noticed only in watermelon juice. The absence of a visible effect in the other juices suggests that the effect of organic acids reported above affected the population cell fraction which was also targeted by carvacrol. In fact, within a microbial population, cells do not have the same resistance to heat treatment. That's why Peleg & Cole [30] considered microbial inactivation kinetics as a cumulative distribution of resistances.

3.4 Quantification of the Effect of Carvacrol during Heat Treatment

Table 4 gives an idea of the impact of carvacrol at the tested concentration (30 µL/L) on the microbial inactivation observed, quantification obtained through the hypothesis decomposition analysis of our raw data. The supplementation of carvacrol to juices had a lower effect on heat inactivation of *L. monocytogenes* than on *Z. bailii*. A greater effect had already been observed in our previous study with *E. coli* [15].

This order of importance in the inactivation is the same as that obtained while assessing the lethal concentration of this compound on each of the three strains (200 µL/L for *E. coli*, 500 µL/L for *Z. bailii* and 1900 µL/L for *L. monocytogenes*). A linear correlation could therefore be made between this effect of carvacrol during heat treatment and the inverse of their MBC or MFC value on the corresponding strain (R^2=0.98).

4. CONCLUSION

Supplementation of fruit juices with natural aroma compounds is helpful in reducing the mild thermal tolerance of acid-adapted microorganisms. The efficiency of this combined process depends on the strength of the antimicrobial effect of the compounds used on the target microorganisms present in the juice processed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Available:http://doi.org/10.1080/10408699891274246

Peer-review history:
The peer review history for this paper can be accessed here:
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