Antimicrobial effects of gallic acid, octyl gallate and propyl gallate on *Carnobacterium divergens* and *Leuconostoc carnosum* originating from meat

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Summary

The antimicrobial activity of gallic acid, propyl gallate and octyl gallate alone, and in binary combination, against reference strains of *Carnobacterium divergens* ATCC 35677 and *Leuconostoc carnosum* ATCC 49367, the two spoilage lactic acid bacteria (LAB) originating from meat, was assayed by checkerboard method. Octyl gallate was the most effective followed by propyl gallate and gallic acid, showing the lowest minimum inhibitory concentrations (*MIC*s) for *Le. carnosum*. Octyl gallate showed lower *MIC*s than gallic acid, both compounds being more effective at pH 5.0. The binary combination of both phenolic compounds was more effective against *Le. carnosum,* showing a synergistic bactericidal effect at pH 5.0. The results of this study could be used to avoid alteration of the meat by spoilage lactic acid bacteria and, due to antioxidant activity, to aid in maintaining the organoleptic properties of the meat product.

Keywords

gallic acid; octyl gallate; propyl gallate; lactic acid bacteria; antimicrobial activity; binary combination; beef extract

The shelf-life of cooked meat product is limited mainly because of microbiological safety and spoilage issues. Most of the spoilage bacteria have been identified as lactic acid bacteria (LAB), which cause unwanted changes in appearance, texture and flavour of the substrate [1].

Carnobacterium is a genus of LAB, which frequently dominates the altering microflora of chilled vaccum- or modified atmosphere-packed meat and related products, as well as on fish and poultry meats [2]. Its growth in these products is favoured due to its tolerance to microaerobic conditions and low pH values [3]. The genus *Carnobacterium* is also found among the altering microorganisms of processed meat products, obtained from whole or chopped muscle or its mixtures with animal fats or vegetable oils [4]. *C. divergens* is the most important species of *Carnobacterium* associated with meat, particularly vaccum-packed meat and fish [2]. The range of growth temperatures is 0–40 °C at pH 7 for this bacterium, which is also resistant to kanamycin, methicillin and nalidixic acid [5].

Leuconostoc spp. are Gram-positive, catalase negative facultative anaerobes present in foods of animal origin, including raw milk and dairy products, meat, poultry and fish. The growth conditions for *Leuconostoc* as a food spoilage bacterium vary depending on particular species and strains, as well as on the specific food system. Psychrotrophic *Leuconostoc* spp., especially *Le. carnosum*, *Le. gasicomitatum* and *Le. gelidum*, are spoilage organisms in package-refrigerated foods, particularly in meat and meat products. *Le. carnosum* is a specific spoilage organism in vaccum-packaged, sliced and cooked meat products, producing sensory changes, gas or slime. Growth of these bacteria occurs at 10 °C but, for most strains,

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not at 37 °C. All leuconostocs are intrinsically resistant to vancomycin and other glycopeptide antibiotics [6]. Many plant-derived compounds, such as essential oils and phenolic compounds, have inhibitory effects on the growth of leuconostocs. For example, thymol and rosemary extract, suitable for meat marinades, were shown to prevent the growth of *Le. carnosum* and *Le. mesenteroides* in a microplate model system [7].

Phenolic compounds, synthetic or natural, were shown to possess antimicrobial activity against a wide range of microorganisms [8], a property that can be exploited to inhibit growth of food-borne bacteria and to extend the shelf life of processed food. These compounds are also considered to have potential human health beneficial effects (due to antioxidant, anticarcinogenic or anti-inflammatory activities [9]) and they are antioxidants of flavouring agents in food [10]. Particularly, gallic acid (3,4,5-trihydroxybenzoic acid) is found in a variety of plants and is included in the database of flavouring substances of the European Union [11]. It possesses a wide range of biological activities such as antibacterial, antiviral, analgesic and anti-apoptotic activities [12]. Octyl gallate (octyl 3,4,5-trihydroxybenzoate) is a synthetic phenolic compound approved to be used in foods as antioxidant by European and United States government agencies [13, 14].

We previously studied the interaction of natural and synthetic phenolic compounds with Gram-positive bacteria and also checked the antioxidant effect of their binary combinations [15–17]. In this work we evaluated the antimicrobial effect of gallic acid and octyl gallate, in combination, against two spoilage LAB, *C. divergens* and *Le. carnosum*, and determined the interactive effect with pH levels and protein concentration of beef extract, in order to optimize the application in meat foods. The effect of the binary combinations on the total antioxidant activity was also evaluated in this study.

MATERIALS AND METHODS

Cultures and microorganism

Carnobacterium divergens ATCC 35677 (originating from vacuum-packed minced beef) and *Leuconostoc carnosum* ATCC 49367 (originating from chill-stored meats) were used in this study, having been purchased from American Type Culture Collection (Manassas, Virginia, USA). Stock cultures of each bacterial strain were maintained either in Eppendorf tubes containing tryptic soya broth (TSB; Oxoid, Basingtoke, United Kingdom) in the presence of 20% (v/v) glycerol, or on a porous bed Cryoinstant 409113/6 (Bioser, Barcelona, Spain) at –80 °C. Frozen stock cultures were activated by transferring 20 µl into 4 ml of TSB with 10 $g \cdot l^{-1}$ yeast extract (Oxoid) and incubating for 24 h at 30 °C ± 1 °C for *C. divergens* or 48 h at 25 °C ± 1 °C for *Le. carnosum*.

Chemicals and preparation of stock solutions for microbiological assays

Gallic acid, propyl gallate, octyl gallate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and potassium persulfate were all obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Iso-Sensitest Broth (ISB) was from Oxoid and beef extract was purchased from Condal Pronadisa (Torrejón de Ardoz, Spain).

Stock solutions of the phenolic compounds used (35 $mg \cdot ml^{-1}$ for gallic acid, 8.5 $mg \cdot ml^{-1}$ for propyl gallate and 0.2 $mg \cdot ml^{-1}$ for propyl gallate) were freshly prepared by dissolving the appropriate amount of the phenolic compound in 2 parts (v/v) of 95% (v/v) ethanol and 8 parts of culture medium used in each experiment (ISB or beef extract). The compounds were first dissolved in alcohol by constant shaking before its addition to the culture medium. The stock solutions were protected from light.

Minimum inhibitory concentration and antimicrobial interaction testing

Minimum inhibitory concentrations (*MIC*s) of gallic acid, propyl gallate and octyl gallate, as well as antimicrobial interaction testing of binary combination of gallic acid with propyl gallate or octyl gallate, were performed by the checkerboard method in microtiter plates with ISB at pH 7.4. Antimicrobial actvity was determined in terms of *MIC* values using a microdilution assay according to the ISO Standard 20776-1 [18]. Briefly, after checking the recovery ability and purity of strains, inoculum for the antimicrobial assays was prepared by diluting the overnight cultures with sterile ISB to obtain a concentration of approximately 5×10^5 $CFU \cdot ml^{-1}$. The concentration of microorganisms was checked by the Miles and Misra technique [19]. The checkerboard method was performed as reported previously [17]. *MIC* of each compound, alone or in combination, was defined as the minimum concentration of the antimicrobial compound that inhibited the visible growth of the strain tested [20]. The growth in each well was quantified by using a visual observation method and the presence of a white point at the bottom of a V-shaped well was interpreted as visible growth of bacteria. At least two trials on

different days were carried out in duplicate for each strain and binary combination.

Individual *MIC* of each compound was estimated in the same microtiter plate from the data of the column or the row in which one of the compound was absent. *MIC* data were transformed to fractional inhibitory concentrations (*FIC*). *FIC* of an individual antimicrobial compound is the ratio of the concentration of the antimicrobial in an inhibitory combination with a second compound to the concentration of the antimicrobial by itself, as follows according to Barry [20]:

$$FIC_A = \frac{MIC_{AB}}{MIC_A} \quad (1)$$

$$FIC_B = \frac{MIC_{BA}}{MIC_B} \quad (2)$$

where FIC_A is the fractional inhibitory concentration for A, FIC_B is the fractional inhibitory concentration for B; MIC_A and MIC_B are the individual minimum inhibitory concentrations for A and B; MIC_{AB} is *MIC* for A determined in the presence of B and MIC_{BA} is *MIC* for B determined in the presence of A. A and B are the phenolic compounds.

FIC index (*FICI*) was calculated with *FIC*s for the individual antimicrobials as follows:

$$FICI = FIC_A + FIC_B \quad (3)$$

The criteria used to determine the type of combined antimicrobial effect were: synergy, $FICI \leq 0.5$; no interaction, $0.5 < FICI \leq 4.0$ and antagonism, $FICI > 4.0$ [21].

Interactive effects of protein and pH in beef extract

The effects of protein and pH on the antimicrobial efficacy of gallic acid and octyl gallate, alone and in binary combination, against *C. divergens* and *Le. carnosum* were studied using ISB or beef extract at concentrations of 30, 60 or 120 g·l^{-1} at pH 5.0 or 6.0 (adjusted with HCl). The effect was evaluated considering the *MIC* values and the antimicrobial interaction of two phenolic compounds, following the checkerboard method described above.

The growth analysis of two bacteria under conditions mentioned above, with gallic acid or octyl gallate at the corresponding *MIC* values, was monitored using 96-well microplates. The wells contained 50 µl of ISB or beef extract, either with 50 µl of gallic acid or octyl gallate alone or in a combination with starting inoculum of approximately 5×10^5 CFU·ml^{-1}. Briefly, 10 µl samples were removed from each culture well at 0 h and 24 h (*C. divergens*) or 48 h (*Le. carnosum*) after inoculation, diluted in peptone water (1 g·l^{-1}), and plated onto Trypticasein soy agar (TSA; Conda Pronadisa, Madrid, Spain) with 10 g·l^{-1} yeast extract. The plates were incubated for 24 h or 48 h at 30 °C or 25 °C (for *C. divergens* or e*L. carnosum*, respectively) and counted for survival estimation. The limit of detection was 1.7 log CFU·ml^{-1} (or 5×10^1 CFU·ml^{-1}). Controls containing culture media were inoculated with each bacterial strain under investigation. At least two trials on different days were carried out in duplicate for each strain.

Bactericidal and bacteriostatic effects were defined as ≥ 3 log CFU·ml^{-1} or < 3 log CFU·ml^{-1} reduction in colony count, respectively, at 24 h (*C. divergens*) or 48 h (*Le. carnosum*) compared with the starting inoculum. Synergism was defined as a decrease in viable counts of ≥ 2 log CFU·ml^{-1}, addition or indifference as a decrease in viable counts of < 2 log CFU·ml^{-1}, and antagonism as an increase in viable counts of ≥ 2 log CFU·ml^{-1} of the combination compared with the most active single phenolic compound after 24 h or 48 h [22].

Antioxidant activity assay and binary interactions of gallic acid and octyl gallate

The antioxidant activity was determined by the ABTS method at pH 4.5 as previously reported [23], with minor modifications [15], using 0.2 mmol·l^{-1} of individual gallic acid or octyl gallate or equimolar binary combinations of these phenolic compounds (0.2 mmol·l^{-1}), dissolved in 0.1 parts of ethanol and 9.9 parts of beef extract (30, 60 or 120 g·l^{-1}) at pH 5 or 6. Antioxidant activity of beef extract without phenolic compounds was also determined. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the antioxidant control and the results were expressed as millimoles of Trolox equivalents per litre. The concentration of Trolox giving the same percentage reduction of absorbance at 734 nm as the 0.2 mmol·l^{-1} antioxidant solution was calculated using a Trolox standard curve (0–3.0 mmol·l^{-1}).

The mixture effect (*ME*) on the antioxidant activity of a binary combination is defined as the ratio of the experimental antioxidant activity of a mixture of two compounds and the the sum of activity of each compound applied separately [24]. *ME* value greater than 1, equal to 1 or lower than 1 defines a synergistic, additive or antagonistic effect between the implicated antioxidants, respectively. Data were presented as means of at least three measurements, each performed in duplicate.

Statistical analysis

Statistical analysis was undertaken using Student's *t*-test for comparison between means of two

different groups, and using one-way analysis of variance (ANOVA) for comparison of more than two different groups, using post hoc Tukey's test. The analyses were performed using SPSS 21.0 package (IBM, Armonk, New York, USA).

RESULTS AND DISCUSSION

Minimum inhibitory concentration and antimicrobial interaction in Iso-Sensitest Broth

Tab. 1 shows *MIC* values of gallic acid and two of its alkyl gallates, propyl gallate and octyl gallate, for *C. divergens* ATCC 35677 and *Le. carnosum* ATCC 49367, the two LAB from meat grown in ISB. The antimicrobial effects of gallic acid and of the two alkyl gallates were similar for the two strains. However, we found significant inter-strain differences among the three assayed phenolic compounds, registering the lowest *MIC* values for *Le. carnosum*, approximately a half of those for *C. divergens*. The antimicrobial effect of these phenolic compounds against the two strains was as follows: octyl gallate > propyl gallate > gallic acid, with the minor *MIC* of octyl gallate being 570- to 20-fold lower than the others.

With regard to propyl gallate and octyl gallate, presence of C3- or C8-alkyl chains in the molecule caused a decrease in *MIC* 12- and 155-fold (for *C. divergens*) and 15- and 350-fold (for *Le. carnosum*), respectively, compared to gallic acid, being the effect of C8-alkyl chain more pronounced for *Le. carnosum*. It is well known that the antimicrobial activity of alkyl gallates increases concomitantly with their chain length [25].

Similar *MIC* values were reported the gallic acid (ranging from 2900 μg·ml^{-1} to 4600 μg·ml^{-1}) against various LAB [15, 17, 26]. However, higher values (reaching 8000 μg·ml^{-1}) and much lower values (200–300 μg·ml^{-1}) were reported [27, 28]. *MIC* values of 22 polyphenols (including gallic acid) against 26 species of bacteria were reported [29, 30], indicating that there was no clear difference between actvity against Gram-negative and Gram-positive.

Tab. 1. Minimum inhibitory concentrations of gallic acid and two of its alkyl derivatives against two strains of lactic acid bacteria.

	MIC [μg·ml^{-1}]	
	Carnobacterium divergens ATCC 35677	*Leuconostoc carnosum* ATCC 49367
Gallic acid	5950.0 ± 1202.1^{A}	3612.2 ± 785.4^{B}
Propyl gallate	503.0 ± 86.2^{A}	233.5 ± 38.7^{B}
Octyl gallate	38.3 ± 7.7^{A}	10.5 ± 1.8^{B}

Values are means of at least two experiments in duplicate ± standard deviation.
Values in the same row with different superscripts are significantly different ($p < 0.05$), using one-way analysis of variance (ANOVA).
MIC – minimum inhibitory concentration determined in Iso-Sensitest broth.

In our study, *MIC* values for octyl gallate ranged between 10.5 μg·ml^{-1} and 38.3 μg·ml^{-1} (Tab. 1), which were lower than the values of 800 μg·ml^{-1} and 1600 μg·ml^{-1} previously reported for *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *B. cereus* [17, 31–33]. *MIC* values of propyl gallate similar to those obtained in this study were previously reported for *Staph. aureus* (methicillin-resistant and methicillin-sensitive) and *Streptococcus mutans* [31, 33–35]. However, *MIC* values as high as 1600 μg·ml^{-1} and 3200 μg·ml^{-1} were reported against to *S. mutans* ATCC 25175, *Staph. aureus* ATCC 12598 and *Staph. aureus* (methicillin-resistant) ATCC 33591 [33, 36].

The interaction of binary combinations of gallic acid with propyl gallate or octyl gallate against *C. divergens* and *Le. carnosum* (*FIC* and *FICI* values) is summarized in Tab. 2. None of the combinations showed antagonism. As seen in the ta-

Tab. 2. Antimicrobial activities of gallic acid in binary combination with propyl gallate or octyl gallate against *Carnobacterium divergens* and *Leuconostoc carnosum* in Iso-Sensitest broth.

Strain	Combination of phenolic compounds	MIC_A [μg·ml^{-1}]	MIC_{AB} [μg·ml^{-1}]	FIC_A	MIC_B [μg·ml^{-1}]	MIC_{BA} [μg·ml^{-1}]	FIC_B	*FICI*
C. divergens ATCC 35677	GA + Propyl gallate	4000	2000	0.50	525.25	331.11	0.63	1.03
	GA + Octyl gallate	2800	1260	0.45	21.87	11.37	0.52	0.97
Le. carnosum ATCC 49367	GA + Propyl gallate	3733	1680	0.45	233.50	100.40	0.43	0.88
	GA + Octyl gallate	3400	952	0.28	14.10	3.67	0.26	0.54

GA – gallic acid, in indices: A – gallic acid, B – propyl gallate or octyl gallate.
MIC – minimum inhibitory concentration, MIC_A – *MIC* of A alone, MIC_B – *MIC* of B alone, MIC_{AB} – *MIC* for A in the presence of B, MIC_{BA} – *MIC* for B in the presence of A; *FIC* – fractional inhibitory concentration, FIC_A – *FIC* for A, FIC_B - *FIC* for B, *FICI* – *FIC* index.

ble, values of FIC_A and FIC_B were lower than 1 for the two bacteria assayed. In the binary combinations assayed, *MIC* values of gallic acid decreased to approximately 1/2 of *MIC* compared with the corresponding *MIC* values of gallic acid alone for both bacteria, except for the combination of gallic acid with octyl gallate against *Le. carnosum*, which decreased to 1/3 *MIC*. Similarly, *MIC* values of propyl gallate or octyl gallate, in binary combinations with gallic acid, decreased to approximately 1/2 *MIC* for both bacteria except for the combination of gallic acid with octyl gallate, which diminished to 1/4 *MIC* against *Le. carnosum*. The results of this study confirmed that, when used in a combination, lower concentrations of synthetic phenolic compounds are sufficient to achieve an antimicrobial effect. This could be of interest for their use as food additives. According to the criteria applied in this study for interpreting the result of the interaction of antimicrobial agents, we have found no interaction, which occurs when two combined antimicrobials give results that are equivalent to the sum of each antimicrobial acting independently. However, as can be seen in Tab. 2, the lowest *FICI* value (0.54), corresponding to the interaction between gallic acid and octyl gallate against *Le. carnosum*, was on the border between no interaction and synergistic effect.

Effect of protein and pH on the antimicrobial activity

The study of the antimicrobial activity of gallic acid and octyl gallate alone and in binary combination on *C. divergens* and *Le. carnosum* was carried out at two pH values (5 and 6) in ISB medium and in beef extract at three concentrations (30 g·l^{-1}, 60 g·l^{-1} or 120 g·l^{-1}). *MIC* values are shown in Tab. 3 and Tab. 4. These two pH values were chosen to reflect the fact that meat of high quality has ultimate pH in the range of 5.4–5.6 and, at pH > 5.8, a decrease in meat delicacy as well as a possibility of maintaining good quality during cooling was mentioned [37].

An overall effect of pH on the antimicrobial activity in ISB medium was observed for the two phenolic compounds and for the two strains, decreasing this activity (reflected by higher *MIC* values) at the highest pH value (Tab. 3). Effect of pH was also observed in beef extract at all concentrations, decreasing the antimicrobial efficacy (reflected by higher *MIC* values) at pH 6 with the two phenolic compounds for both strains. Addition of gallic acid caused a three-fold increase in *MIC* values in both strains at any concentration of beef extract. In the case of octyl gallate, this increase was related to protein concentration, ranging from 3-fold (in 30 g·l^{-1} beef extract) to 12-fold (in 120 g·l^{-1} beef extract) for *C. divergens* and from 3-fold (in 30 g·l^{-1} beef extract) to 7-fold (in 120 g·l^{-1} beef extract) for *Le. carnosum*.

The effect of pH on the antimicrobial efficacy was more remarkable than that of protein. Acidification of the medium increased the antimicrobial efficacy of gallic acid and octyl gallate for both bacteria in all studied conditions, i.e. at two pH values (5 and 6) in ISB medium and in beef extract (30 g·l^{-1}, 60 g·l^{-1} or 120 g·l^{-1}). No significant interspecies differences were detected. Gallic acid is a weak phenolic acid ($pK_a \approx 4.0$ [38], where pK_a is the negative logarithm of the dissociation con-

Tab. 3. Effect of protein and pH on the antimicrobial activity of gallic acid and octyl gallate against *C. divergens* and *Le. carnosum*.

Growth medium	pH	Minimum inhibitory concentration [μg·ml^{-1}]			
		C. divergens ATCC 35677		*Le. carnosum* ATCC 49367	
		Gallic acid	Octyl gallate	Gallic acid	Octyl gallate
Iso-Sensitest broth	5	1487.50 ± 300.52^{A}	6.84 ± 1.94^{AB*}	1581.12 ± 319.43^{AB*}	4.78 ± 0.97^{A*}
	6	4250.00 ± 1202.08^{a}	19.14 ± 3.87^{a}	3612.18 ± 785.37^{a}	10.48 ± 1.78^{a}
Beef extract (30 g·l^{-1})	5	1400.00 ± 230.94^{A*}	10.94 ± 1.81^{B*}	685.71 ± 106.90^{A*}	9.38 ± 2.55^{A*}
	6	2800.00 ± 461.88^{a}	43.75 ± 7.22^{b}	2000.00 ± 462.00^{b}	26.25 ± 4.33^{b}
Beef extract (60 g·l^{-1})	5	2450.00 ± 495.00^{B*}	9.57 ± 1.93^{AB*}	2000.00 ± 462.00^{B*}	9.38 ± 2.95^{A*}
	6	5600.00 ± 923.76^{ab}	43.75 ± 7.22^{b}	4000.00 ± 924.00^{a}	43.75± 7.22^{c}
Beef extract (120 g·l^{-1})	5	2450.00 ± 495.00^{B*}	4.79 ± 0.97^{A*}	2500.00 ± 945.00^{B*}	8.20 ± 3.47^{A*}
	6	8000.00 ± 847.52^{b}	57.78 ± 8.98^{b}	5600.00 ± 924.00^{c}	55.00 ± 5.77^{d}

Values are means of at least 2 experiments in duplicate ± standard deviation.
Different letter in superscript in each column indicates significant differences for each pH value ($p < 0.05$) using one-way analysis of variance (ANOVA). Uppercase letters for pH 5 and lowercase letters for pH 6.
* – significant differences ($p < 0.05$) between pH 5 and 6; p values were calculated using Student's t-test.

Tab. 4. Antimicrobial activities of gallic acid combined with octyl gallate against *C. divergens* and *Le. carnosum* in beef extract medium.

Strains	Beef extract	pH	MIC_A [μg·ml^{-1}]	MIC_{AB} [μg·ml^{-1}]	FIC_A	MIC_B [μg·ml^{-1}]	MIC_{BA} [μg·ml^{-1}]	FIC_B	*FICI*
C. divergens ATCC 35677	30 g·l^{-1}	5	1400	672	0.48	10.93	6.56	0.60	1.08
		6	2800	1316	0.47	43.75	14.87	0.34	0.81
	60 g·l^{-1}	5	2450	1151	0.47	9.58	5.27	0.55	1.02
		6	5600	2408	0.43	43.75	22.75	0.52	0.95
	120 g·l^{-1}	5	2450	833	0.34	4.78	2.48	0.52	0.86
		6	8000	3120	0.39	57.78	26.58	0.46	0.85
Le. carnosum ATCC 49367	30 g·l^{-1}	5	700	238	0.34	9.37	4.68	0.50	0.84
		6	2000	780	0.39	26.25	9.71	0.37	0.76
	60 g·l^{-1}	5	2000	620	0.31	9.37	3.75	0.40	0.71
		6	4000	1480	0.37	43.75	14.44	0.33	0.70
	120 g·l^{-1}	5	2500	725	0.29	8.20	3.77	0.46	0.75
		6	5600	1960	0.35	55.00	18.15	0.33	0.68

In indices: A – gallic acid, B – propyl gallate or octyl gallate.
MIC – minimum inhibitory concentration, MIC_A – *MIC* of A alone, MIC_B – MIC of B alone, MIC_{AB} – *MIC* for A in the presence of B, MIC_{BA} – *MIC* for B in the presence of A. *FIC* – fractional inhibitory concentration, FIC_A – *FIC* for A, FIC_B – *FIC* for B, *FICI* – *FIC* index.

stant of gallic acid) and, at pH 5.0, the undissociated form of gallic acid accounts for 9%, while at pH 6, this percentage is 9-fold lower. It has been established that the antimicrobial activity of weak phenolic acids is pH dependent and that the concentration of the undissociated form, being more lipophilic, rises with the decreasing pH. This undissociated form is able to cross the cell membrane by passive diffusion, disturbing the cell membrane structure and possibly acidifying the cytoplasm and causing protein denaturation [8, 26, 39].

Regarding the effect of pH on the antimicrobial activity of octyl gallate, it was reported that the hydrophilic pyrogallol moiety first binds by intermolecular hydrogen bonds to the hydrophilic portion of the bacterial membrane (polar part of the phospholipid and some proteins that are membrane-bound), and the hydrophobic alkyl portion of the molecule is then able to enter into the membrane lipid bilayers, which disturbs several cellular functions [33, 35]. At pH more acidic, the polarity of pyrogallol increases, which would explain the higher antimicrobial efficacy of this compound at pH 5.0. The negative effect of pH increase on the antimicrobial activity of octyl gallate is potentiated with the increase in the protein concentration in the beef extract. The hydrophobic interactions between octyl gallate and peptones, the main components of beef extract, could explain this negative effect [40, 41]. It is usually assumed that the increase in protein concentration in food protects bacteria from the essential oils action [42], although both positive and negative impacts on the effectiveness of essential oils have been described by the presence of protein [40, 43].

In this study, the combined effect of gallic acid and octyl gallate (Tab. 4) did not appear to be generally affected by either pH or protein concentration for the two bacteria. A reciprocal effect of the presence of each of the antimicrobials on the other in the binary combination was detected, decreasing the respective values of the individual *MIC* values of gallic acid and octyl gallate approximately to 1/2 in *C. divergens* and approximately to 1/3 in *Le. carnosum*. This decrease in *MIC* values means that gallic acid (the natural compound with a higher *MIC*) and also the synthetic octyl gallate are present in lower concentrations in the combination, so this could be of interest for using them as additives in foods. According to the criteria applied in this study, none of the combinations tested showed antagonism. In all the binary combinations analysed, no interaction was found ($0.5 < FICI \leq 4$; $FICI \approx 1$), which occurs when two combined antimicrobials give results equivalent to the sum of both antimicrobial acting independently [17], even though in *Le. carnosum FICI* was approximately the same (0.75) in all conditions.

Effect of protein and pH on microbial growth

The checkerboard method merely reflects the bacteriostatic effects whereas the study of growth allows to test both bacteriostatic and bactericidal activities. In this study, we determined the growth

Tab. 5. Effect of gallic acid and octyl gallate on the growth of *C. divergens* and *Le. carnosum* under various conditions.

Growth conditions		*C. divergens* [log CFU·ml^{-1}]				*Le. carnosum* [log CFU·ml^{-1}]			
		pH 5		pH 6		pH 5		pH 6	
		ΔVC	ΔVC_{comb}	ΔVC	ΔVC_{comb}	ΔVC	ΔVC_{comb}	ΔVC	ΔVC_{comb}
Iso-Sensitest broth	+ GA	–0.32		–0.44		0.6		–0.38	
	+ OG	–1.95		–3.69		0.54		–0.03	
	+ GA + OG	–1.59	–0.36	–3.69	0	–0.51	–1.05	–3.73	–3.35
Beef extract (30 g·l^{-1})	+ GA	–0.28		–0.08		0.38		0.95	
	+ OG	–1.62		–1.51		–1.04		–3.73	
	+ GA + OG	–2.77	–1.15	–3.69	–2.18	–3.73	–2.69	–3.73	0
Beef extract (60 g·l^{-1})	+ GA	–0.23		–0.34		–0.11		–0.77	
	+ OG	–2.35		–3.69		–1.25		–3.73	
	+ GA + OG	–1.87	0.48	–3.69	0	–3.73	–2.48	–3.73	0
Beef extract (120 g·l^{-1})	+ GA	–0.23		0.14		1.8		–0.23	
	+ OG	–0.91		–3.69		0.78		–3.73	
	+ GA + OG	–0.23	0.36	–3.69	0	–1.99	–2.77	–3.73	0

GA – gallic acid, OG – octyl gallate, ΔVC – differences between viable counts before and after the application of phenolic compounds, ΔVC_{comb} – differences between viable counts after the application of phenolic compounds in a combination and after the application of the most effective single phenolic compound.

of *C. divergens* (at 0 h and 24 h) and *Le. carnosum* (at 0 h and 48 h) without the presence of phenolic compounds or with gallic acid, octyl gallate or both of them in a binary combination at *MIC* values of each phenolic compound. This allowed us to test the antimicrobial activity of each compound as well as the effect of the interaction between them (Tab. 5). In all tested conditions, the growth of two bacteria in ISB and in beef extract was similar in the absence of the phenolic compounds used.

As demontrated in this study, gallic acid is a bacteriostatic agent (< 3 log CFU·ml^{-1} reduction in colony counts compared to the starting inoculum) against *C. divergens* and *Le. carnosum*, regardless of pH, growth medium and protein concentration in beef extract. Generally, a decrease in viable counts was observed with respect to the initial inoculum in practically all conditions for *C. divergens.* However, for *Le. carnosum* this decrease was only observed at pH 6.0. Our results are in agreement with the bacteriostatic effect of gallic acid reported for other Gram-positive bacteria [44, 45].

Octyl gallate was bacteriostatic at pH 5 for the two species and practically bactericidal (≥ 3 log CFU·ml^{-1} reduction in colony counts compared to the starting inoculum) at pH 6 (*MIC* equivalent to minimum bactericidal concentration, *MBC*). So, the results obtained at pH 6.0 show that the differences between *MIC* and *MBC* values of octyl gallate against Gram-positive bacteria were not greater than 2-fold, suggesting that residual bacteriostatic activity was unlikely involved [33, 45].

In our study, the antibacterial effect of the combination of the two phenolic compounds on the growth of *C. divergens* seemed to depend on pH but not of protein concentration. This effect was bacteriostatic at pH 5 and bactericidal at pH 6. However, for *Le. carnosum* the antibacterial effect of the combination seemed to be independent on both pH and protein concentration, always being bactericidal. For *C. divergens* at pH 5, there was an inverse correlation between the decrease in viable counts (with respect to the initial inoculum) and the protein concentration of beef extract (from 2.77 log CFU·ml^{-1} to 30 g·l^{-1} and 0.23 log CFU·ml^{-1} to 120 g·l^{-1}, respectively), suggesting that the protein concentration of beef extract could exert a protective effect regarding the antibacterial action of the combination. However, for *Le. carnosum* and at pH 5 this protective effect was only observed in media with 120 g·l^{-1} beef extract.

The effect of both compounds in a combination against *C. divergens* was synergistic (≥ 2 log CFU·ml^{-1} reduction in viable counts in the combination compared to the most active single phenolic compound) at pH 6 and at 30 g·l^{-1} beef extract. For *Le. carnosum*, the effect of the combination of gallic acid and octyl gallate was mostly synergistic under conditions of the study. On the

other hand, under conditions in which octyl gallate alone showed a bactericidal effect, no synergic effect was detected in the combination with gallic acid, but rather additive effect was observed (Tab. 5, 0 values). No antagonistic effect was detected in the combination of both phenolic compounds in any of the conditions studied. These results are in agreement with those determined by the checkerboard method where non-interaction (equivalent to additive effect) was determined for the binary combination in all conditions for *C. divergens*. However, for *Le. carnosum* the antibacterial synergistic effect of the combination was clearly detected in the growth study but not in the chekerboard analysis (Tab. 4) although this showed the lowest *FICI* values (close to 0.5).

Antioxidant activity of binary combination of gallic acid and octyl gallate

We determined antioxidant activity of individual phenolic compounds used at a concentration of 0.2 mmol·l^{-1} alone and in binary combination, dissolved in beef extract (at 30 g·l^{-1}, 60 g·l^{-1} and 120 g·l^{-1}) at pH 5 and pH 6 by the ABTS assay (Tab. 6). The antioxidant activity appears to be affected by pH and the beef extract concentration in absence of the phenolic compounds. An increase in the antioxidant activity was observed after the addition of gallic acid or octyl gallate, alone or in binary combination. Under these conditions, the activity seemed not to be affected by pH, although it increased with the increase in beef extract concentration. In general, the antioxidant activity of octyl gallate was slightly lower than that of gallic acid in all conditions of this study. A much lower antioxidant activity of octyl gallate than gallic acid was detected when they were dissolved in ethanol. This decrease could be due to esterification of the carboxy group of gallic acid, which is dependent on the length of the alkyl chain [46]. The lower difference in the antioxidant activity obtained for octyl gallate with respect to gallic acid in beef extract was probably due to interactions of some component of the medium (e.g. protein) either with these phenolic compounds and/or with the ABTS assay, since the phenolic compounds used are soluble, as indicated above.

The effect of the binary combination of gallic acid and octyl gallate was antagonistic in all conditions studied. Previously, we had also found antagonistic effect with these phenolic compounds dissolved in ethanol, with gallic acid (with higher antioxidant activity) regenerating octyl gallate (with lower antioxidant activity) [46]. In this case, since the antioxidant activity values of the two phenolic compounds are very similar, antagonism could not be explained by a mechanism of regeneration [24, 46]. Possibly, the presence of proteins in the beef extract could mask the effect of regeneration, which would be interesting since gallic acid would regenerate octyl gallate, the most effective antimicrobial compound against *C. divergens* and *Le. carnosum*.

In conclusion, octyl gallate was the most effective antimicrobial followed by propyl gallate and gallic acid in ISB growth medium, showing the lowest *MIC* values for *Le. carnosum*. For both LAB, the antimicrobial efficacy of gallic acid and octyl gallate alone and in binary combination were influenced by pH variation and not by the protein concentration in the beef extract. Both phenolic compounds were more effective at pH 5.0, and

Tab. 6. Antioxidant activity of gallic acid and octyl gallate alone and in binary combination in beef extract solution.

Beef extract	pH	Antioxidant activity at addition [mmol·l^{-1}]				Effect of binary combination	
		None	Gallic acid	Octyl gallate	Gallic acid + octyl gallate	*ME*	Description of interaction
30 g·l^{-1}	5	0.88 ± 0.05^{A}	1.57 ± 0.11^{A}	$1.13 \pm 0.06^{A*}$	1.99 ± 0.03^{A}	0.74 ± 0.01	Antagonism
	6	0.87 ± 0.01^{a}	1.55 ± 0.14^{a}	1.25 ± 0.08^{a}	1.98 ± 0.11^{a}	0.71 ± 0.05	Antagonism
60 g·l^{-1}	5	$1.38 \pm 0.06^{B*}$	1.79 ± 0.08^{A}	1.45 ± 0.05^{B}	2.12 ± 0.13^{A}	0.65 ± 0.04	Antagonism
	6	1.14 ± 0.09^{b}	1.79 ± 0.12^{a}	1.60 ± 0.18^{b}	2.23 ± 0.05^{b}	0.66 ± 0.01	Antagonism
120 g·l^{-1}	5	$2.21 \pm 0.07^{C*}$	2.38 ± 0.22^{B}	2.05 ± 0.25^{C}	2.66 ± 0.19^{B}	0.60 ± 0.04	Antagonism
	6	1.70 ± 0.08^{c}	2.44 ± 0.05^{b}	2.40 ± 0.01^{c}	2.71 ± 0.08^{c}	0.56 ± 0.02	Antagonism

Antioxidant activity is expressed as millimoles of Trolox equivalent per litre.
Different superscripts in each column indicate significant differences for the respective pH value ($p < 0.05$) calculated using one-way analysis of variance (ANOVA). Uppercase letters regard pH 5, lowercase letters regard pH 6.
* – significant differences ($p < 0.05$) between pH 5 and pH 6; p values were calculated using Student's t-test.
ME – mixture effect on the antioxidant activity in binary combinations of gallic acid and octyl gallate at equimolar concentrations of 0.2 mmol·l^{-1}.

only octyl gallate showed a bactericidal effect at pH 6.0. The binary combination of both phenolic compounds was more effective against *Le. carnosum,* showing a synergistic bactericidal effect at pH 5.0. In general, the use of the binary combination of octyl gallate and gallic acid could be considered to avoid the alteration of the hygienic quality of meat by the two bacteria. It would also provide antioxidant activity, which could aid to maintaining the organoleptic properties of meat.

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