1 # Antimicrobial effects of modified chitosan based coating containing
2 nanoemulsion of essential oils, modified atmosphere packaging and gamma
3 irradiation against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on
4 green beans

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Abstract

The antibacterial activity of modified chitosan-based coatings containing nanoemulsion of essential oils (EOs), gamma irradiation, modified atmosphere packaging (MAP), alone or in combinations, against *Escherichia coli* O157: H7 and *Salmonella* Typhimurium was evaluated on inoculated green bean samples .

Firstly, four different nanoemulsions, made of carvacrol, mandarin, bergamot and lemon Eos, respectively, were compared in terms of minimum inhibitory concentration (MIC) against the two bacteria evaluated *in vitro* using the micro-broth dilution method. Carvacrol nanoemulsion resulted to be the most effective antibacterial agent and was therefore selected to be incorporated into modified chitosan (MC) to form a bioactive coating.

Secondly, the radiosensitivity of *E. coli* and *S*. Typhimurium to gamma irradiation was evaluated on inoculated green beans after coating deposition and MAP. Results showed that, without MAP, MC-based coating containing carvacrol nanoemulsion significantly increased the radiosensitization of *E. coli* and *S.* Typhimurium by 1.32-fold and 1.30-fold, respectively. Remarkably, the use of bioactive coating under MAP caused a synergistic effect with an increase in radiosensitivity by 1.80-fold and 1.89-fold for *E. coli* and *S.* Typhimurium, respectively.

Thirdly, the antibacterial effects of the antimicrobial coating, gamma irradiation, MAP alone and their combinations were evaluated against these two bacteria during a 13-days storage of green beans at 4 °C. Bioactive coating deposition or gamma irradiation treatment resulted effective in controlling the growth of the two bacteria during the entire shelf-life. Moreover, it was also found that the combined treatment of antimicrobial coating, gamma irradiation and MAP caused the reduction of microbial population to undetectable levels during the whole storage period for *E. coli* and from day 7 to the end of storage or *S. typhimurium*.

The obtained results are of great interest to food companies aiming to ensure the safety of ready-to-eat food products with a prolonged shelf life.

46 **Key words**: modified atmosphere packaging, essential oils, gamma irradiation, antimicrobial
47 coating, *Escherichia coli* O157: H7, *Salmonella* Typhimurium

48 ## 1. Introduction

49 During recent years there has been an increasing demand for healthy and safe foods, as a
50 consequence of some serious food illnesses caused by consumption of foods contaminated by
51 foodborne pathogens. The Center for Disease Control and Prevention (CDC) estimates that each
52 year in the United States 48 million people get sick due to foodborne diseases. Thermal processing
53 is able to efficiently inactivate spoilage and pathogen bacteria in food, but with a huge impact on
54 nutritional and organoleptic food properties (Raso et al., 2003). Therefore, researchers are
55 investigating non thermal methods to reduce pathogens and simultaneously to ensure the safety and
56 quality of the produce (Birmpa et al., 2013). The use of irradiation to control foodborne pathogens
57 in vegetables is well documented (Caillet et al., 2006; Takala et al., 2011); however the use of
58 radiation to kill pathogens is limited because radiation may induce adverse effects on the sensory
59 quality of the food products, especially, at high irradiation doses. Because degradation of sensory
60 quality by irradiation is dose dependent, reduction of the treatment dose would result in improved
61 sensory quality of the treated products (Lacroix et al., 1991).An increase in the radiation sensitivity
62 of the target microorganisms would therefore result in lower doses required for lethality. The
63 combination of irradiation in presence of active compounds, like bioactive edible coating, and in
64 presence of modified atmosphere packaging would help to increase the radiosensitization of food
65 pathogens, such as *Escherichia coli* O157: H7 and *Salmonella* Typhimurium, without affecting the
66 sensory quality of food products.
67 Several studies showed the efficacy of chitosan, a polycationic polymer, as matrix for food coatings
68 to preserve food quality against microorganisms (Vu et al., 2011; Rabea et al., 2003). It has also
69 been reported the antimicrobial activity of chitosan and its derivatives in coating formulations
70 (Kanatt et al., 2013; Bordenave et al., 2010; Severino et al., 2014). The mechanism of action of

71 chitosan against bacteria has not been completely explained yet, but several hypotheses have been
72 postulated: most likely, the antimicrobial activity can be attributed to a change in cell permeability
73 due to interactions between the amine groups of chitosan and the electronegative charges on the
74 bacterial cell surface. This interaction leads to the leakage of intracellular electrolytes and
75 proteinaceous constituents (Papineau et al., 1991). A new formulation was recently developed,
76 based on chitosan acylation with fatty acids derivatives, with the aim of enhancing the hydrophobic
77 properties of the polymer (Han et al., 2008).
78 Essential oils (EOs) have also gained interests as natural antimicrobial agents for food preservation
79 against foodborne pathogens and spoilage bacteria (Caillet et al., 2006). However, being EOs
80 constituents characterized by low solubility in water, they need to be encapsulated in appropriate
81 delivery systems to promote their efficiency (Weiss et al., 2009). Recently, the encapsulation of
82 EOs in nanoscale delivery systems was shown to offer the potential of improving EOs bioactivity
83 through the activation of passive mechanisms of cell absorption, owing to their subcellular size,
84 therefore enabling the reduction of the dose of essential oils required to ensure antimicrobial
85 activity in foods, minimizing the impact on aroma, flavor and taste (Donsì et al., 2011, 2012).
86 Nanoscale encapsulation can also increase the concentration of bioactive compounds in food areas
87 where microorganisms are preferably located, for example water-rich phases or liquid-solid
88 interfaces (Weiss et al., 2009).
89 Modified atmosphere packaging (MAP) has been used to control bacterial populations in several
90 food products, especially ready-to-use vegetables, because the change in package atmosphere
91 composition could lead to the reduction of respiration of vegetables/fruits, therefore increasing their
92 shelf-lives (Farber, 1991; Genigeorgis, 1985). Moreover, the combination of MAP and irradiation
93 has been found to increase the radiosensitization of bacteria (Chiasson et al., 2004; Lacroix et al.,
94 2004).
95 The aim of this study was to develop a hurdle approach to the preservation of vegetable products,
96 based on the combination of a bioactive coating containing essential oils, gamma irradiation and

97 MAP. In particular, two target Gram-negative pathogenic bacteria were selected, *E. coli* O157: H7
98 and *S.* Typhimurium, which were inoculated on green beans. Initially, the bioactive coating was
99 developed, made of a palmitoylated chitosan (modified chitosan) incorporating the most active EO
100 nanoemulsion, and subsequently the antibacterial effects of the edible coating, gamma irradiation
101 treatment and MAP, alone and in combinations, were evaluated on green bean samples inoculated
102 with *E. coli* and *S.* Typhimurium over a 13-day shelf-life at 4 °C.
103

104 **2. Materials and methods**

105 **2.1. Bacterial preparation**

106 *E. coli* O157:H7 and *S.* Typhimurium were stored at -80 °C in TSB medium containing glycerol
107 (10% v/v). Before each experiment, stock cultures were propagated through two consecutive 24 h
108 growth cycles in TSB medium at 37 °C. The cultivated cultures were centrifuged at 4000 g for 15
109 minutes and the obtained pellets were washed twice in sterile saline water (0.85% w/v) to obtain
110 working cultures containing approximately at 10^9 CFU/mL.

111 **2.2. Fabrication of essential oils nanoemulsion**

112 Carvacrol (≥98% FCC Sigma-Aldrich, Germany), bergamot , mandarin and lemon essential oils
113 (kind gifts from the Stazione Sperimentale per le Industrie delle Essenze e dei Derivati dagli
114 Agrumi, Italy) are the four antimicrobial agents used in nanoemulsions preparation. A mixture of
115 sunflower oil (Sagra, Italy), glycerol monooleate (Sigma-Aldrich, Germany) and essential oil was
116 dispersed in bidistilled water containing Tween 20 (Sigma-Aldrich, Germany), using an Ultra
117 Turrax T25 (IKA Labortechnik, Germany) at 24000 rpm for 5 min, to form a primary emulsion.
118 Subsequently, the primary emulsions were subjected to 5 cycles of high pressure homogenization
119 treatment in an in-house developed system, equipped with a 80 μm diameter orifice valve (model
120 WS1973, Maximator JET GmbH, Schweinfurt, Germany), operated at 200 MPa through an air-
121 driven Haskel pump model DXHF-683 (EGAR S.r.l., Milano, Italy)., to reach a nanometric size.

122 A photon correlation spectrometer (HPPS, Malvern Instruments, Malvern, UK) was used for the
123 particle size measurement of the nanoemulsion droplets. The droplet size distribution was
124 characterized in terms of the mean droplet size (z-diameter) and polydispersity index (PDI) by
125 measuring the backscattered (173°) light through samples diluted 1:100 with bidistilled water to
126 avoid multiple scattering effects within polystyrene cuvettes. Measurements were carried out at 25
127 °C. Each measurement was replicated twice, with the means and the standard deviations being
128 calculated. The composition, z-diameter and PDI of the different nanoemulsions tested are reported
129 in Table 1.

130 **2.3. Minimum Inhibitory Concentration (MIC) determination**

131 The MIC of four different nanoemulsions of EO against *E. coli* and *S.* Typhimurium was evaluated
132 *in vitro* using micro-broth dilution method (Turgis et al., 2012; Dussault et al., 2014). The final
133 concentrations of antimicrobial agents in culture media were varied from 4 g/l to 0.008 g/l. The
134 samples were inoculated with 15 µl of a microbial suspension (10^6 CFU/ml) and incubated for 24 h
135 at 37 °C. The MIC value was determined as the lowest concentration of the antimicrobial agent that
136 inhibited the growth of the tested microorganism, evaluating the absorbance of the sample with a
137 microplate reader (EL x 800, Bio-Tek, Winooski, VT, USA) at the wavelength of 595 nm (Turgis et
138 al., 2012; Dussault et al., 2014).

139 **2.4. Preparation of bioactive coating**

140 In this study, modified chitosan (MC) was used as coating matrix for incorporation of an
141 antimicrobial emulsion. Modified chitosan (3% N-palmitoyl chitosan) was prepared by N-acylation
142 of native chitosan (Kitomer^{TM}, M*w* 1600 kDa, 83 % deacetylation, Marinard Biotech, Canada)
143 using palmitoyl chloride, using a method previously developed (Le Tien et al., 2003). The
144 functionalization of MC was characterized by FTIR structural analysis. The MC exhibited changes
145 in band intensities that were correlated to a chemical modification in the presence of palmitoyl
146 chains linked to the polymers by acylation (data not shown) (Le Tien et al., 2003; Han et al., 2008).

147 MC was dissolved in 1% (v/v) acetic acid solution and was stirred for 24 h to ensure total solubility.
148 The final concentration of MC was 1 % (w/v). Carvacrol nanoemulsion (CN) was added into
149 coating suspensions and mixed vigorously using an Ultra Turrax T25 at 19000 rpm for 5 min. The
150 final concentration of CN in the coating formulations was 0.05 % (w/v) (Severino et al., 2014).

151 **2.5. Samples preparation**

152 Fresh green beans (*Phaseolus vulgaris L.*) were purchased from a local supermarket (IGA, Laval,
153 Quebec, Canada). Samples were packaged in 3-mil nylon EVA copolymer bags. The packaged
154 green beans were sterilized by gamma-irradiation at the Canadian Irradiation Center using a UC-15
155 A (SS canister) underwater calibrator (Nordion Inc., Kanata, Ontario, Canada) equipped with a ^{60}Co
156 source. A radiation dose of 10 kGy was delivered at a dose rate of 16.74 kGy/h to sterilize the green
157 bean samples. The packages were then stored at 4°C.

158 **2.6. Coating application**

159 The coating was applied on green beans samples (20-22 g) using the a previously tested procedure
160 (Severino et al., 2014): the samples were sprayed with the coating formulation for 5 seconds for
161 each side using a compressed air-assisted sprayer, set at the pressure of 20 psi (1.4 bar);
162 subsequently, the coated samples were allowed to dry for 1 hour on sterile aluminium sheets placed
163 in a biological safety cabinet.

164 **2.7. Effect of combined treatments on D_{10} value of *E. coli* and *S*. Typhimurium**

165 Green bean samples (20 g) were first coated with the coating formulation (MC+CN) and then
166 inoculated with 500 µl of diluted working culture of selected bacteria to reach a final concentration
167 of 10^6 CFU/g. Subsequently, samples were packaged in 3-mil nylon EVA copolymer bags (oxygen
168 transmission rate at 23°C: 52 cm^3/m^2/24 h; water vapor transmission rate at 37.8°C: 8.0g/m^2/24 h)
169 and sealed under air (78.1% N_2, 20.9% O_2, and 0.036% CO_2) and modified atmosphere (MA)
170 conditions (60% O_2, 30% CO_2, and 10% N_2). Samples were stored for 24 h at 4 °C and then were
171 irradiated at the Canadian Irradiation Center using a UC-15 A (SS canister) underwater calibrator
172 (Nordion Inc., Kanata, Ontario, Canada) equipped with a ^{60}Co source at room temperature. The

173 radiation treatments were conducted at doses ranging from 0 to 0.7 kGy for samples inoculated with
174 *E. coli*, and from 0 to 2.4 kGy for samples inoculated with *S.* Typhimurium. Microbial analysis of
175 samples was conducted after irradiation.

176 D_{10} values (irradiated dose required to reduce microbial population of one log CFU) were calculated
177 from the linear regression of the kinetics of bacterial destruction. Bacterial counts (log CFU/g) were
178 plotted against radiation doses, and the reciprocal of the slope of the trendline was extracted from
179 the plot. Moreover, the relative radiation sensitivity (S_R) was also determined using the following
180 equation:

$$S_R = \frac{D_{10}^{control}}{D_{10}^{coating}}$$

181 where S_R is the relative radiation sensitivity, $D_{10}^{control}$ is the radiation D_{10} value of the control sample
182 and $D_{10}^{coating}$ is the radiation D_{10} value of sample treated in the presence of antimicrobial coating.

183

184 **2.8. Antimicrobial effect of combined treatment of coating, MAP and gamma-irradiation**
185 **against *E. coli* and *S.* Typhimurium on green beans during storage.**

186 Green bean samples were coated with the developed formulation (MC+CN) and then inoculated
187 with 500 µl of diluted working culture of selected bacteria to reach a final concentration of 10^3
188 CFU/g. Subsequently, samples were packaged in 3-mil nylon EVA copolymer bags (oxygen
189 transmission rate at 23°C: 52 $cm^3/m^2/24$ h; water vapor transmission rate at 37.8°C: 8.0$g/m^2/24$ h)
190 and sealed under air (78.1% N_2, 20.9% O_2, and 0.036% CO_2) and MA conditions (60% O_2, 30%
191 CO_2, and 10% N_2). Finally, the samples were irradiated with a dose of 0.25 kGy.

192 All samples were stored at 4°C and for all the different combined treatments, microbial analysis
193 was conducted at days 1, 3, 5, 7, 9, 11, 13 during storage.

194 **2.9. Microbial analysis**

195 Samples were homogenized for 2 min at 230 rpm in 80 ml peptone water (0.1% w/v) using a Lab-
196 blender 400 Stomacher (Laboratory Equipment, London, UK). From the homogenate, serial
197 decimal dilutions were prepared and plated on petri dishes containing Tryptic Soy Agar (Alpha
198 Biosciences, Baltimore, MD, USA). The petri plates were incubated at 37 °C for 48 h.

199 **2.10. Statistical analysis**

200 All experiments were conducted in duplicates. For each replicate, two samples were analyzed
201 (n=4). The data were analyzed using STATISTICA (StatSoft, Tulsa, OK, USA), and the means
202 comparison among treatments was based on Tukey's HSD (Honestly Significantly Difference) tests
203 ($P \leq 0.05$).

204

205 **3. Results and discussion**

206 **3.1. MIC determination**

207 MIC values of four different nanoemulsions of EOs, measured against *E. coli* and *S.* Typhimurium,
208 are shown in Table 2. CN showed a MIC value, for both *E. coli* and *S.* Typhimurium, equal to 0.5
209 g/l. In contrast, citrus oil nanoemulsions exhibited significantly higher MIC values: MIC value of
210 bergamot nanoemulsion was 4 g/l against *E. coli* and 2 g/l against *S.* Typhimurium, MIC value of of
211 mandarin and lemon nanoemulsions were higher than 4 g/l for both *E. coli* and *S.* Typhimurium.
212 Based on the obtained data, CN was considered the most effective of the antimicrobial agents
213 against *E. coli* and *S.* Typhimurium, and was selected to be further used in the coating formulations.

214 Many studies previously demonstrated the antibacterial activity of EOs against pathogenic bacteria,
215 such as *E. coli, L. monocytogenes* and *S.* Typhimurium (Oussalah et al. 2006; Cosentino et al.,
216 1999). The major active components of EOs are phenols, terpenes and aldehydes; they act
217 principally against the cell cytoplasmic membrane, due to their hydrophobic nature, affecting the
218 unsaturated fatty acid on the bacterial membrane and thus altering its structure (Severino et al.,
219 2014). In particular, phenolic compounds act as protonophore, a carrier of protons across the lipid

220 bilayers, causing the dissipation of the proton motive force (Ultee et al., 2002); terpenes
221 compounds, such as limonene, cause the loss of membrane integrity and dissipation of the proton-
222 motive force (Sikkema et al., 1994) while the mechanism of action of aldehyde is based on the
223 dissipation of the proton motive force due to the leakage of small ions (Gill and Holley, 2004).
224 Several studies showed the antimicrobial efficacy of mountain savory EO and its main component,
225 carvacrol, against a wide range of pathogen bacteria (Friedman et al. 2002, Pol et al., 1999). Ultee
226 et al. (2000) showed the efficacy of carvacrol against *Bacillus cereus* on rice, while Ndoti-Nembe et
227 al. (2013) showed the efficacy of a mixture of carvacrol and nisin in increasing the
228 radiosensitization of *L. monocytogenes* and *S.* Typhimurium on mini carrots.
229 Donsì et al. (2011) showed that the encapsulation in nanoemulsion formulation of a terpenes
230 mixture and limonene increased the antimicrobial activity of the pure compounds against *E. coli*,
231 *Lactobacilus delbrueckii* and *Saccharomyces cerevisiae,* therefore suggesting the enhancement of
232 transport mechanisms through the cell membrane of the target microorganisms. In fact, the use of
233 nanoscale delivery systems is able to improve not only the physico-chemical stability of
234 encapsulated bioactive compounds in foods, but also their bioactivity through the activation of
235 passive mechanisms of cell absorption (Donsì et al., 2011, 2012).

236

237 **3.2. Relative radiosensitivity of *E. coli* and *S.* Typhimurium in green bean coated by**
238 **antimicrobial coating**

239 Figure 1 shows the radiosensitization of *E. coli* on green bean samples treated with the bioactive
240 coating (MC containing CN) and packaged with air or modified different atmosphere. The presence
241 of the bioactive coating increased the radiosensitivity of *E. coli*, reported as microbial inactivation
242 as a function of gamma-ray dose for the uncoated and coated samples. Modified atmosphere did not
243 significantly impact on radiosensitization of *E. coli*: radiation D_{10}- value were 0.110 kGy for
244 samples sealed under air and 0.102 kGy for samples sealed under MAP. However, the addition of
245 antimicrobial compound significantly increased the radiosensitization of *E. coli* , both when

246 packaged under air, with a D_{10}- value of 0.083 kGy, and when sealed under MAP, with a D_{10}- value
247 of 0.061 kGy. In air, the increase of S_R caused by the bioactive coating was 1.32-fold, while under
248 MAP the increase of S_R caused by the bioactive coating was 1.80-fold.
249 The radiosensitization of *S.* Typhimurium are instead shown in Figure 2. A radiation D_{10}- value of
250 0.383 kGy was obtained for samples sealed under air, while samples sealed under MAP exhibited a
251 radiation D_{10}- value of 0.332 kGy, with a consequent increase in S_R of 1.15-fold
252 When coating was applied, samples sealed under air exhibited a radiation D_{10}- value of 0.295 kGy,
253 therefore increasing S_R by 1.30-fold. Coated samples, sealed under MAP, showed instead a
254 radiation D_{10}- value of 0.202 kGy, causing an increase in S_R of 1.89- fold.
255 From the experimental data it clearly emerges that on green beans the effect of MAP on radiation
256 sensitivity was negligible for *E. coli*, while it was significant for *S.* Typhimurium. The use of MC
257 based coating containing CN was able to significantly increase the radiosensitization of both *E. coli*
258 and *S.* Typhimurium, with an increase in S_R by 1.32-fold and 1.30-fold, respectively. The use of
259 bioactive coating together with MAP resulted in a synergistic effect, which amplified the impact of
260 gamma irradiation, with an increase in S_R of 1.80-fold for *E. coli* and of 1.89-fold for *S.*
261 Typhimurium.
262 Several studies have already showed the efficacy of bioactive coating and MAP in increasing the
263 radiosensitization of pathogenic bacteria in food. In a previous work from our group (Severino et
264 al., 2014), the radiosensitization of *L. monocytogenes* inoculated on broccoli florets, coated with
265 palmitoylated chitosan containing mandarin EO nanoemulsion was proved. Caillet et al. (2006)
266 showed the increased radiosensitization of *L. monocytogenes* in the presence of *trans*-
267 cinnamaldehyde, Spanish oregano, winter savory, and Chinese cinnamon on peeled minicarrots,
268 sealed under air as well as under MAP (60% O_2, 30% CO_2, and 10% N_2).
269 The storage under MAP is in fact recommended to control or eliminate foodborne pathogens in
270 food and to improve the shelf life of minimally processed vegetables (Lacroix et Lafortune, 2004;
271 Monk et al., 1995). According to Amanatidou et al. (2000), the use of a MAP (50% O_2, 30% CO_2,

272 and 20% N_2) prolonged the shelf life of sliced carrots by 3 days compared to samples stored under
273 air. The application of 0.6 kGy under MAP resulted in a complete inhibition of *E. coli* in grated
274 carrots, whereas a dose of 0.9 kGy was necessary to achieve the same effect when the treatment was
275 applied under air (Lacroix et Lafortune, 2004).

276

277 **3.3. Antimicrobial effect of combined treatment of coating, MAP and gamma-irradiation**
278 **against *E. coli* and *S.* Typhimurium on green beans during storage.**

279 The antimicrobial effects of the developed bioactive coating in combination with gamma irradiation
280 and MAP (60% O_2, 30% CO_2, and 10% N_2) against *E. coli* on green bean samples was evaluated
281 during 13 day of refrigerated storage at 4°C.

282 Data reported in Table 3 show that during 13 days of storage *E. coli* population inoculated on
283 control samples increased from 2.98 log CFU/g to 3.95 log CFU/g.

284 Green bean samples sealed under MAP did not show any significant microbial reduction, but *E. coli*
285 population was stable during the 13 days of storage, reaching a final value of 3.01 log CFU/g,
286 which was 0.94 log CFU/g lower than control samples. Therefore, MAP resulted effective in
287 controlling bacterial growth during 13 days of refrigerated storage, showing a prolonged
288 bacteriostatic effect.

289 The deposition of the bioactive coating on green bean samples caused an immediate reduction in *E.*
290 *coli* population, which reached the value of 2.45 log CFU/g already on day 1 of storage. After 7
291 days of storage, *E. coli* population on coated samples was significantly lower (of about 1.7 log
292 CFU/g) than in control samples at the same day. Remarkably, after 11 days of storage there were no
293 detectable bacteria on coated samples, highlighting the strong bactericidal effect of the developed
294 coating formulation, based on MC containing CN.

295 The combined treatment of MAP and bioactive coating showed a significant 1.5 log CFU/g
296 reduction of *E. coli* population after 7 days of storage, as compared to control samples, with no
297 detectable bacteria after 11 days of storage. Green bean samples treated with gamma irradiation
298 doses of 0.25 kGy showed an *E. coli* population of 1.71 log CFU/g on day 1 of storage, with a
299 significant reduction of 1.27 log CFU/g, as compared to control samples. Gamma irradiation
300 treatment showed a strong residual antimicrobial effect already after 5 days of storage, with a
301 microbial load reduction of 2 log CFU/g as compared to control samples, while after 7 days of
302 refrigerated storage there were no detectable bacteria on treated samples.

303 The use of combined treatment of gamma irradiation and MAP did not significantly affect the
304 effectiveness of gamma irradiation treatment alone: samples treated with gamma irradiation under
305 MAP showed an *E. coli* population of 1.62 log CFU/g on day 1 of storage, and no detectable
306 bacteria after 7 days of storage. Therefore, no significant difference can be noticed between
307 irradiated samples packaged under air and irradiated samples packaged under MAP.

308 The combined treatment of gamma irradiation and bioactive coating reduced *E. coli* population to
309 1.3 log CFU/g on day 1 of storage, with a significant reduction of 1.7 log CFU/g, as compared to
310 control samples. This combined treatment also showed a strong residual antimicrobial effect, with
311 no detectable bacteria after 7 days of refrigerated storage. The combined treatment of on green bean
312 samples with gamma irradiation, bioactive coating and MAP exhibited the strongest antimicrobial
313 effect against *E. coli*, with no detectable bacteria over the entire storage period.

314 The antimicrobial effects of bioactive coating in combination with gamma irradiation and MAP
315 against *S.* Typhimurium on green bean samples was similarly evaluated during 13 day of
316 refrigerated storage at 4°C. Results, presented in Table 4, show that during 13 days storage at 4°C,
317 *S.* Typhimurium load on control samples increased from 3.12 log CFU/g to 4.43 log CFU/g. MAP
318 alone was not able to significantly reduce *S.* Typhimurium load : microbial load on green bean
319 samples sealed under MAP reached 3.95 log CFU/g.

320 Samples treated with the bioactive coating reduced *S.* Typhimurium population to 2.70 log CFU/g
321 on day 1 of storage. The bioactive coating was able to reduce *S.* Typhimurium by 1 log as compared
322 to control samples after 7 days of refrigerated storage. After 13 days, coated samples exhibited a
323 microbial load reduction of 1.78 log CFU/g as compared to control samples. It can be therefore
324 inferred that the bioactive coating explicate a bacteriostatic action, maintaining at a constant level
325 the *S.* Typhimurium population during 13 days storage.

326 Samples treated with the bioactive coating and sealed under MAP reduced the microbial load to
327 2.65 log CFU/g on day 1 of storage. Moreover, after 7 days of storage a significant reduction of
328 microbioal population was observed as compared to control samples. Experimental results therefore
329 highlight that the antimicrobial effectiveness of the bioactive coating on green beans is not
330 significantly enhanced by MAP, with no differences being observed between the combination of
331 bioactive coating with packaging under air or MAP, both against *E. coli* and *S.* Typhimurium.

332 The microbial load of green bean samples inoculated with *S.* Typhimurium and treated with gamma
333 irradiation dose of 0.25 kGy (Table 4) was 2.40 log CFU/g after 1 day of storage, and was a
334 significantly reduced after 7 days of storage, as compared to control samples. Gamma irradiation
335 treatment also showed a strong residual antimicrobial effect, with an increase of *S.* Typhimurium
336 population of only 0.35 log CFU/g on treated samples during all 13 days of storage. The combined
337 treatment of gamma irradiation and MAP caused a significant reduction of *S.* Typhimurium
338 population, which was already of 1 log CFU/g reduction on day 1 of storage. It also exhibited a
339 strong residual antimicrobial activity, and on day 13 of storage the load reduction was of
340 approximately 2 log CFU/g, as compared to control samples. Samples treated with combined
341 treatments of gamma irradiation and bioactive coating caused a significant reduction of *S.*
342 Typhimurium by 1.4 log CFU/g on day 1 of storage. In addition, it also showed a strong residual
343 antimicrobial activity, with a microbial load reduction of 2.07 log CFU/g, as compared to control,
344 being observed after 13 days of storage.

345 The combined treatment of gamma irradiation, coating formulation and MAP not only caused the
346 highest reduction of *S.* Typhimurium after 1 day of storage (approximately 1.5 log CFU/g reduction
347 as compared to control), but also expressed a remarkable residual antimicrobial activity: already
348 after 7 days of storage, there were no detectable bacteria on treated samples.

349 The reported data confirm the occurrence of a synergistic effect between gamma irradiation,
350 bioactive coating and MAP. In fact, several authors in previous works on gamma irradiation
351 treatment in combination with other non-thermal treatments, suggested that microorganisms that are
352 able to survive after irradiation treatment are more sensitive than untreated cells to adverse
353 environmental conditions, such as the presence of antimicrobial compounds or modified atmosphere
354 (Severino et al., 2014; Caillet et al., 2006). Takala et al. (2011) showed the effect of antimicrobial
355 coating containing citrus and rosemary extracts, spice mixture and lactic acid in increasing the
356 radiosensitization of *E. coli, S*. Typhimurium and *L. monocytogenes* in broccoli florets. Borsa et al.
357 (2004) also evaluated the increased radiation sensitization of *E. coli* and *S.* Typhi due to the addition
358 of carvacrol, thymol, *trans*-cinnamaldehyde and the use of MAP. Damages caused by gamma
359 irradiation on bacterial cells, such as breakdown of chemical bonds in DNA molecules, alteration of
360 membrane permeability, as well as alteration of cellular function, may facilitate the contact between
361 antimicrobial compounds and cell membranes, therefore increasing their antimicrobial effect
362 (Lopez-Gonzalez et al., 1999). In addition, encapsulation of bioactive compounds in
363 nanoemulsions, may potentially further enhance the synergies with gamma irradiation, by
364 promoting the dispersion of antimicrobial agents in aqueous phase and activating passive
365 mechanisms of cell adsorption (Donsì et al., 2012). The antimicrobial efficacy of carvacrol, a
366 predominant monoterpenic phenol which occurs in aromatic plants and in many essential oils of the
367 family Labiatae including *Origanum*, *Satureja*, *Thymbra*, *Thymus* and *Corydothymus* species
368 (Nostro et al., 2012), has been deeply studied. Ultee et al. (2000) examined the antimicrobial
369 activity of carvacrol against *Bacillus cereus* on rice; Esteban et al. (2013) estimated the combined
370 effect of a previous mild heat treatment (15 min at 55 °C) with the use of antimicrobials, nisin and

371 carvacrol, on the growth of *Salmonella* Enteritidis and *Salmonella* Senftenberg. Pérez-Conesa
372 (2006) documented the efficacy of encapsulated carvacrol against *E. coli* and *L. monocytogenes*
373 cells aggregated in a biofilm. The hypothesized mechanism of action of carvacrol is related to its
374 phenolic hydroxyl group: carvacrol acts as a trans-membrane carrier of monovalent cations by
375 exchanging its hydroxyl proton for another ion, such as potassium ion. These events result in the
376 absence of a proton motive force; depletion of ATP pools leads to impairment of essential processes
377 in the cell and finally to cell death (Ultee et al., 2002).
378 The combined treatment of gamma irradiation, bioactive coating and MAP (60% O_2, 30% CO_2, and
379 10% N_2) significantly affected *E. coli* and *S.* Typhimurium population on green bean samples,
380 giving a strong residual antimicrobial effect, therefore ensuring a strong antimicrobial effect during
381 13 days storage. Several work found that the radiosensitivity of bacteria varies depending on the
382 packaging atmosphere used (Caillet et al., 2006). Amanatidou et al. (1999) found that the combined
383 treatment of high O_2 and 10 to 20% CO_2 can provide adequate suppression of microbial growth of
384 vegetable- associated microorganisms. According to Daniels et al. (1985) and Dixon and Kell
385 (1989), the germicidal effect of MAP is principally attributed to the carbon dioxide. However,
386 bacteria are also very sensitive to irradiation in the presence of oxygen (Thakhur et al., 1994). The
387 presence of 60% oxygen in a MAP might be expected to enhance the lethal effect of radiation
388 because of oxygen radical and ozone formation during the treatment. Oxygen has been implicated
389 in the creation of free radicals during irradiation, which affects the DNA and hence the reproduction
390 of bacteria (Caillet et al., 2006). However, in this study MAP (60% O_2, 30% CO_2, and 10% N_2) was
391 not able to significantly improve the antimicrobial efficacy of the coating formulation or gamma
392 irradiation, but showed a synergistic effect only when combined simultaneously with gamma
393 irradiation and bioactive coating.

394 **4. Conclusion**

395 This study confirmed the strong antimicrobial activity, measured in terms of MIC, of carvacrol
396 nanoemulsions against two Gram negative pathogenic bacteria, such as *E. coli* O157:H7 and *S.*

Typhimurium, in comparison to other nanoemulsions of essential oils, derived from mandarin, lemon and bergamot. The incorporation of carvacrol nanoemulsions into modified chitosan enabled the development of a bioactive coating to be deposited on green beans, which resulted active against the two tested pathogens during storage. Modified atmosphere packaging alone, consisting of 60% O_2, 30% CO_2, and 10% N_2, was instead not very efficient in reducing the growth of *E. coli* and *S*. Typhimurium. Gamma irradiation alone, at low dose (0.25 kGy) resulted in the effective growth control of *E. coli* and *S*. Typhimurium. The combination treatment of γ-irradiation, coating and MAP resulted extremely effective against both tested bacteria during storage, not only in controlling their growth , but also in reducing their inoculated population.

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Table 4. Effect of bioactive coating in combination with modified atmosphere packaging and gamma irradiation on population of *S.* Typhimurium on green beans samples during storage at 4 °C

559

Table 1. Composition and droplet size distribution of the EO nanoemulsions

EO	**Formulation**	**z-diameter (nm)**	**PDI**
Carvacrol	Carvacrol 1%wt, Sunflower oil 3%wt, Tween 20 0.75%wt, Glycerol monooleate 0.75%wt, Water 94.5%wt	133.4 ± 5.8	0.21 ± 0.01
Bergamot oil	Bergamot oil 2%wt, Sunflower oil 2%wt, Tween 20 0.75%wt, Glycerol monooleate 0.75%wt, Water 94.5%wt	161.5 ± 7.2	0.19 ± 0.07
Lemon oil	Lemon oil 2%wt, Sunflower oil 2%wt, Tween 20 0.75%wt, Glycerol monooleate 0.75%wt, Water 94.5%wt	163.7± 6.3	0.21± 0.05
Mandarin oil	Mandarin oil 2%wt, Sunflower oil 2%wt, Tween 20 0.75%wt, Glycerol monooleate 0.75%wt, Water 94.5%wt	176.4± 14.5	0.22± 0.02

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561

562 **Table 2**. *In vitro* MIC values of the tested nanoemulsions against *E. coli* and *S.* Typhimurium

	MIC values (g/l)	
	E. coli	*S.* Typhimurium
Carvacrol nanoemulsion	0.5	0.5
Bergamot nanoemulsion	4	2
Mandarin nanoemulsion	> 4	> 4
Lemon nanoemulsion	> 4	> 4

563

564

565 **Figure 1**. Radiosensitization of *E. coli* on green bean samples as affected by coating formulation

566 under various atmospheres. Symbols are survival fractions as a function of irradiation dose. Lines

567 are linear regression of experimental data, with D_{10}-values indicated for each line

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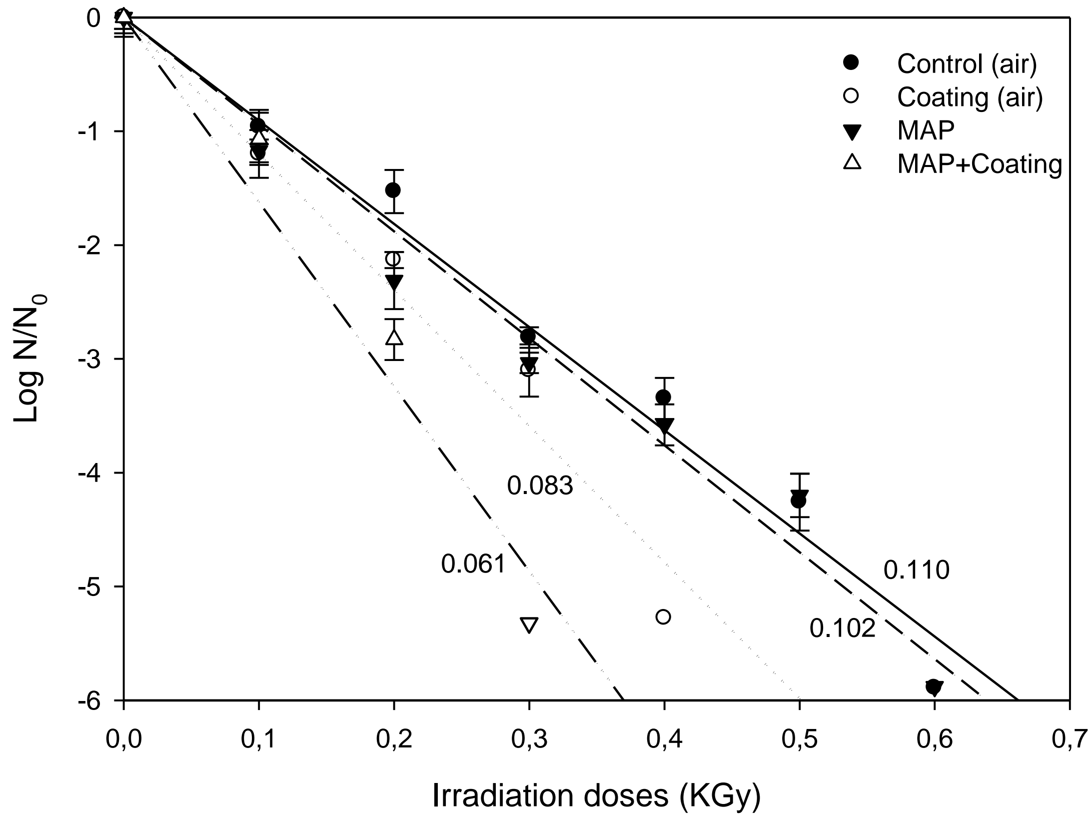

569

570

571 **Figure 2**. Radiosensitization of *S.* Typhimurium on green bean samples as affected by coating

572 formulation under various atmospheres. Symbols are survival fractions as a function of irradiation

573 dose. Lines are linear regression of experimental data, with D_{10}-values indicated for each line

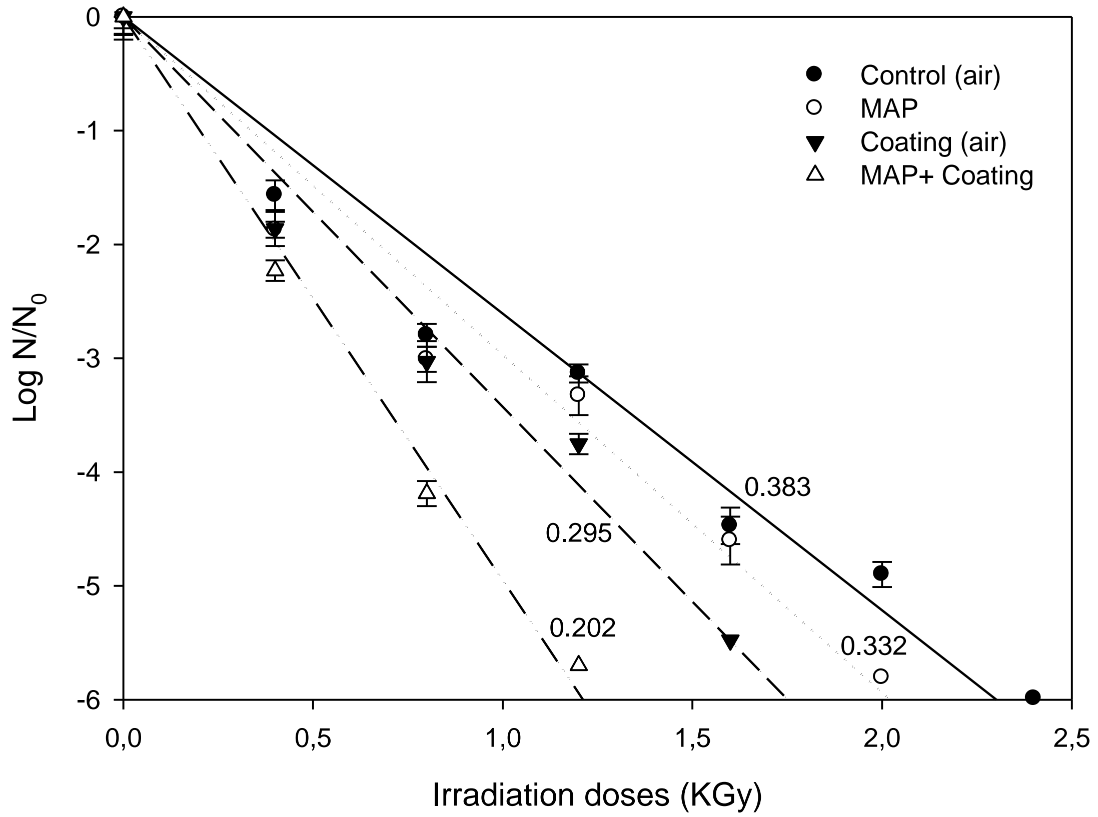

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Table 3. Effect of bioactive coating in combination with modified atmosphere packaging and gamma irradiation on population of *E. coli* on green beans samples during storage at 4 °C

	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Day 13**
Control	$2.98\pm0.08^{A}_{a}$	$3.03\pm0.11^{A}_{a}$	$3.10\pm0.07^{AB}_{a}$	$3.14\pm0.09^{AB}_{a}$	$3.18\pm0.21^{B}_{a}$	$3.41\pm0.19^{C}_{a}$	$3.95\pm0.10^{D}_{a}$
MAP	$3.02\pm0.31^{A}_{a}$	$3.19\pm0.07^{A}_{a}$	$3.05\pm0.10^{AB}_{a}$	3.01 ± 0.15^{ABa}	$2.80\pm0.15^{B}_{b}$	$2.98\pm0.11^{AB}_{b}$	$3.01\pm0.19^{AB}_{b}$
Coating (air)	$2.45\pm0.10^{AB}_{b}$	$2.15\pm0.08^{A}_{b}$	$2.57\pm0.22^{B}_{b}$	$1.40\pm0.29^{C}_{b}$	$1.25\pm0.12^{C}_{c}$	ND	ND
Coating+MAP	$2.64\pm0.14^{A}_{b}$	$2.59\pm0.12^{AB}_{c}$	$2.30\pm0.15^{B}_{b}$	$1.66\pm0.12^{C}_{b}$	$1.19\pm0.10^{D}_{c}$	ND	ND
γ (air)	$1.71\pm0.26^{A}_{c}$	$1.26\pm0.16^{B}_{d}$	$1.18\pm0.26^{B}_{c}$	ND	ND	ND	ND
γ +MAP	$1.62\pm0.15^{A}_{cd}$	$1.45\pm0.21^{B}_{e}$	$1.19\pm0.25^{C}_{c}$	ND	ND	ND	ND
γ+coating (air)	$1.30\pm0.26^{A}_{d}$	$1.35\pm0.15^{A}_{de}$	$1.25\pm0.43^{A}_{c}$	ND	ND	ND	ND
γ+coating+MAP	ND	ND	ND	ND	ND	ND	ND

Values are means ± standard deviations. Means with different lowercase letters within the same column are significantly different ($P \leq 0.05$), while means with different uppercase letters within each treatment lot are significantly different ($P \leq 0.05$)

Table 4. Effect of bioactive coating in combination with modified atmosphere packaging and gamma irradiation on population of *S.* Typhimurium on green beans samples during storage at 4 °C

	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Day 13**
Control	$3.12\pm0.12^{A}_{a}$	$3.18\pm0.03^{A}_{a}$	$3.25\pm0.05^{A}_{a}$	$3.45\pm0.07^{B}_{a}$	$3.87\pm0.09^{C}_{a}$	$4.10\pm0.04^{D}_{a}$	$4.43\pm0.13^{E}_{a}$
MAP	$3.00\pm0.10^{A}_{a}$	$2.97\pm0.14^{A}_{ab}$	$2.99\pm0.2^{A}_{b}$	$3.13\pm0.06^{A}_{b}$	$3.59\pm0.05^{B}_{b}$	$3.78\pm0.03^{BC}_{b}$	$3.95\pm0.08^{C}_{b}$
Coating (air)	$2.70\pm0.09^{A}_{b}$	$2.55\pm0.10^{ABC}_{c}$	$2.50\pm0.16^{ABC}_{cd}$	$2.53\pm0.06^{ABC}_{c}$	$2.35\pm0.09^{C}_{c}$	$2.40\pm0.04^{BC}_{c}$	$2.65\pm0.20^{AB}_{cd}$
Coating+MAP	$2.65\pm0.19^{AB}_{b}$	$2.80\pm0.08^{B}_{bd}$	$2.72\pm0.07^{B}_{bc}$	$2.45\pm0.32^{A}_{c}$	$2.60\pm0.12^{AB}_{d}$	$2.78\pm0.13^{B}_{d}$	$2.80\pm0.07^{B}_{d}$
γ (air)	$2.40\pm0.06^{AB}_{c}$	$2.65\pm0.05^{BC}_{cd}$	$2.34\pm0.12^{AD}_{d}$	$2.12\pm0.31^{D}_{d}$	$2.30\pm0.04^{AD}_{c}$	$2.48\pm0.12^{AB}_{c}$	$2.75\pm0.05^{C}_{cd}$
γ +MAP	$2.10\pm0.14^{A}_{d}$	$2.30\pm0.16^{ABC}_{e}$	$2.40\pm0.10^{BC}_{d}$	$2.25\pm0.11^{AB}_{cd}$	$2.18\pm0.16^{AB}_{ce}$	$2.38\pm0.11^{BC}_{c}$	$2.52\pm0.09^{C}_{ce}$
γ+coating (air)	$1.75\pm0.07^{A}_{e}$	$2.02\pm0.17^{A}_{f}$	$1.79\pm0.21^{A}_{e}$	$2.00\pm0.24^{A}_{d}$	$2.04\pm0.06^{A}_{e}$	$2.35\pm0.10^{B}_{c}$	$2.38\pm0.18^{B}_{e}$
γ+coating+MAP	$1.68\pm0.08^{A}_{e}$	$1.75\pm0.06^{A}_{g}$	$1.44\pm0.17^{B}_{f}$	ND	ND	ND	ND

Values are means ± standard deviations. Means with different lowercase letters within the same column are significantly different ($P \leq 0.05$), while means with different uppercase letters within each treatment lot are significantly different ($P \leq 0.05$)