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Abstract

The purpose of this study was to investigate the effect of dietary vitamin E or EconomasE™ 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™. 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™ 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™ *in vivo* had a protective antioxidant role, while EconomasE™ *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

DL α tocopherol acetate (PubChem CID: 86472); ethanol (PubChem CID: 702).

1. Introduction

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

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

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

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

2. Materials and methods

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

2.1. Animals and diets

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

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

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

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

2.2. Microbiological analyses

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

2.3. Feed and carcass hygiene

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

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

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

2.4. Trend of natural background bacteria on rabbit hamburgers

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

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

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

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

2.5. Listeria challenge testing

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

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

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

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

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

2.7. Statistical analysis

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

(1) Logistic model

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

(2) Gompertz model

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

(3) Baranyi model

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

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

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

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

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

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

j=1: Ethanol percentage=0.1, VE=0;

j=2: Ethanol percentage=0.1, VE=100;

j=3: Ethanol percentage=1, VE=0;

j=4: Ethanol percentage=1, VE=1000;

j=5: Ethanol percentage=10, VE=0;

j=6: Ethanol percentage=10, VE=10000.

The ANOVA model is specified as follows:

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

where α denotes the general intercept and captures the $\log_{10}$ concentration at the baseline, parameters μ denote the main effects, parameters γ denote the second-order interactions. Superscripts *I*, *V* and *T* refer respectively to experimental variables inoculum, vitamin E and time. The model was parameterized such that the baseline represents the $\log_{10}$ concentration at the first level of each variable; i.e. all the considered effects are equal to 0 when $j = k = t = 1$ (baseline). A stepwise selection procedure (not shown) suggested to ignore third-order interaction term.

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

$$Y_{ijkt} = \alpha + \beta^E E_j + \mu_k^I + \mu_t^T + \beta_k^{E:I} E_{jk} + \beta_t^{E:T} E_{jt} + \gamma_{kt}^{I:T} + \varepsilon_{ijkt} \qquad \varepsilon_{ijkt} \sim N\left(0,\sigma^2\right) \qquad (2)$$

Parameter α denotes the general intercept: in order to maintain the interpretation of this intercept as the $\log_{10}$ concentration at baseline, variable *E* was shifted by subtracting its minimum value. The main effects of variables *I* and *T* were captured by parameter μ, while γ denotes their second-order interaction. Parameter β^E is the slope of the linear relationship between EcoE and bacterial concentration when $k = 1$ and $t = 1$, while parameters $\beta_t^{E:T}$ and $\beta_k^{E:I}$ are the effect modifiers referring to time and inoculum, respectively.

3. Results and discussion

3.1. Feed and carcass hygiene

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

Rabbit meat is not mentioned in the European Regulation No. 2073/2005 on the microbiological criteria for the acceptability of carcasses (EC, 2005); for this reason, dressing of rabbit carcasses was compared with that of beef carcasses. The $\log_{10}$ means mesophilic aerobes and *Enterobacteriaceae* were in agreement with other reports and, even if they were slightly high for hygienically processed meat, values fell within the European limits for red meat. *Pseudomonas* spp., most of them fluorescent strains, mesophilic aerobes and psychrotrophic aerobes, were the 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 Soultos, 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*

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

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

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

The interpretation of the results regarding VE was complex because the effect of the ethanol used as solvent must also be considered. VE, in fact, is insoluble in water, while it is soluble in organic solvents such as ethanol which at concentrations up to 1.25% does not inhibit bacteria growth, but bacteria are strongly inhibited in the presence of 5% ethanol (Oh & Marshall, 1993). Referring to the experimental design explained above and summarized in Table 2, analyses concerning *P. aeruginosa* ATCC 27853 refer to data collected at grid cells enclosed in the dashed line, while analyses concerning *L. monocytogenes* ATCC 7644 refer to the whole table. In fact, *P. aeruginosa* growth was inhibited at 10% ethanol, regardless of the starter inoculum. The results obtained for *L. monocytogenes* are reported in Table 4. The main effects capture the difference between the $\log_{10}$ counts at baseline and the $\log_{10}$ counts in experimental blocks, where all but one experimental variable was kept constant. The estimate of μ_2^I highlights a statistically significant increase corresponding to the increase in starter inoculum while the estimate of μ_2^T denotes a statistically significant reduction after 48 h. As regards the main effects μ_j^V, $j>1$, results show a highly significant reduction in log_{10} counts in experimental blocks where Ethanol=10. Second-order interactions measure the variation of $\log_{10}$ counts with respect to the sum of the baseline and the main effects involved; as an example, an estimate of $\gamma_{62}^{V:I}$ equal to 1.628 means that the expected $\log_{10}$ count where Ethanol=10, VE=10000 and inoculum=100 is equal to 9.432+0.954-9.868+1.628=2.146. Estimates concerning second-order interactions were statistically significant only in blocks where Ethanol=10. VE seemed to protect bacterial cells since it allowed for the

survival and growth of *L. monocytogenes* in the presence of toxic levels of ethanol. The results obtained for *P. aeruginosa* are summarized in Table 5. Parameter interpretation was analogous to the previous model. It is worth noting that all parameters were statistically significant, except μ_2^T and $\gamma_{22}^{I:T}$; while time had a non-significant main effect, estimates of interactions between time and experimental factor *V* ($\gamma_{22}^{V:T}$, $\gamma_{32}^{V:T}$ and $\gamma_{42}^{V:T}$) demonstrated an increase in $\log_{10}$ counts after 48 h at non-baseline values of *V*. Inoculum showed a positive main effect, but interactions with experimental factor *V* were all negative.

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

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

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

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

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

4. Conclusions

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

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

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