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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Albonetti, S., Minardi, P., Trombetti, F., Savigni, F., Mordenti, A.L., Baranzoni, G.M., et al. (2017). In vivo and in vitro effects of selected antioxidants on rabbit meat microbiota. MEAT SCIENCE, 123, 88-96 [10.1016/j.meatsci.2016.09.004].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/566319> since: 2016-11-03

*Published:*

DOI: <http://doi.org/10.1016/j.meatsci.2016.09.004>

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This is the final peer-reviewed accepted manuscript of:

Albonetti, Sabrina, Paola Minardi, Fabiana Trombetti, Fabiana Savigni, Attilio Luigi Mordenti, Gian Marco Baranzoni, Carlo Trivisano, Fedele Pasquale Greco, e Anna Badiani. 2017. «In Vivo and in Vitro Effects of Selected Antioxidants on Rabbit Meat Microbiota». *Meat Science* 123 (gennaio): 88–96.

The final published version is available online at:  
<https://doi.org/10.1016/j.meatsci.2016.09.004>.

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# ***In vivo and in vitro* effects of selected antioxidants on rabbit meat microbiota**

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## **Abstract**

The purpose of this study was to investigate the effect of dietary vitamin E or EconomasE<sup>TM</sup> supplementation on the growth of several background/pathogenic bacteria on rabbit carcasses and hamburgers during refrigerated storage. For 51 days, 270 New Zealand rabbits received either a basal diet, or experimental diets enriched with 100 or 200 mg/kg of vitamin E or EconomasE<sup>TM</sup>. The bacteria studied were *Salmonella*, *Listeria monocytogenes*, *Pseudomonas*, *Enterobacteriaceae*, *Escherichia coli*, coagulase-positive staphylococci, plus both mesophilic and psychrotrophic aerobes. The growth of *Listeria monocytogenes* on contaminated patties was evaluated through a challenge test. The potential protective or antimicrobial effect of vitamin E or EconomasE<sup>TM</sup> on *Listeria monocytogenes* or *Pseudomonas aeruginosa* were assessed *in vitro*. Diet did not influence the concentrations of bacteria found on rabbit carcasses and developing on hamburgers. Vitamin E (*in vivo* and *in vitro*) and EconomasE<sup>TM</sup> *in vivo* had a protective antioxidant role, while EconomasE<sup>TM</sup> *in vitro* had strong antibacterial activity against *Listeria monocytogenes*, but not against *Pseudomonas aeruginosa*.

**Keywords:** Meat safety; Antioxidants; Rabbit meat microbiota; Challenge test; *Listeria monocytogenes*; *Pseudomonas aeruginosa*

## **Chemical compounds studied in this article**

35 DL  $\alpha$  tocopherol acetate (PubChem CID: 86472); ethanol (PubChem CID: 702).

36

37

## 38 1. Introduction

39

40 Rabbit meat presents excellent nutritional and dietetic properties and meets the current  
41 demand for low-fat meat (Dalle Zotte & Szendrő, 2011). However, rabbit meat is expensive, time-  
42 consuming to prepare and rather perishable because it is prone to oxidative damage due to its high  
43 level of polyunsaturated fatty acids (Abdel-Khalek, 2013). Consequently, in order to expand the  
44 market, in addition to retail fresh cuts, many rabbit meat industries have tried to approach the  
45 consumer through the production of meat preparations, such as hamburgers or patties, which may  
46 benefit from antioxidants. Given the above, a relevant question might be raised, as already  
47 suggested by Sofos, Cabedo, Zerby, Belk, & Smith (2000): are those antioxidants able to protect  
48 bacterial cellular membranes as well, when present on the very same meat or meat preparation?  
49 There is plenty of literature regarding the effects of antioxidants on rabbit meat and meat  
50 preparations, but special attention has been paid to vitamin E, i.e. DL- $\alpha$ -tocopherol (VE) (Castellini,  
51 Dal Bosco, & Bernardini, 2001; Castellini, Dal Bosco, Bernardini, & Cyril, 1998; Dal Bosco,  
52 Castellini, Bianchi, & Mugnai, 2004; Lo Fiego et al., 2004). Due to its high antioxidant activity,  
53 VE, especially as  $\alpha$ -tocopherol acetate, is commonly used in animal feed to promote growth and to  
54 improve meat quality; VE is deposited in muscle cell membranes and lipid depots, thus reducing  
55 lipid oxidation, which is one of the most significant causes of meat deterioration during  
56 refrigeration (**Hu et al., 2015**). Moreover, among the antioxidants it is possible to include the  
57 EconomasE<sup>TM</sup> (EcoE), a patented commercial premixture of nutritional additives consisting mainly  
58 of L-ascorbic acid (50 000 mg/kg) and organic selenium produced by *Saccharomyces cerevisiae*  
59 CNCMI-3060 (750 000 mg/kg). Selenium in yeast is incorporated into organic compounds, mainly  
60 selenomethionine, and low molecular weight seleno-components. Selenium is an essential trace  
61 element involved in various physiological functions; as an integral part of selenoproteins, it plays an  
62 important role in the antioxidant defense system against reactive molecules and free radicals  
63 (**Ahmad et al., 2012**; Mehdi, Hornick, Istasse, & Dufresne, 2013). Despite its importance, to our  
64 knowledge, information about the effect of dietary supplementation of antioxidant on microbial  
65 growth in rabbit carcasses and meat preparations is almost non-existent. In detail, carcasses and  
66 meat preparations from rabbits fed additional levels of VE or EcoE have not been studied so far in  
67 terms of their microbial status.

68 At each step of the food chain, meat and meat preparations might be contaminated and cold  
69 storage does not always inhibit the growth of bacteria. In particular, *Listeria monocytogenes* is a  
70 ubiquitous pathogen, and is especially dangerous because it is able to grow also at refrigeration  
71 temperatures, unlike most other foodborne pathogens (EFSA, 2014; Swaminathan, Cabanes, Zhang,

72 & Cossart, 2007). A wide variety of meats and processed products have been associated with *L.*  
73 *monocytogenes* contamination at a prevalence which can be high because of various conditions of  
74 storage, distribution and handling in addition to inadequate bacterial inactivation (Swaminathan et  
75 al., 2007). Furthermore, *L. monocytogenes* survives in foods for a long time, even under adverse  
76 conditions (Ramaswamy et al., 2007; Rocourt, BenEmbarek, Toyofuku, & Schlundt, 2003) and it  
77 causes severe symptoms and diseases (meningitis, septicemia and abortion) (**Ramaswamy et al.,**  
78 **2007**). It must also be remembered that human listeriosis cases in Europe have been increasing in  
79 recent years (EFSA & ECDC, 2014). In contrast, the *Pseudomonas* genus represents the dominant  
80 contaminant on rabbit carcasses and other packed meat (Bobbitt, 2002; Rodríguez-Calleja, Santos,  
81 Otero, & García-López, 2004). In particular, *Pseudomonas aeruginosa* is a food spoilage agent  
82 included in the list of bacteria carrying a biological risk, unlike all other species of *Pseudomonas*. In  
83 fact, the public health interest in these two microorganisms stems from the fact that they are both  
84 human pathogens and, according to regulations in Europe and the United States, these two bacteria  
85 are classified in risk group 2 on the basis of biohazard (EC, 2000; HHS, 2013).

86 The purpose of this work was to investigate the effect of dietary VE or EcoE  
87 supplementation on the growth of eight types of background or pathogenic bacteria on rabbit  
88 carcasses and rabbit meat preparations (hamburgers) during refrigerated storage. The growth of  
89 *Listeria monocytogenes* on contaminated patties was also evaluated through a challenge test. The  
90 potential protective or antimicrobial effect of VE or EcoE on *L. monocytogenes* or *Pseudomonas*  
91 *aeruginosa* were also assessed *in vitro*.

92

## 93 **2. Materials and methods**

94 This work represents the microbiological part of a multidisciplinary research project designed to  
95 evaluate the shelf-life of rabbit meat, including the study of carcass quality and the technological,  
96 nutritional and sensory quality of rabbit meat.

97

### 98 *2.1. Animals and diets*

99 Two hundred and seventy commercial New Zealand white rabbits (*Oryctolagus cuniculis*)  
100 provided by the Rabbit Genetic Centre of the Martini Group were selected for this study. Thirty-  
101 five-day-old males from a single breeding were randomly divided into five experimental *units* (*e.u.*)  
102 of 54 animals each. Every *e.u.* was housed under controlled temperature and light conditions (12 h  
103 light/12 h dark photoperiod cycle), equally and randomly divided into three cages (= *replicates*)  
104 having provision of *ad libitum* feeding and watering. A starter complete basal diet for growing  
105 rabbits and a subsequent finisher diet for fattening rabbits were formulated to meet the nutrient

106 requirements of the animals during the experimental period (Table 1).

107 Two antioxidants in two different concentrations were tested in this work. The basal diets of  
108 two *e.u.* were supplemented with 100 or 200 mg/kg of DL- $\alpha$ -tocopherol acetate (Sigma-Aldrich, St.  
109 Louis, MO, USA) (indicated as VE 100 and VE 200, respectively) while the diets of other two *e.u.*  
110 were supplemented with 100 or 200 mg/kg of EcoE (Alltech Ireland Ltd., Dunboyne, Ireland)  
111 (indicated as EcoE 100 and EcoE 200, respectively), as suggested by the producer. The remaining  
112 *e.u.* was fed a normal diet and used as a control (CTRL). After 51 days, 256 animals (mortality  
113 5.2%) were slaughtered in the Ma.Ge.Ma abattoir (Savignano sul Rubicone, FC, Italy); rabbits  
114 underwent electrical stunning followed by cutting of the carotid arteries and jugular veins. Two  
115 carcasses were discarded due to abscesses. Slaughter weights (g)  $\pm$  standard errors (SE) were:  
116 2991 $\pm$  35.09 (CTRL); 2934  $\pm$  29.18 (VE 100); 2867  $\pm$  38.85 (VE 200); 2905 $\pm$  37.15 (EcoE 100);  
117 2981 $\pm$  30.31 (EcoE 200). The abattoir structure, layout and hygiene procedures were in compliance  
118 with European Union requirements (EC, 2004). All handling procedures followed the  
119 recommendations of the European Council Directive 86/609/EEC for the protection of animals used  
120 for experimental and other scientific purposes (EEC, 1986).

121 Ten carcasses were randomly selected out of each *e.u.* (total number = 50). The selected  
122 carcasses were transported to the DIMEVET laboratory of Food Hygiene and Technology in  
123 accordance with traceability and cold chain. After 24 h at 4°C, carcass hygiene was tested and then  
124 carcasses were used to produce hamburgers and patties.

125

## 126 2.2. Microbiological analyses

127 Microbiological assays on rabbit carcasses and meat preparations were performed using  
128 international standard methods. Samples were prepared according to the ISO standard 6887-1 (ISO,  
129 1999) and 6887-2 (ISO, 2003a) and were diluted with a solution of 0.1% tryptone (Oxoid Ltd.,  
130 Basingstoke, England) and 0.85% NaCl (Oxoid Ltd.) in distilled water. ISO standard 6579 (ISO,  
131 2007) and ISO 11290-1 (ISO, 2004a) were used respectively to detect *Salmonella* spp. and *L.*  
132 *monocytogenes*, while ISO standard 4833-2 (ISO, 2013), 17410 (ISO, 2001b), 21528-2 (ISO,  
133 2004c), 16649-2 (ISO, 2001a), 13720 (ISO, 2010), 6888-1 (ISO, 2003b), and 11290-2 (ISO, 2004b)  
134 were used respectively to enumerate aerobic mesophilic bacteria, aerobic psychrotrophic bacteria,  
135 *Enterobacteriaceae*, *Escherichia coli*, *Pseudomonas* spp., coagulase-positive staphylococci, and *L.*  
136 *monocytogenes*.

137

## 138 2.3. Feed and carcass hygiene

139 The feeds for growing and fattening rabbits were preliminarily sampled and examined for *L.*

140 *monocytogenes* and *Salmonella* spp., as described in the specific ISO standards (subsection number  
141 2.2).

142 After 24 h at 4°C, 15 random post-chill rabbit carcasses (three from each *e.u.*), according to  
143 European Regulation No. 2073/2005 which rules carcass sampling on the slaughter line (EC, 2005),  
144 were tested for the detection of *Salmonella* spp. and *L. monocytogenes*, and for the enumeration of  
145 aerobic mesophilic bacteria, aerobic psychrotrophic bacteria, *Enterobacteriaceae*, *E. coli*,  
146 *Pseudomonas* spp. and coagulase-positive staphylococci, as described in the specific ISO standards  
147 (subsection number 2.2). The whole carcasses were sampled according to standards 17604 (ISO,  
148 2003c) and 6887-2 (ISO, 2003a), using the excision method. In particular, 50 g of surface tissue (~  
149 2 mm deep) from the neck, both external scapular regions, thorax, brisket, flanks, fore rib, and hind  
150 limbs were obtained with sterile scalpels and forceps. Then, 10 g of tissue were cut using sterile  
151 scissors, placed in sterile stomacher bags, diluted ten-fold, and blended for two minutes in a  
152 stomacher (Lab blender 400, Abbot Park, USA) for enumeration procedures; 25 g was used for  
153 detection procedures.

154

#### 155 2.4. Trend of natural background bacteria on rabbit hamburgers

156 ***Ten post-chill carcasses from each e.u. were boned and minced using a refrigerated***  
157 ***mincer (TC 32 Frozen, Sirman Spa, Padua, Italy) to generate a single batch of minced meat from***  
158 ***which all the necessary hamburgers and patties were produced, without any seasoning or***  
159 ***additive to avoid interferences with the tested antioxidants. The “single batch approach” was***  
160 ***regarded as mandatory within the e.u., in that it was necessary to prepare a pabulum as***  
161 ***homogeneous as possible and therefore not affected by intra e.u. differences, in order both to***  
162 ***study the growth of natural background bacteria and to perform the Listeria challenge testing,***  
163 ***each determination being repeated as many times as requested by the relevant ISO standard.***

164 Ninety hamburgers ( $100 \pm 3$  g) and 135 patties ( $30 \pm 3$  g) (18 and 27 respectively for each  
165 *e.u.*) were produced, aerobically packaged, two by two (hamburgers) or three by three (patties) in  
166 polystyrene trays, and wrapped with food plastic film.

167 All the trays were stored at 0-2°C in a cabinet (Quartet 200, Costan, Belluno, Italy) which  
168 was closed with a lid every night to reproduce retail storage conditions. On the day of production  
169 (Time 0) and after 1, 2, 4, 6, 8, 10, 12, and 16 days, one tray with two hamburgers per *e.u.* was  
170 tested for the detection of *Salmonella* spp. and *L. monocytogenes*, and for the enumeration of  
171 aerobic mesophilic bacteria, aerobic psychrotrophic bacteria, *Enterobacteriaceae*, *E. coli*,  
172 *Pseudomonas* spp., and coagulase-positive staphylococci as described in the specific ISO standards  
173 (subsection number 2.2). After each day of analysis, these ten hamburgers were discarded.

174 Patties were used for the *Listeria* challenge testing described in subsection 2.5.

175

### 176 2.5. *Listeria* challenge testing

177 Challenge testing was performed to assess the growth of *L. monocytogenes* in artificially  
178 contaminated patties of rabbit meat during and beyond the commercial shelf-life period. Four *L.*  
179 *monocytogenes* strains were used for the artificial contamination: a reference strain ATCC 7644  
180 (clinical isolate) and three strains from the internal collection (87-1771 and 115-1924 from pork  
181 meat; 88-1777 from frozen chicken). All strains were stored in vials containing trypticase soy broth  
182 (TSB, Becton, Dickinson and company, Le Pont de Claix, France) with 0.6% yeast extract (YE,  
183 Becton Dickinson France SA, Le Pont de Claix, France) and 20% glycerol (Carlo Erba Reagents,  
184 Milan, Italy) at - 20°C. The inocula for each strain were prepared adding 100 µL of stock solution  
185 to 10 mL TSB-YE. The tube was incubated overnight at 37°C then 100 µL from every grown  
186 culture were added in 10 mL TSB-YE and incubated at 7°C for 96 h. *L. monocytogenes* strains  
187 cultures were grown at low temperatures to reduce the lag time period after inoculation in food  
188 samples (Uyttendaele et al., 2004). Appropriate volumes of diluted bacterial strains were mixed  
189 together and used to prepare an inoculum of 50 CFU/g. Contamination was performed by spreading  
190 the mixed bacterial cultures on the surface of patties using sterile glass rods. Contaminated patties  
191 were kept at 20°C for five minutes to allow adhesion of the bacterial cells on the product surface  
192 (Pal, Labuza, & Diez-Gonzalez, 2008) and then they were stored in the cabinet. The inoculum  
193 concentration was verified by plate counting on trypticase soy agar supplemented with 0.6% YE  
194 (TSA-YE). A tray with three patties for each *e.u.* was tested for the enumeration of *L.*  
195 *monocytogenes* on the same day as the artificial contamination (Time 0) and after 1, 2, 4, 6, 8, 10,  
196 12, and 16 days. The patties were discarded after analysis.

197

### 198 2.6. In vitro effect of vitamin E and EconomasE™ on the growth of *L. monocytogenes* and *P.* 199 *aeruginosa*

200 The effect of VE or EcoE supplementation on microbial growth in rabbit meat was further  
201 studied by evaluating the growth of two bacteria in TSB-YE with different concentrations of the  
202 two antioxidants. The strains *L. monocytogenes* ATCC 7644 (stored in vials containing TSB-YE  
203 supplemented with 20% glycerol at - 20°C) and *P. aeruginosa* ATCC 27853 (stored in dry pellet  
204 disk at 4°C) were used for these experiments. The inocula were prepared adding 100 µL of the  
205 stock solution or dissolving one pellet disk in 10 mL of TSB-YE followed by overnight incubation  
206 at 37°C. Then, 100 µL of each grown culture were transferred to a new tube containing 10 mL of  
207 TSB-YE and incubated at 37°C for 24 h. The culture was appropriately diluted and a volume of

208 approximately 100-200  $\mu\text{L}$  added to a tube containing 10 mL of TSB-YE supplemented with one of  
 209 the antioxidants to obtain a final concentration of 10 CFU/mL or 100 CFU/mL. VE (Sigma-Aldrich,  
 210 St. Louis, MO, USA) was dissolved in ethanol 96% (solubility of 100  $\mu\text{L}/\text{mL}$ ) and diluted in TSB-  
 211 YE to obtain concentrations of  $10^4$ ,  $10^3$ , and  $10^2$  ppm (Table 2). The effect of ethanol at 10%, 1%,  
 212 and 0.1% on bacterial growth and without VE was checked as well. For both bacterial strains, the  
 213 three concentrations of VE along with the three controls were contaminated with both 10 CFU/mL  
 214 and 100 CFU/mL. All the tubes were incubated at  $37^\circ\text{C}$  for *L. monocytogenes* and  $25^\circ\text{C}$  for *P.*  
 215 *aeruginosa*, and after 24 h and 48 h the bacteria were quantified, as described in subsection 2.2. The  
 216 experiment was repeated three times (Table 2).

217 EcoE was dissolved in TSB-YE through sonication for 45 minutes (Ultrasonic UTA Falc  
 218 Instruments Srl, Treviglio, Italy);  $4 \times 10^3$  ppm was the highest concentration achieved. Differential  
 219 thermal analyses were also performed before and after sonication to verify this process did not lead  
 220 to physical and chemical changes in the additive. After appropriate dilutions in TSB-YE, the  
 221 concentrations  $4 \times 10^3$ ,  $3 \times 10^3$ ,  $2 \times 10^3$ ,  $10^3$ , and  $10^2$  ppm were contaminated with 10 CFU/mL or 100  
 222 CFU/mL for both bacteria. All the tubes were incubated at  $37^\circ\text{C}$  for *L. monocytogenes* and  $25^\circ\text{C}$  for  
 223 *P. aeruginosa*, and after 24 h and 48 h the bacteria were quantified as explained in subsection 2.2.  
 224 The experiment was repeated three times.

225

## 226 2.7. Statistical analysis

227 Bacterial concentrations were transformed to a  $\log_{10}$  scale. The analysis of data concerning  
 228 natural background bacteria on rabbit hamburgers and *Listeria* challenge testing (see sections 2.4  
 229 and 2.5) was performed by comparing growth curves estimated on the basis of microbial  
 230 concentrations observed at times  $t=0, 1, 2, 4, 6, 8, 10, 12, 16$ . For each microorganism and for each  
 231 diet, three different growth models were compared, namely the logistic model, the Gompertz model  
 232 with the parameterizations proposed in Zwietering, Jongenburger, Rombouts, & Van't Riet (1990),  
 233 and the Baranyi model with the parameterization proposed in Baranyi and Roberts (1994). These  
 234 growth models are described in equations (1) - (3).

235 (1) Logistic model

$$236 \log_{10} N_t = \log_{10} N_0 + \frac{\log_{10}(N_\infty / N_0)}{1 + \exp\left(4\mu_m(\lambda - t)\ln(10)\left(\log_{10}(N_\infty / N_0)\right)^{-1} + 2\right)}$$

237 (2) Gompertz model

$$238 \log_{10} N_t = \log_{10} N_0 + \log_{10}(N_\infty / N_0) \exp\left(-\exp\left(\frac{e\mu_m(\lambda - t)}{\log_{10}(N_\infty / N_0)\ln(10)} + 1\right)\right)$$

239

240 (3) Baranyi model

241 
$$\log_{10} N_t = \log_{10} N_0 + \log_{10} \left( \frac{-1 + \exp(\mu_m \lambda) + \exp(\mu_m t)}{\exp(\mu_m t) - 1 + \exp(\mu_m \lambda) 10^{\log_{10}(N_\infty/N_0)}} \right)$$

242 Estimates of model parameters were obtained by means of the function *nls* (non-linear least  
243 squares) implemented in the statistical software R version 3.1.0 (Copyright© 2011 The R  
244 Foundation for Statistical Computing).

245 For each diet and microorganism, the best fitting model was selected on the basis of the  
246 residual sum of squares (RSS) statistic. Since different growth models could be selected, direct  
247 comparison in terms of parameters estimates is not meaningful; indeed, parameter interpretation is  
248 different across models. The rationale for this approach is to favour model fitting over the  
249 comparability of parameters.

250 In order to compare estimated curves for each microorganism, Confidence Intervals (CI) for  
251 growth curves were obtained at each observational time following a bootstrap approach (Efron &  
252 Tibshirani, 1993), as implemented in the R package *nlstool*. This is a distribution-free procedure  
253 which is preferable to the usual approximation based on the normality assumption that holds in non-  
254 linear regression only for large samples.

255 With regard to the *in vitro* effect of VE or EcoE on the growth of *L. monocytogenes* ATCC  
256 7644 and *P. aeruginosa* ATCC 27853 (subsection number 2.6), only two observational times were  
257 available: for this reason, growth curve estimation was not feasible and experimental results were  
258 analyzed by means of an ANOVA model where observational time was considered as a  
259 dichotomous experimental factor. Experimental vials were assigned to two experimental groups  
260 defined by two different bacterial inoculum values and observed after 24 h and 48 h.

261 Let  $Y_{ijk}$  be the  $\log_{10}$  concentration, at time  $t$ , of *L. monocytogenes* or *P. aeruginosa* in the  $i$ -  
262 the experimental vial ( $i=1,2,3$ ) assigned to the  $k$ -th ( $k=1,2$ ) level of inoculum ( $I$ ), and the  $j$ -the  
263 treatment defined as follows:

- 264  $j=1$ : Ethanol percentage=0.1, VE=0;  
265  $j=2$ : Ethanol percentage=0.1, VE=100;  
266  $j=3$ : Ethanol percentage=1, VE=0;  
267  $j=4$ : Ethanol percentage=1, VE=1000;  
268  $j=5$ : Ethanol percentage=10, VE=0;  
269  $j=6$ : Ethanol percentage=10, VE=10000.

270 The ANOVA model is specified as follows:

$$271 Y_{ijkl} = \alpha + \mu_j^V + \mu_k^I + \mu_t^T + \mu_{jk}^{V:I} + \mu_{jt}^{V:T} + \mu_{kt}^{I:T} + \varepsilon_{ijkl} \quad \varepsilon_{ijkl} \sim N(0, \sigma^2) \quad (1)$$

272 where  $\alpha$  denotes the general intercept and captures the  $\log_{10}$  concentration at the baseline,  
273 parameters  $\mu$  denote the main effects, parameters  $\gamma$  denote the second-order interactions.

274 Superscripts  $I$ ,  $V$  and  $T$  refer respectively to experimental variables inoculum, vitamin E and time.  
275 The model was parameterized such that the baseline represents the  $\log_{10}$  concentration at the first  
276 level of each variable; i.e. all the considered effects are equal to 0 when  $j = k = t = 1$  (baseline). A  
277 stepwise selection procedure (not shown) suggested to ignore third-order interaction term.

278 Regarding EcoE, it was possible to manage the experimental variable EcoE ( $E$ ) as a  
279 continuous variable. As a consequence the specification of the model, selected using a stepwise  
280 procedure, is:

$$281 Y_{ijkl} = \alpha + \beta^E E_j + \mu_k^I + \mu_t^T + \beta_{jk}^{E:I} E_{jk} + \beta_{jt}^{E:T} E_{jt} + \gamma_{kt}^{I:T} + \varepsilon_{ijkl} \quad \varepsilon_{ijkl} \sim N(0, \sigma^2) \quad (2)$$

282 Parameter  $\alpha$  denotes the general intercept: in order to maintain the interpretation of this intercept as  
283 the  $\log_{10}$  concentration at baseline, variable  $E$  was shifted by subtracting its minimum value. The  
284 main effects of variables  $I$  and  $T$  were captured by parameter  $\mu$ , while  $\gamma$  denotes their second-order  
285 interaction. Parameter  $\beta^E$  is the slope of the linear relationship between EcoE and bacterial  
286 concentration when  $k = 1$  and  $t = 1$ , while parameters  $\beta_{jt}^{E:T}$  and  $\beta_{jk}^{E:I}$  are the effect modifiers  
287 referring to time and inoculum, respectively.

288

### 289 3. Results and discussion

290

#### 291 3.1. Feed and carcass hygiene

292 The feeds for growing and fattening rabbits were negative for *L. monocytogenes* and  
293 *Salmonella* spp.. The microbiota on 24 h post-chilled rabbit carcasses is summarized in Table 3; *L.*  
294 *monocytogenes*, *Salmonella* spp., and coagulase-positive staphylococci were not reported since they  
295 were absent in all carcasses, indicating good slaughtering practice.

296 Rabbit meat is not mentioned in the European Regulation No. 2073/2005 on the  
297 microbiological criteria for the acceptability of carcasses (EC, 2005); for this reason, dressing of  
298 rabbit carcasses was compared with that of beef carcasses. The  $\log_{10}$  means mesophilic aerobes and  
299 *Enterobacteriaceae* were in agreement with other reports and, even if they were slightly high for  
300 hygienically processed meat, values fell within the European limits for red meat. *Pseudomonas*  
301 spp., most of them fluorescent strains, mesophilic aerobes and psychrotrophic aerobes, were the  
302 main microorganisms of all groups after slaughter and revealed similar mean counts regardless of

303 the dietary treatment. *E. coli* counts were low, in accordance with the data of Bobbitt (2003).  
304 Relative variability among *e.u.* was observed for *E. coli* and *Enterobacteriaceae* with a variation  
305 within 1.5 log CFUs. *Pseudomonas* genus predominated on rabbit meat (Table 3), as already  
306 observed by Bobbitt (2002) and Rodríguez-Calleja et al. (2004). *Pseudomonas* is commonly the  
307 dominant meat spoilage bacteria at refrigerated temperatures, driven by enhanced catabolism of  
308 glucose and lactate (García-López, Prieto, & Otero, 1998).

309

### 310 3.2. Evolution of microbial growth on rabbit hamburgers

311 The hamburger background bacteria trend was determined using the counts of several  
312 microbiological indicators plotted as a function of time to monitor microbial population dynamics  
313 throughout refrigerated storage. The results of the microbiological analyses are shown in Fig. 1.  
314 Regarding the growth curves obtained, the choice of the best fitting model implies the selection of  
315 different growth curve families for different *e.u.*. As a consequence, direct comparison in terms of  
316 parameter estimates is not feasible, since parameter interpretation differs across models; the  
317 rationale for this approach is to favour model fitting over the comparability of parameter estimates.  
318 Comparison between growth curves is based on graphical examination of the estimated curves,  
319 along with the CIs obtained at the observational times.

320 First, it is important to note the absence of bacteria of public health significance: *Salmonella*  
321 spp., *L. monocytogenes*, and coagulase-positive staphylococci were absent in all samples, indicating  
322 good handling and meat processing practices during hamburger preparation. Initial concentrations  
323 of the other microbial populations investigated at time 0 fell within the acceptability limits set out in  
324 EC Regulation No. 2073/2005 (EC, 2005), although referring to other animal species. As expected,  
325 all microbial counts of all *e.u.* considerably increased throughout refrigerated storage. On average,  
326 growth rates for mesophiles, psychrotrophic bacteria, and *Pseudomonas* spp. were similar, reaching  
327 the plateau phase at day 4, independent from the tested diets. The storage flora of rabbit hamburgers  
328 was dominated by the genus *Pseudomonas*, in agreement with data reported by Soutos, Tzikas,  
329 Christaki, Papageorgiou, & Steris (2009). Regarding *E. coli*, hamburgers derived from the control  
330 *e.u.* (CTRL) showed the highest initial bacterial concentrations but the lowest and slowest growth.  
331 The most rapid and abundant growth was sustained by VE 200, followed by EcoE 200 and EcoE  
332 100. Therefore, except for *E. coli*, there were no significant differences among the dietary  
333 treatments: the curves and the corresponding CIs overlapped considerably. This result was primarily  
334 influenced by the high initial concentrations of *E. coli* commonly present on rabbit meat.

335

### 336 3.3. *Listeria monocytogenes* challenge test

337 Fig. 2 shows the growth of *L. monocytogenes* in contaminated rabbit patties during and  
338 beyond the commercial shelf-life period. On the first day of analysis (Time 0), CTRL presented the  
339 highest amount of bacteria, significantly different from VE 100 and EcoE 200, probably because of  
340 slight contamination during patty manipulation, but the bacterial growth was the slowest throughout  
341 the subsequent period of analysis. EcoE 200 significantly allowed the highest and fastest growth  
342 rate, followed by EcoE 100 and VE 200, which overlapped. The relatively low initial level of *L.*  
343 *monocytogenes* suggested differences among dietary fortifications, confirming, as for *E. coli*, that  
344 EcoE (both amounts) and VE 200 supported the highest and fastest bacterial growth.

345

346 3.4. In vitro effect of vitamin E and EconomasE™ on the growth of *L. monocytogenes* and *P.*  
347 *aeruginosa*

348 The absence of information on the biological role of antioxidants on microbial growth in  
349 foods required *in vitro* assays investigating contact between VE or EcoE and the two chosen  
350 bacterial strains.

351 The interpretation of the results regarding VE was complex because the effect of the ethanol  
352 used as solvent must also be considered. VE, in fact, is insoluble in water, while it is soluble in  
353 organic solvents such as ethanol which at concentrations up to 1.25% does not inhibit bacteria  
354 growth, but bacteria are strongly inhibited in the presence of 5% ethanol (Oh & Marshall, 1993).  
355 Referring to the experimental design explained above and summarized in Table 2, analyses  
356 concerning *P. aeruginosa* ATCC 27853 refer to data collected at grid cells enclosed in the dashed  
357 line, while analyses concerning *L. monocytogenes* ATCC 7644 refer to the whole table. In fact, *P.*  
358 *aeruginosa* growth was inhibited at 10% ethanol, regardless of the starter inoculum. The results  
359 obtained for *L. monocytogenes* are reported in Table 4. The main effects capture the difference  
360 between the  $\log_{10}$  counts at baseline and the  $\log_{10}$  counts in experimental blocks, where all but one  
361 experimental variable was kept constant. The estimate of  $\mu_2^I$  highlights a statistically significant  
362 increase corresponding to the increase in starter inoculum while the estimate of  $\mu_2^T$  denotes a  
363 statistically significant reduction after 48 h. As regards the main effects  $\mu_j^V$ ,  $j > 1$ , results show a  
364 highly significant reduction in  $\log_{10}$  counts in experimental blocks where Ethanol=10. Second-order  
365 interactions measure the variation of  $\log_{10}$  counts with respect to the sum of the baseline and the  
366 main effects involved; as an example, an estimate of  $\gamma_{62}^{V,I}$  equal to 1.628 means that the expected  
367  $\log_{10}$  count where Ethanol=10, VE=10000 and inoculum=100 is equal to  $9.432+0.954-$   
368  $9.868+1.628=2.146$ . Estimates concerning second-order interactions were statistically significant  
369 only in blocks where Ethanol=10. VE seemed to protect bacterial cells since it allowed for the

370 survival and growth of *L. monocytogenes* in the presence of toxic levels of ethanol. The results  
371 obtained for *P. aeruginosa* are summarized in Table 5. Parameter interpretation was analogous to  
372 the previous model. It is worth noting that all parameters were statistically significant, except  $\mu_2^T$   
373 and  $\gamma_{22}^{I:T}$ ; while time had a non-significant main effect, estimates of interactions between time and  
374 experimental factor  $V$  ( $\gamma_{22}^{V:T}$ ,  $\gamma_{32}^{V:T}$  and  $\gamma_{42}^{V:T}$ ) demonstrated an increase in log<sub>10</sub> counts after 48 h at  
375 non-baseline values of  $V$ . Inoculum showed a positive main effect, but interactions with  
376 experimental factor  $V$  were all negative.

377 Regarding EcoE assays, it is important to emphasize that the contents of selenium were  
378 equal (for EcoE 100 ppm) or one log unit higher (for EcoE 1000 ppm) than the average level of  
379 selenium reported in rabbit muscle (0.18 ppm) by Puls (1988). Results concerning the *in vitro* effect  
380 of EcoE on the growth of *L. monocytogenes* were not analyzed by means of a statistical model since  
381 no appreciable variations were observed. As can be seen from Fig. 3, bacterial growth was  
382 completely inhibited in the presence of 1000 ppm and subsequent doses of EcoE for the inoculum  
383 10 CFU/mL (a), while with 100 CFU/mL there was a slight growth only at 24 h (b). On the  
384 contrary, *P. aeruginosa* showed growth at every dose of EcoE. Table 6 summarizes parameter  
385 estimates concerning the linear model (2).

386 Marginal effects of inoculum and time were significantly greater than zero. The estimate of  
387 the interaction term  $\gamma_{22}^{I:T}$  highlights a lower growth when inoculum =100 and time =48 with respect  
388 to the growth expected, considering only marginal effects (note that the sum of the two main effects  
389 and the interaction term is positive). The negative sign of the estimate of  $\beta^E$  shows that, when  
390 inoculum =10 CFU/mL and time =24 h, the commercial additive had an inhibitory effect on  
391 microbial growth. However, when time =48 h, the estimate of  $\beta_2^{E:T}$  shows that EcoE had a positive  
392 effect on bacterial growth. It can be assumed that the bacterium adapted over time.

393 According to the reported results, the microbiota found on rabbit carcasses and developing  
394 on rabbit hamburgers did not diversify on the basis of the different antioxidants added to the diet.  
395 However, dietary treatment with EcoE (both amounts) and VE 200 corresponded to the highest  
396 amount and the fastest growth rate of *E. coli* and *L. monocytogenes* on rabbit hamburgers and  
397 patties, respectively. VE is a powerful chain-breaking antioxidant with an essential role in  
398 maintaining the structural integrity of biological membranes, in which it primarily resides (Sun et  
399 al., 2012). It can therefore be assumed that antioxidant protection of tissue against oxidative damage  
400 also promotes microorganism growth. There is no clear evidence of the role of vitamins in  
401 improving the survival of bacteria, as these supplements are used to enhance the quality of the final  
402 product and expand the shelf-life period, but Shan, Ding, Fallourd, & Leyer (2010) reported that the

403 addition of ascorbic acid (vitamin C) can protect probiotic cells. According to Murata, Tanaka,  
404 Kubo, & Fujita (2013), VE protects *S. aureus* cells from oxidative stress via free radical generation  
405 induced by cardol (C<sub>15:3</sub>). The role and effects of selenium as an antioxidant in most rabbit studies is  
406 unclear (Abdel-Khalek, 2013), but it is important to emphasize that selenium has been investigated  
407 for medical applications: Yang et al. (2009) reported a strong inhibitory activity of selenium-  
408 enriched probiotics against pathogenic *E. coli* *in vivo* and *in vitro*; a series of organoselenium  
409 compounds were successfully tested as antibacterial agents.

410 In the present study EcoE completely inhibited the Gram-positive *L. monocytogenes* *in vitro*  
411 with doses  $\geq 1000$  ppm (corresponding to a toxic level of selenium of 1.5 ppm), but it had no effect  
412 against the Gram negative *P. aeruginosa*. The resistance of *P. aeruginosa* can be explained by the  
413 inability of selenium to cross the protective outer membrane of Gram-negative bacteria and  
414 accumulate at the cell membrane or within the cytoplasm. However, this may not be the only  
415 explanation, since low molecular weight components are able to reach the periplasm through the  
416 porin proteins on the outer membranes (Helander et al., 1998). Another reason could be that  
417 selenium is pumped out from the periplasm exceeding its penetration rate, but it is clear that further  
418 studies are needed to better elucidate the effects of selenium on bacterial metabolism. Instead, EcoE  
419 showed only a protective antioxidant role *in vivo*.

420

#### 421 **4. Conclusions**

422

423 VE (*in vivo* and *in vitro*, since it allowed for the survival of bacteria at a toxic level of  
424 ethanol) and EcoE *in vivo* had a protective antioxidant effect on bacteria. EcoE *in vitro* showed  
425 strong antibacterial activity against Gram positive *L. monocytogenes* but not against Gram negative  
426 *P. aeruginosa*. Diet did not influence the concentrations of bacteria found on rabbit carcasses and  
427 developing on hamburgers.

428 This work is the first to study the effect of these selected antioxidant dietary supplements on  
429 the microbiological status of rabbit carcasses and rabbit meat preparations.

430

431

432 **Acknowledgments**

433

434           This work was funded by the Department of Veterinary Medical Science (University of  
435 Bologna). The authors are grateful to Martini Group (Martini Spa, Budrio di Longiano, FC, Italy)  
436 who provided both animals and feeds and to Alltech (Alltech Ireland Ltd., Dunboyne, Ireland) who  
437 provided EcoE. Thanks are extended to Isidoro Giorgio Lesci and Loretta Antoni for their skilful  
438 technical assistance, Andrea Capitani (Alltech Italy) for his helpful attitude, and Proof-Reading-  
439 Service.com for English language editing.

440

441

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