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Response of *Bacillus cereus* to γ-Irradiation in Combination with Carvacrol or Mild Heat Treatment

Samia Ayari,[†,‡] Dominic Dussault,[§] Mathieu Millette,[§,∥] Mokhtar Hamdi,[‡] and Monique Lacroix*[,§]

[†]National Center for Nuclear Sciences and Technologies (CNSTN), Tunis Cedex, 2020, Tunisia, [‡]National Institute for Applied Sciences and Technologies (INSAT), B.P. 676, Tunis Cedex, 1080, Tunisia, [§]Research Laboratory in Sciences Applied to Food, Canadian Irradiation Center, INRS-Institut Armand-Frappier, 531 Boulevard des Prairies, Laval, Quebec H7V 1B7, Canada, and [∥]Bio-K+ International Inc., 495, Boulevard Armand-Frappier, Laval, Quebec H7V 4B3, Canada

Carvacrol and mild heat treatment were tested for their efficiency to increase the radiosensitivity of *Bacillus cereus* in broth. The bacterium was treated with γ-irradiation alone or in combination with carvacrol at its minimal inhibitory concentration or mild heat treatment for 10 min at 45 °C. The effects of this combination of treatments were studied on various parameters: the bacterial viability, the modifications of the cell morphology with scanning electron microscopy (SEM), the cellular fatty acids composition of the membrane quantified by gas chromatography, the intracellular and extracellular adenosine 5′-triphosphate (ATP) concentrations, and the DNA degradation. Combined treatments resulted in additive or synergistic effects as compared to γ-irradiation alone. A significant modification ($P \leq 0.05$) of the fatty acid composition and unsaturation ratios was observed. Pretreatment with mild heat or carvacrol before irradiation disturbed the membrane integrity of *B. cereus* and induced a significant decrease ($P \leq 0.05$) of the intracellular ATP concentration. SEM observations revealed that the cell membrane was more severely affected with combined treatment than irradiation alone. The electrophoresis analysis showed that DNA degradation by combined treatments was greater than the γ-irradiation alone.

KEYWORDS: γ-Irradiation; *Bacillus cereus*; carvacrol; mild heat treatment; combined treatment

INTRODUCTION

Bacillus cereus is a spore-forming food-borne pathogen, facultative anaerobe, and Gram-positive rod. Many authors have widely documented the presence of *B. cereus* in raw and processed meat, vegetables, rice, and dairy products (*1*−*4*). *B. cereus* is associated with two kinds of food-borne illnesses: diarrheal and emetic types, caused by two distinct toxins (*5*). Because of its capacity to secrete these toxins, *B. cereus* is becoming one of the most important causes of food poisoning in industrialized countries (*6*).

Several food preservation systems such as heating, refrigeration, and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of *B. cereus* food poisoning. Current trends toward improvement in safety, quality, and convenience of foods and saving energy in food processing and distribution have increased the interest in developing new combinations of methods for food preservation. A combination of radiation treatment with other preservative agents also seems to be of potential importance in enhancing the effectiveness and reducing the energy or dose requirement of food preservation while retaining/improving product quality.

*To whom correspondence should be addressed. Tel: +1 450 687 5010, ext. 4489. Fax: +1 450 687 5792. E-mail: Monique.lacroix@iaf.inrs.ca.

Processing at high temperatures is often detrimental to product quality as it causes a significant reduction in nutritional value and changes in organoleptic properties (*7*). Therefore, less severe heat application during processing is desirable. Several investigations have demonstrated, however, the usefulness of mild heat treatment prior to low-dose irradiation in extending the shelf life of certain fresh fruits and cereal products without affecting their normal quality attribute (*8*). The usefulness of mild heat treatment prior to low-dose irradiation has been demonstrated for the preservation of fruit juices and some other processed fruit products and for inactivation of toxigenic molds on nuts, dried fruits, cocoa beans, and maize. Much research has also been carried out on the antimicrobial effects of heat in combination with modified environments such as low A_w, pH (*9*), and ultrahigh pressure up to 200 MPa (*10*).

The utilization of plant essential oils is a novel way to preserve foods. The antifungal and antibacterial effects of these volatile oils toward different microorganisms have been described in several studies (*11*−*15*). Herbs and spices have been known for their antimicrobial activity since antiquity. The safe use of herbs and spices and their components (such as thymol and cymene) has led to their current status of Generally Recognized as Safe (GRAS) food ingredients. By example, carvacrol is an antimicrobial compound present in the essential oil fraction of oregano. Carvacrol is the major component of the essential oil

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fraction of oregano (60−74%) and thyme (45%) (*16*, *17*). Much research is devoted to the antimicrobial activity of carvacrol and its mechanism of action (*15*, *18*), but little has been done on the combination of γ-irradiation with this natural antimicrobial.

The bacterial membrane could be identified as an important target of applied treatment combinations. The lipid composition of microorganisms can display considerable variations with changes in chemical and physical properties of the environment (*19*, *20*). These modifications of membrane lipids are important in maintaining membrane fluidity, integrity, and functionality in the face of external perturbations (*21*). Such changes in microbial lipid composition may result in membranes with altered physical characteristics and may be important in controlling the ionic permeability (*22*).

The main objective of this study was to assess whether combining less severe single treatments caused inhibitory effects on *B. cereus* cells and was strong enough to substitute efficiently for the more extreme single measures, which usually resulted in degradation of food quality. An attempt was also made to elucidate some elements of the underlying physiological mechanisms of combined treatment effects by determining the changes in growth, fatty acid composition, cell membrane morphology, adenosine 5′-triphosphate (ATP) concentration, and DNA degradation. To our knowledge, no one has described the combined effects of irradiation with mild heat treatment or carvacrol treatment on the growth inhibition of *B. cereus*. In addition, we are unaware of reports demonstrating how these agents may act together to inactivate the bacterium. Therefore, the present study was designed to define the interaction between these treatments and to elucidate how they may influence the cell membrane of *B. cereus*.

MATERIAL AND METHODS

Bacterial Strain and Growth Conditions. *B. cereus* strain LSPQ 2872 (Laboratoire de Santé Publique du Québec, Canada) was used throughout this study. Cells were grown at 30 °C in brain heart infusion (BHI) medium (Difco Laboratories, Sparks, MD) supplemented with 0.5% (w/v) glucose. The bacterium was stored at −80 °C in BHI containing 25% glycerol as a cryoprotectant. Prior to the experiment, 1 mL of culture was inoculated in 9 mL of BHI and incubated through two successive cycles at 30 °C. Preceding the treatment procedures, the working culture was diluted 100 times in fresh BHI supplemented with 0.5% (w/v) glucose and incubated overnight at 30 °C with agitation at 150 rpm.

Determination of Minimal Inhibitory Concentration (MIC) Value of Carvacrol. Carvacrol was obtained from Sigma Aldrich Chemicals (St. Louis, MO). Carvacrol was emulsified in sterile, distilled water, and sterile Tween 80 (Laboratoire Mat Inc., Quebec, Canada) was added (1% as highest final concentration) to stabilize the emulsion. A stock solution was stored at 4 °C. The antimicrobial activity was determined using a modified critical dilution assay (*23*) using 96-well microtiter plates. The first well contained 58 μL of the stock solution of carvacrol and was diluted in Mueller Hinton broth (MH; Oxoid, England). The range of the initial concentration was 5000 ppm. The remainder of the wells was filled with 100 μL of the MH broth. The first wells of each row were then diluted 2-fold along each column. Finally, 15 μL of bacterial culture (10^7 CFU/mL) was added to all of the wells to obtain 10^6 CFU/mL in each well. All analyses were conducted in triplicate, and two columns per plate were used for positive and negative controls. Positive controls (growth controls) containing MH broth and Tween 80 were inoculated with *B. cereus*. Negative controls (sterility controls) contained carvacrol, Tween 80, and MH broth. After 18 h of incubation at 30 °C, the absorbance readings were taken at 570 nm using a microplate reader (Biorad model 450, Mississauga, Canada). The MIC was defined as the lowest concentration of carvacrol required to completely inhibit the growth of *B. cereus*.

Antibacterial Treatment Procedures. Preliminary tests were conducted to determine the lethal and sublethal doses of irradiation for *B. cereus* LSPQ 2872 in BHI broth supplemented with 0.5% (w/v) glucose. The lethal dose was defined as the dose required that caused cell death to the whole bacterial population and at which no growth was observed on BHI agar after 24 h of incubation at 30 °C. To create cell damage, a 1.2 kGy was selected as a sublethal ionizing radiation dose. Immediately before applying the irradiation treatment, bacterial cultures were divided in three parts: The first part of the bacterial cells was separately irradiated with 1.2 kGy and with 3.6 kGy. The second part of the cell suspension was exposed to a mild heat treatment at 45 °C for 10 min and subsequently irradiated at 1.2 kGy. The third part of the cells was exposed to carvacrol at 312 ppm (MIC) final concentration for 10 min at 30 °C and subsequently irradiated at 1.2 kGy. Mild heat treatment was performed using a shaking water bath. Immediately following the treatments, the samples were enumerated for colony-forming units (CFU) by preparing serial dilutions in peptone water (0.1%, w/v; Difco Laboratories), pour-plating in BHI agar, and incubating at 30 °C for 24 h. Seven treatments were studied as follows: (1) untreated, treated with (2) 1.2 and (3) 3.6 kGy radiations doses, (4) treated with carvacrol at the MIC, (5) treated with carvacrol in combination with sublethal γ-irradiation (1.2 kGy), (6) treated with a mild heat treatment (45 °C for 10 min), and (7) preheated (45 °C for 10 min) and then irradiated at 1.2 kGy.

Irradiation Treatment. The γ-irradiation treatment was performed using an underwater irradiator UC-15A (MDS Nordion International Inc., Kanata, Ontario, Canada) equipped with a ^{60}Co source at a dose rate of 15.6 kGy/h. This irradiator was certified by the National Institute of Standards and Technology (Gaithersburg, MD), and the dose rate was established using a correction for decay of the source. Amber Perpex 3042D (Atomic Energy Research Establishment, Harwell, Oxfordshire, United Kingdom) was used to validate the dose distributions. The irradiation treatment was carried out at the Canadian Irradiation Center (Laval, Québec, Canada) at room temperature (20 °C).

D_{10} Determination. For the D_{10} determination, a volume of 5 mL of bacterial culture was irradiated with various doses ranging from 0.25 to 1.25 kGy. Immediately after irradiation, serial dilutions were prepared in sterile peptone water (0.1%, w/v), and appropriate dilutions were pour-plated in BHI agar and incubated at 30 °C during 24 h. The D_{10} value was defined as the dose required to inactivate 1 $\log_{10}$ cycle of the initial population and was calculated as the reciprocal of the slope of the inactivation curve.

Lipid Extraction. The total lipid extraction procedure was done as described by Evans et al. (*24*) with slight modification. The bacterial cultures were centrifuged at 2500*g* for 15 min, and the resulting bacterial pellet was washed twice using NaCl (0.85%, w/v). The pellet was then resuspended in 5 mL of sterile distilled water, and 5 mL of methanol/chloroform (2:1, v/v) was added. The mixture was shaken for 1 min on a mechanical vortexer and left for 2 h at room temperature. Thereafter, the mixture was centrifuged at 2500*g* for 5 min. The supernatant (SN1) was retained by decantation. The pellet was resuspended in 12 mL of methanol/chloroform/water (2:1:0.8; v/v/v), and the extraction was operated as described previously for SN1 to obtain supernatant 2 (SN2). The supernatants (SN1 and SN2) were combined, and a volume of 7 mL of chloroform/water (1:1) was added. The mixture was allowed to separate in two phases, and the lower phase containing the lipids was harvested. The chloroform was then evaporated under a stream of nitrogen, and the resulting precipitate was resuspended in hexane. The resulting solution was stored at −20 °C until further analysis.

Methylation of Fatty Acid. Lipid samples were trans-methylated for analysis of their acyl groups as fatty acid methyl ester (FAMEs) forms by gas chromatography. The samples of total lipid extract were trans-methylated as previously described by Ayari et al. (*25*). The samples of total lipid extract were evaporated under a stream of nitrogen gas to remove chloroform. The dry extracts were resuspended in 1 mL of hexane, and to these was added 2 mL of boron trifluoride (BF_3) in methanol (14%, v/v) containing 250 mg of sodium sulfate. The tubes were capped tightly and heated at 60 °C for 10 min. A volume of 1 mL of Milli-Q water and 1 mL of hexane was then added. The upper phase was retained and dried under a stream of nitrogen. The fatty acid extracts were resuspended in a minimal volume of hexane. The samples were stored at −20 °C until further analysis.

Gas−Liquid Chromatography. Analysis of FAMEs was performed using a Varian gas chromatograph (model 3400, Varian Inc., Palo Alto, CA) equipped with a flame ionization detector and a capillary column of

fused silica Equity-1 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Supelco, Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). A volume of 1 μL of sample was injected using the following operation temperature program: injector at 280 °C; detector at 290 °C; initial oven at 70 °C for 1 min; ramp 1, 15 °C min^{-1} to 170 °C; ramp 2, 10 °C min^{-1} to 270 °C and hold for 15 min. The FAME peaks were identified by comparison to those of a standard FAME solution (Supelco, 37 component FAME standards). The peaks were integrated with the Varian Star Chromatography Workstation version 5 (Varian Inc.). The areas of the peaks of interest were added together, and individual peaks were expressed as a percentage of this total.

Scanning Electron Microscopy Finite Element Method (SEM-FEM). After the treatments, cells were fixed with glutaraldehyde (2.5%) (Sigma Chemical), attached on a glass cover slide with poly-L-lysine (0.1%) (Sigma) for 1 h, and then dehydrated in water−alcohol solutions at various alcohol concentrations (30, 50, 70, 80, 90, and 100%) and in amyl acetate−alcohol solutions (30, 50, 70, and 100%) for 10 min each. After that, samples were dehydrated with a critical point dryer (EMS 850, Electron Microscopy Sciences, Hatfield, PA) at 42 °C and 1300 psi. Samples were fixed on a SEM support and sprayed with Au−Pd prior to observation using a field emission gun SEM (FEG-SEM, Hitachi S-4700, Tokyo, Japan).

Determination of Intra- and Extracellular ATP Concentrations. The intra- and extracellular ATP concentrations were measured according to the method described by Lee et al. (*26*). Cells from an overnight culture of *B. cereus* containing approximately 10^9 CFU/mL were divided into seven different groups. Immediately after treatments, cell suspensions were centrifuged for 10 min at 4000*g*. The cell pellets were washed three times in 0.1 M sodium phosphate buffer (pH 7) and then collected by centrifugation under the same conditions. Cell suspensions were prepared with 2 mL of sodium phosphate buffer (0.1 M, pH 7), and the subsamples were placed into 1.5 mL tubes and incubated in ice to prevent ATP loss until measurement. The extracellular (upper layer) and the intracellular (lower layer) ATP concentrations were measured using an ATP assay kit (Calbiochem, EMD Biosciences Inc., San Diego, CA). The ATP concentration of the supernatants (extracellular ATP) was determined using a luminometer (EG&G Berthold Lumat model LB 9507-2, Mandel Scientific Co. Inc., Guelph, ON, Canada) after the addition of 100 μL of nucleotide releasing buffer to 10 μL of supernatant and 1 μL of luciferase (1 mg/mL). The ATP concentration in the cell pellets (intracellular ATP) was determined by washing the cell pellets with 0.85% (w/v) sodium chloride solution and removing the supernatant. The resulting cell pellets were incubated in 20 μL of 5X passive lysis buffer (PLB; Promega, Madison, WI), at room temperature for 15 min, to disrupt the microorganisms, and then centrifuged at 8000*g* for 1 min. Subsequently, 1 μL of luciferase and 100 μL of nucleotide releasing buffer were added to 10 μL of resulting supernatants, and the ATP concentrations were measured after 1 min using a luminometer. To calculate the intra- and extracellular ATP concentrations, a standard ATP curve ranging from 0 to 10^4 ng/mL was used to obtain a linear relationship between ATP concentration (ng/mL) and the relative light unit, which resulted in an r^2 (coefficient of determination) value of 0.99.

DNA Extraction and Electrophoresis. The DNA extraction was performed according to a modified method described by Le Tien et al. (*27*). Untreated and treated cells of *B. cereus* were harvested by centrifugation at 2000*g* for 15 min, and cells were washed twice with TEN buffer (50 mM Tris-HCl, 100 mM EDTA, and 150 mM NaCl; pH 8.0). Afterward, the cells were suspended in the same buffer containing 10 mg/mL of freshly prepared lysosyme and incubated at 37 °C for 30 min. Complete lysis was obtained by the addition of 50 μL of 10% sodium dodecyl sulfate followed by several inversions of the microtube. An amount of 10 μL of RNase A (20 μg/mL final concentration) was added in the precipitate, to purify DNA from the RNA contamination, and incubated for 15 min at 37 °C. Proteins were denaturated by successive additions of phenol−chloroform−isoamylalcohol (1:1:1), and centrifugations were done until the interface was cleared of any cloudy material. The DNA was precipitated from the aqueous phase with 2 volumes of ethanol (−20 °C) and Na−acetate 10 N (1/4 of volume). The precipitate was harvested by centrifugation at 2000*g* for 15 min and washed with 70% ethanol. The final precipitate was dissolved in 100 μL of distilled water for the determination of the concentration and the purity of DNA using a nanodrop instrument (ND-1000 spectrophotometer). For the electrophoresis analysis, purified DNA extract was solubilized in 6X loading buffer (30% glycerol and 0.25% cyanol xylene), and DNA fragments were separated by migration on 1% agarose gel dissolved in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) in the presence of ethidium bromide (0.5 μg/mL). DNA kiloBase and Lambda-*Pst*1 were used as markers. Migration was done overnight at 30 V using an electrophoresis system (mini DNA SUB CELL, Bio-Rad, Mississauga, Canada). The DNA fragments were visualized under UV using a Gel Doc 1000 (Bio-Rad).

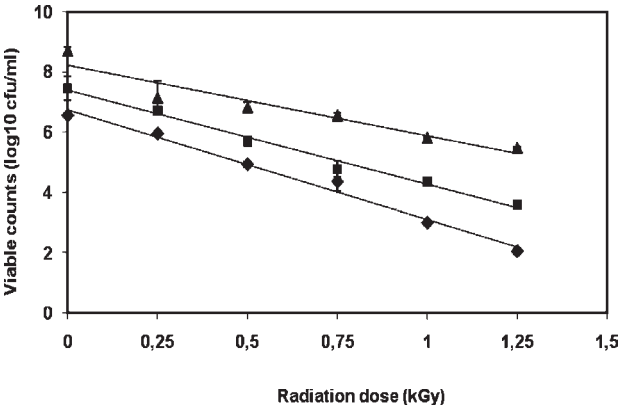

Figure 1. Inactivation curve by γ-irradiation for *B. cereus* LSPQ 2872. Control untreated cells (▲), cells treated with mild heat for 10 min at 45 °C (■), and cells treated with 312 ppm of carvacrol for 10 min at 30 °C (♦). The standard errors of the means are indicated by the error bars.

Statistical Analysis. An analysis of variance (one-way) and Duncan's multiple range tests were employed to determine the effect of γ-irradiation alone or in combination with mild heat or carvacrol treatment on fatty acid compositions and ATP concentrations. Calculations were performed using SPSS software Base 13.0 (SPSS, Inc., Chicago, IL). The data were obtained from two different replicates, and for each replicate, three samples were analyzed. Differences between means were considered significant at $P \leq 0.05$.

RESULTS

Relative Radiation Sensitivity. Figure 1 shows the radiation sensitivity of *B. cereus* after a mild heat or carvacrol treatment. This section was performed as a preliminary study to demonstrate the effect of mild heat for 10 min at 45 °C or carvacrol with MIC for 10 min at 30 °C on the relative radiation sensitivity (D_{10}) in comparison with control (untreated cells). D_{10} was used most frequently as an indicator to assess antimicrobial combinations with irradiation (*28*). On the basis of the results obtained, a pretreatment of *B. cereus* cells with a mild heat treatment or with carvacrol at MIC, before irradiation, strongly affected the radiosensitivity. Radiation D_{10} of 0.428 kGy was observed for control samples. When cells were preheated before irradiation, the radiation D_{10} decreased to 0.320 kGy. In the presence of carvacrol, an increase of the radiation sensitivity by 1.56 times was observed.

Influence of Irradiation in Combination with Carvacrol or Mild Heat Treatment on the Membrane's Fatty Acid Profile. Individual (irradiation, mild heat treatment, and carvacrol treatment) and selected dual combinations (mild heat + irradiation and carvacrol + irradiation) of antimicrobial treatments were assessed for their impact on the membrane's FA profiles of *B. cereus* LSPQ 2872. The FA composition of *B. cereus* LSPQ 2872 is shown in **Table 1**. Data analysis has shown that the FA composition was significantly ($P \leq 0.05$) influenced by the presence of the environmental stresses. At the sublethal radiation dose of 1.2 kGy, moderate changes to the overall composition were observed, but an increase of the unsaturated fatty acid (UFA) with 14 carbons at the expense of C14 saturated fatty acid (SFA) was noticed.

Table 1. Effect of γ-Irradiation Alone or in Combination with Mild Heat or Carvacrol Treatment on Fatty Acid Composition (%) of *B. cereus* LSPQ 2872[a]

	C12:0	C13:0	C14:1	C14:0	C15:1	C15:0	C16:0	C18:1	C20:0	TUFAs	TSFAs
0 kGy	2.226 ± 0.366 bc	1.501 ± 0.307 a	1.028 ± 0.067 a	8.846 ± 0.328 e	2.772 ± 0.530 a	2.173 ± 0.165 b	2.053 ± 0.211 cd	61.726 ± 1.290 bc	17.673 ± 0.989 b	65.527 ± 1.430 ab	34.473 ± 1.430 cd
1.2 kGy	2.169 ± 0.187 bc	2.170 ± 0.244 c	1.756 ± 0.175 c	6.560 ± 0.269 a	2.956 ± 0.135 a	2.239 ± 0.197 b	2.231 ± 0.257 d	61.281 ± 1.012 b	18.634 ± 1.479 b	65.994 ± 1.143 ab	34.033 ± 1.155 cd
3.6 kGy	1.932 ± 0.116 ab	1.707 ± 1.388 ab	1.679 ± 0.382 c	7.423 ± 0.521 bc	3.771 ± 0.497 b	2.214 ± 0.093 b	1.733 ± 0.229 b	61.408 ± 1.002 b	18.129 ± 0.939 b	66.859 ± 0.605 bc	33.140 ± 0.605 bc
heat treatment (45 °C, 10 min)	2.466 ± 0.283 cd	1.972 ± 0.152 bc	1.356 ± 1.267 b	8.047 ± 0.245 cd	2.711 ± 0.187 a	2.228 ± 0.096 b	1.271 ± 0.183 a	64.451 ± 1.072 d	15.495 ± 1.664 a	68.519 ± 1.343 d	31.480 ± 1.343 a
heat (45 °C, 10 min) + irradiation (1.2 kGy)	2.590 ± 0.362 d	2.166 ± 0.145 c	1.830 ± 0.173 c	8.729 ± 0.698 de	2.771 ± 0.056 a	2.709 ± 0.423 c	1.928 ± 0.226 bc	62.954 ± 2.239 c	14.321 ± 1.145 a	67.555 ± 2.169 cd	32.444 ± 2.169 ab
carvacrol treatment (312 ppm)	1.588 ± 0.119 a	1.659 ± 0.156 a	2.422 ± 0.241 d	7.092 ± 0.427 ab	3.460 ± 0.197 b	1.483 ± 0.150 a	ND	59.138 ± 0.540 a	23.154 ± 1.265 c	65.021 ± 0.742 a	34.978 ± 0.742 d
carvacrol (312 ppm) + irradiation (1.2 kGy)	1.800 ± 0.439 a	2.042 ± 0.349 c	2.152 ± 0.345 d	7.490 ± 1.172 bc	3.752 ± 0.478 b	1.745 ± 0.365 a	ND	58.513 ± 0.552 a	22.501 ± 2.238 c	64.419 ± 0.508 a	35.581 ± 0.539 d

[a] The values are the means ± standard deviations of six measurements, and those within a column that bear the same letter are not significantly different ($P > 0.05$). ND, not detected; TSFAs, total saturated fatty acids; and TUFAs, total unsaturated fatty acids.

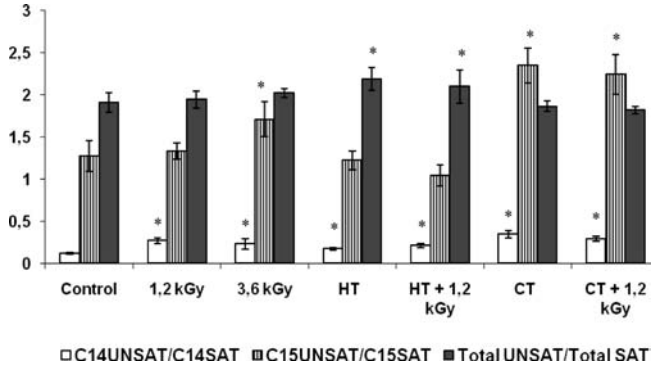

Figure 2. Fatty acid ratios (unsaturated/saturated) of *B. cereus* after γ-irradiation alone or in combination with mild heat (HT) or carvacrol treatment (CT). *Significantly different ($P \leq 0.05$) as compared with control (untreated). SAT, saturated; and USAT, unsaturated.

At 3.6 kGy, results showed an increase of unsaturated myrestoleic (C14:1) and *cis*-10 heptadecanoic (C15:1) FAs, while a lowering of saturated myrestic (C14:0) and palmitic (C16:0) FAs was observed. However, no significant changes ($P > 0.05$) occurred in the total FAs. It is interesting to note that irradiation at low doses induced less modifications in FA composition.

After a mild heat treatment for 10 min at 45 °C, a tendency in the increase of the total UFAs was observed. When the bacterial cells were preheated and subsequently irradiated with a sublethal dose of γ-irradiation, the increase of UFAs in the membrane was still noticeable. This increase was because of the significant increase ($P \leq 0.05$) in the percentage of C18:1 and the lowering of the SFA C20 and C16. After irradiation of preheated cells, the shift to smaller carbon chains seemed to take place. The percentage of C12, C13, C14, and C15 increased, but during this time, the longer chained C20 and C16 FAs decreased.

The treatment with carvacrol at the MIC modified the FA composition by decreasing the percentage of SFA with a 16 carbon chain length (C16) to nondetectable level. A significant increase ($P \leq 0.05$) of SFA C20 at the expense of monounsaturated FA C18:1 was also noticed. After irradiation in combination with carvacrol, the shift seemed to take place in the length of the FA chain itself. The concentration of the C20 FA increased at the expense of shorter chained C15:0, C14:0, and C12:0 but not C13:0.

The FA ratio (unsaturated/saturated) present in *B. cereus* LSPQ 2872 membrane is shown in **Figure 2**. This proportion demonstrates more easily the relation between the effects of combined treatment with γ-irradiation and the unsaturation level of the FA. For the C14 ratio, significant modifications ($P \leq 0.05$) seemed to take place when bacterial cells were under environmental stresses, and an increase of this ratio was observed as compared with untreated control. However, for the C15 ratio, a significant increase ($P \leq 0.05$) was only observed with the samples treated at 3.6 kGy and cells treated with carvacrol alone or in combination with γ-irradiation at 1.2 kGy. Before an exposure to γ-irradiation stress, the pretreatment of *B. cereus* cells with mild heat for 10 min at 45 °C induced a significant increase ($P \leq 0.05$) in the total unsaturation ratio. However, for the cells pretreated with carvacrol and irradiated, there was no significant effect ($P > 0.05$) in the total unsaturation ratio. With regard to the control, only irradiated at 1.2 kGy, more modifications were observed when cells were preheated or pretreated with carvacrol.

Morphology of the Bacterial Cells. SEM observations of *B. cereus* cells under various antimicrobial treatments are shown in **Figure 3**. Concerning the untreated bacteria, SEM reveals that cells with a striated membrane and rod-shaped form were

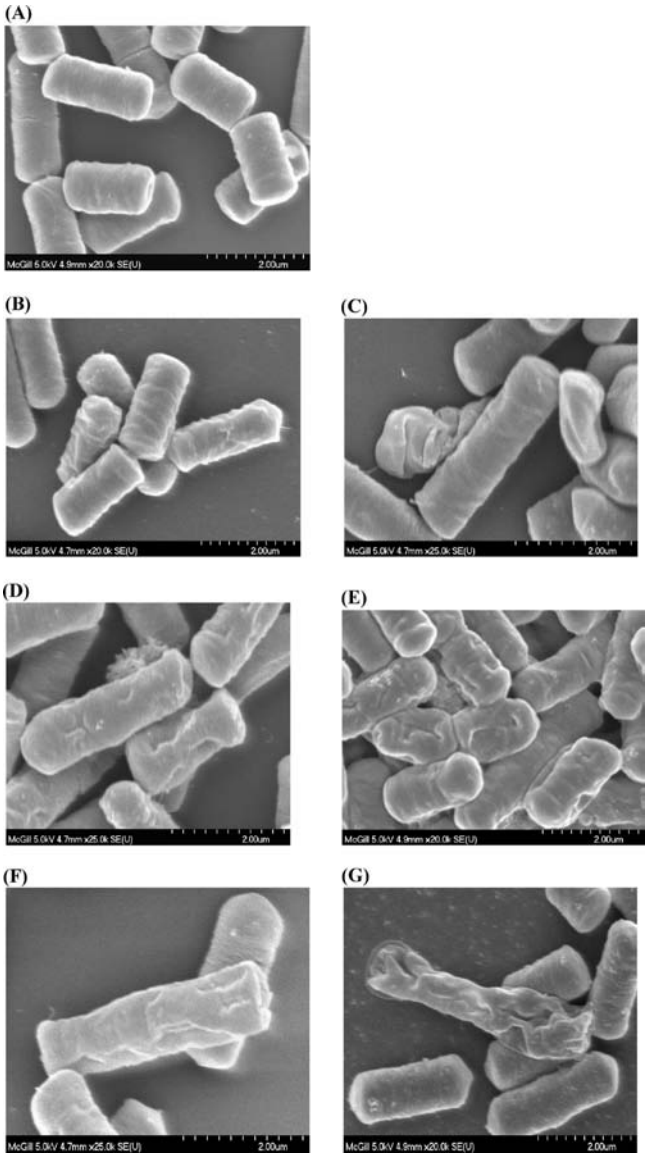

Figure 3. Scanning electron microphotographs of *B. cereus* cells: untreated (**A**), irradiated at 1.2 kGy (**B**), irradiated at 3.6 kGy (**C**), heat treated at 45 °C for 10 min (**D**), preheated cells at 45 °C for 10 min and irradiated at 1.2 kGy (**E**), treated with 312 ppm of carvacrol (**F**), and pretreated with 312 ppm of carvacrol and irradiated at 1.2 kGy (**G**).

observed. Cells only irradiated at 1.2 and 3.6 kGy showed no deformation of the shape, and the entire morphology was similar to the control cells. However, with a γ-irradiation dose of 3.6 kGy, some totally impaired cells were observed.

The bacterial cell membrane was disrupted when treated with carvacrol. Moreover, elongated *B. cereus* was observed by SEM. This phenomenon was more pronounced with cells treated with carvacrol and consecutively irradiated at 1.2 kGy. The electron micrograph of cells treated with a mild heat treatment showed injured membranes and deformed shapes. In addition, the appearance of nonseparated injured cells was observed with preheated and irradiated cells. A synergistic or additive effect on the cell morphology destruction was observed with combined treatment, and the membrane was more severely affected and damaged than the single treatment.

Intra- and Extracellular ATP Concentrations. Cytoplasmic membrane disruption is expected to have a large impact on the membrane-associated energy-transducing system. Therefore, the effect of irradiation treatment alone or in combination with

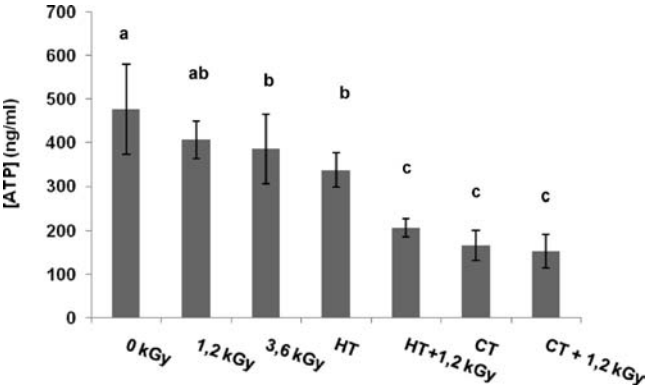

Figure 4. Intracellular ATP of *B. cereus* after γ-irradiation alone or in combination with mild heat (HT) or carvacrol treatment (CT). Values represent the means of six measurements. The standard errors of the means are indicated by the error bars. Means of the antimicrobial treatments with different letters above their bars are significantly different ($P \leq 0.05$).

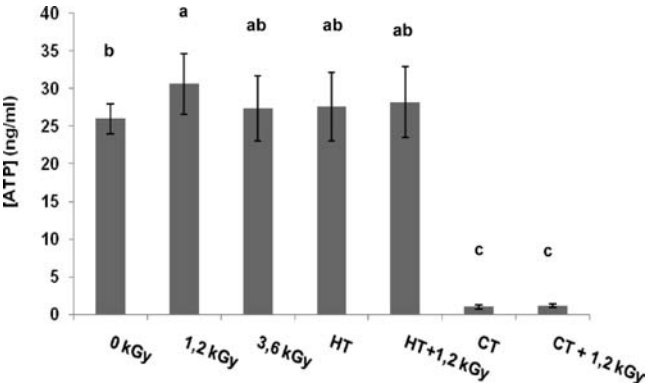

Figure 5. Extracellular ATP of *B. cereus* after γ-irradiation alone or in combination with mild heat (HT) or carvacrol treatment (CT). Values represent the means of six measurements. The standard errors of the means are indicated by the error bars. Means of the antimicrobial treatments with different letters above their bars are significantly different ($P \leq 0.05$).

carvacrol or a mild heat treatment on the intra- and extracellular ATP pools was studied. The effects of individual and combined treatments on intra- and extracellular ATP concentrations are presented in **Figures 4** and **5**. *B. cereus* nonirradiated controls had intra- and extracellular ATP concentrations of 476.7 and 25.9 ng/mL, respectively. Irradiation at 1.2 kGy increased significantly ($P \leq 0.05$) the extracellular ATP concentration without a significant ($P > 0.05$) decrease in the intracellular ATP concentration. In contrast, the results indicated that irradiation at 3.6 kGy decreased significantly ($P \leq 0.05$) the intracellular ATP concentration, but no significant increase ($P > 0.05$) of the extracellular ATP pool was observed. After a mild heat treatment at 45 °C for 10 min, a significant decrease ($P \leq 0.05$) of the intracellular ATP concentration from 476.73 to 337.47 ng/mL was obtained. The combination of mild heat treatment with irradiation had a significant ($P \leq 0.05$) effect on the decrease of the intracellular ATP concentration in comparison with the sample only irradiated. A small increase of the extracellular ATP was observed in the presence of mild heat treatment, although this was not significant ($P > 0.05$) and was not proportional to the decrease of the intracellular ATP. The addition of carvacrol at MIC decreased significantly ($P \leq 0.05$), and simultaneously, the intracellular and the extracellular amount of ATP values compared to

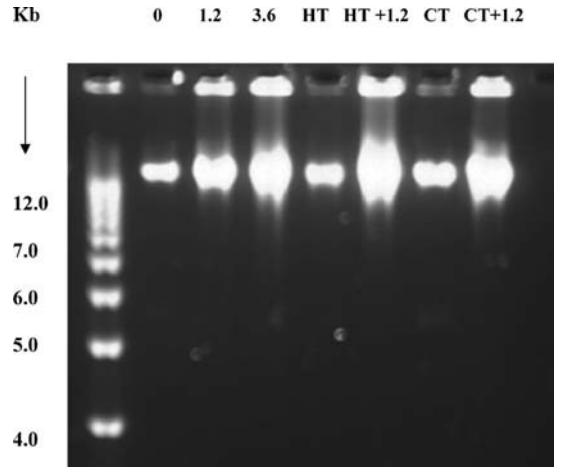

Figure 6. Agarose gel (1%) electrophoresis of DNA isolates from untreated and treated *B. cereus* LSPQ 2872 cells. Mild heat (HT) and carvacrol treatment (CT).

samples not treated. Similar results were obtained with the combination of carvacrol and irradiation.

DNA Analysis. Results have shown that there was no significant difference between the DNA treated only with carvacrol at MIC or with mild heat treatment at 45 °C as compared to the control DNA samples (untreated sample) (**Figure 6**). However, DNA degradations were observed with all of the irradiated samples, and this was more obvious with samples treated with 3.6 kGy and with 1.2 kGy in combination with carvacrol or mild heat treatment. In addition, it appeared that the DNA of irradiated cells underwent an agglomeration as observed in the wells of the agarose gel. This event of agglomeration was not found in control and in the DNA isolated from cells treated only with carvacrol and mild heat treatment.

DISCUSSION

New trends in food irradiation technology consist to develop combined treatments to reduce the irradiation doses required to kill pathogenic bacteria and/or reduce overall microbial load (*29*, *30*). Before applying improved or novel preservation techniques in food media, it is important to gain further insight into physiological bases of the organisms' survival of and responses to food-relevant stresses. In many cases, the physiological effects of single stresses or preservatives are reasonably well understood, but the physiological bases of the successful combination techniques have still hardly been studied. Therefore, it is important to obtain more information about the physiological bases of synergistic systems that are already empirically applied or that have future potential (*9*).

In the present study, *B. cereus* LSPQ 2872 was used to investigate the mechanism of defense toward combined treatment. According to the obtained results, combination of mild heat or carvacrol with γ-irradiation, in less extreme applications, results in additive or synergistic reductions in the viability of *B. cereus* to levels, which are comparable with the effects of severe single treatments. It was also noticed that modifications of the FA composition by γ-irradiation alone were not important when compared with cells treated with mild heat treatment or carvacrol antimicrobial agent targeting directly on the membrane (*31*−*34*). The ability of bacteria to multiply under suboptimal conditions requires membrane fluidity modification, a phenomena known as homeoviscous adaptation (*35*). Membrane fluidity can be altered through membrane fatty acid profile modification (*36*) to ensure vital functions such as the maintenance of a proton−motive force or the active transport of metabolites (*37*). The adaptation can occur in different ways, such as changes in type of branching, chain length, and degree of fatty acid unsaturation. However, the overall effect of the changes in lipid composition on the membrane fluidity is determined by the sum of the effect of every single lipid on the membrane fluidity.

Temperature is one of the most important environmental factors that significantly affects the FA composition of most microorganisms. Irradiation of preheated cells induced more modifications than the cells submitted to irradiation alone. An increase of the percentage of the total UFA was observed. The increase of UFA in the cell membrane is a general response of certain thermotolerant strains or species when exposed to superoptimal temperatures and in combination with other stresses, especially oxidative stress (*38*, *39*). The increase of unsaturation, as a consequence of the reduction of growth temperature, has been described for several microbes (*40*, *41*). The desaturation of the FA could be related to an increase or activation of desaturase activity (*42*, *43*). Contrary to irradiation in the presence of carvacrol, the shift to a shorter carbon chain seemed to take place after combination of mild heat treatment with irradiation. The change in the length of the FA could be the direct consequence of the bacterium remodeling its membrane composition.

The hydrophobic character of carvacrol and its mode of action suggest interaction with the cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains (*44*). The irradiation treatment of *B. cereus* cells pretreated with 312 ppm of carvacrol significantly changed ($P \leq 0.05$) the membrane lipid composition, but there was no significant effect ($P > 0.05$) in the total unsaturation ratio. Furthermore, an increase of the longer chained FA concentration at the expense of shorter chained was noticed. According to Ultee et al. (*44*), the measurement of the average phase transition temperature of the *B. cereus* lipids confirmed that membranes instantaneously became more fluid in the presence of carvacrol, which in turn would increase passive permeability (*18*). This could be some kind of defense mechanism exhibited by the cell.

The passage of *B. cereus* cell metabolites across the cell membrane on exposure to different treatments has also been investigated. The results indicated that there was an additive or synergistic effect of combined treatments in decreasing the intracellular ATP concentration of *B. cereus* as compared to irradiation alone. These results are similar to those obtained by Caillet and Lacroix (*45*) who observed that the combination of oregano oil treatment with irradiation at 1.2 kGy caused a significant reduction of the intracellular ATP concentration of *Listeria monocytogenes*. Extracellular ATP measurements showed a small increase with mild heat treatment in combination with irradiation, although this was not proportional to the decrease of the intracellular ATP. After exposure to carvacrol, a significant decrease in extracellular ATP was observed. It is therefore presumed that the rate of ATP synthesis was reduced or that the rate of ATP hydrolysis was increased. Ultee et al. (*15*) reported that the addition of carvacrol at 2 mM produces a significant depletion of the intracellular ATP to values close to the limit of detection, and no proportional increase of the extracellular ATP was observed. According to Ultee et al. (*18*), carvacrol destabilizes the cytoplasmic membrane and, in addition, acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. The resulting collapse of the proton−motive force and depletion of the ATP pool eventually lead to impairment of essential processes in the cell, to disruption of the cell membrane, and finally to cell death.

Observations with SEM showed a significant effect of combined treatments on the destruction of the cell membrane.

As compared to irradiation alone, combined treatment causes a disruption and damage of the cell membrane. Similar observations have been obtained with allyl isothiocyanate against *L. monocytogenes* (*46*), and the authors reported destruction of the cell membrane using a transmission electron microscope. Kwon et al. (*47*) observed that the treatment of *B. cereus* with cinnamic aldehyde (300 ppm) inhibits cell separation, and the bacterial cells became filamentous.

The use of techniques in combinations delivers an additive or synergistic effect in the DNA degradation to a level that was observed with the lethal dose of 3.6 kGy. It could be hypothesized that mild heat or carvacrol treatments act by disruption and destabilization of the cytoplasmic membrane, which may facilitate the direct effect of ionizing radiation, leading to an increase in the bacterial radiosensitivity. After ionizing radiation, the mechanism of defense toward environmental stresses could be the bacterial response to the destructive effect of the reactive oxygen species (ROS). The ROS produced by ionizing radiation are generally superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$) and can affect the whole cell by inducing damages to its DNA and other important molecules such as the proteins and lipids (*48*, *49*). According to Bing Tian et al. (*50*), most of the damaging effects of ionizing radiation on biological macromolecules are due to ROS produced by water radiolysis. ROS produced introduced some heavy lesions in DNA molecules preventing the DNA from functioning normally and inducing alteration of protein expression (*51*), which could change the enzymatic activity and membrane biosynthesis. From these experiments, we conclude that the mode of action of the combined treatment using γ-irradiation with mild heat or carvacrol involves several targets, which are affecting directly or indirectly the cell membrane, in the bacterial cell. Therefore, its antimicrobial effect was more important than the single treatments.

It has been reported that antimicrobial results obtained in vitro are correlated to the in situ model (food) but would require from 2 to 100 times more concentrated compound, depending on the food matrix, to obtain the same effect (*52*). The present research implies that it is possible to radiosensitize the bacteria using natural compound already used in the food industry, thus limiting the impact of higher irradiation doses on the food. Further research in this field would greatly benefit the use of food irradiation and assess the growing concern of food safety.

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