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Inactivation of *Listeria monocytogenes* and *Salmonella* Typhimurium in strawberry juice enriched with strawberry polyphenols

Running title: Strawberry polyphenols as natural antibacterial agent in juice

Claudia Verónica Vallejo[1,2], Carlos Javier Minahk[3], Graciela Celestina Rollán[4] and María José Rodríguez-Vaquero[1,2*]

[1]Institute of Microbiology, Faculty of Biochemistry, Chemistry and Pharmacy (FBQF), National University of Tucumán (UNT) and [2]CONICET, Ayacucho 471, 4000, Tucumán, Argentina, [3]INSIBIO, [4]CERELA, Chacabuco 145, Tucumán, Argentina.

(*) Author to whom correspondence should be addressed: M.J. Rodríguez-Vaquero mariajo@fbqf.unt.edu.ar - Tel: 54-381-4247752 ext. 7067. Ayacucho 471, 4000, Tucumán, Argentina.

ABSTRACT

Background: To extract and identify the low molecular weight phenolic fractions (LMPFs) from *Albion* (LMPF-A) and *Camarosa* (LMPF-C) strawberry cultivars, investigate their antibacterial activities against *Listeria monocytogenes* and *Salmonella* Typhimurium cocktails *in vitro* and *in vivo* using strawberry juice as food model; and also determine the antibacterial mechanism. **Results:** Quercetin was identified as a principal compound in both phenolic fractions and their MIC and MBC values were around 600 and 950 μg mL^{-1} against *S.* Typhimurium and *L. monocytogenes*, respectively. The possible antibacterial activity of phenolic extracts could be related with the release of phosphate and potassium ions mL^{-1}, the dissipation in membrane potential and the disruption of membrane integrity on *L. monocytogenes;* and with the inhibition of NADH oxidase activity on *S.* Typhimurium. Quercetin and kaempferol were the most active compounds in produce bacterial damage. The bacterial viability in strawberry juice supplemented with phenolic fractions and incubated at 37, 20 and 4 ºC reduced bacterial viability, moreover with the combined treatment of phenolic fraction and the lowest temperature no viable cells were detected after 7 days incubation. *Salmonella* was more sensitive than

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Listeria in strawberry juice. **Conclusions:** This study could set the basis for the development of natural antibacterial agents could be included in natural juice or for pharmaceutical industry.

Keywords: phenolic extracts; antibacterial action mechanism; *Salmonella* Typhimurium; *Listeria monocytogenes*, strawberry juice.

INTRODUCTION

The production and export of berries have a relevant place in the economic activities of Argentine and many other countries in the world. In Argentine, during strawberries harvest a large amounts of crushed strawberries are produced and some of them are rot away every year due to the lack of processing [1]. The consumption of fresh strawberry juice in our country is high, and the inherent characteristics of this fruits render them susceptible to microbial contamination and potential vehicles for foodborne illness transmission [2]. Many studies have suggested that *Listeria monocytogenes* and *Salmonella* Typhimurium are the major causes of foodborne outbreaks [3-4].

Conventionally, thermal pasteurization has been used to achieve microbial safety and preservation of fruit juices. However, temperatures used during thermal pasteurization can affect sensory properties and cause deterioration of fruit juice nutritional values [5].

Application of naturally occurring antimicrobial compounds and bio-preservatives is gaining popularity for fruit juice processing as a replacement for thermal pasteurization [6]. Phenolic compounds represent a common constituent of the human diet, they are found in fruit, vegetables and derived products and some authors reported their beneficial properties on human health, including anti-inflammatory activity, enzyme inhibition, antimicrobial activity and anti-oxidant activity [7, 8, 9]. Berries are rich in phenolic compounds and there are many reports about beneficial properties of phenolic compounds for human health, including antimicrobial activity [7-8]. But there are few studies about the antibacterial mechanism of phenolic extracts, especially strawberry extracts. The hypothesis of this investigation is that phenolic compounds from strawberry have antibacterial activity against *L. monocytogenes* and *Salmonella* Typhimurium and the antibacterial mechanism could be related with disruption on membrane integrity or inhibition of NADH oxidase activity.

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The aims of this study were 1) to extract and identify the phenolic fractions from *Albion* and *Camarosa* strawberry cultivars, 2) to determine the antibacterial activity of the phenolic fraction from strawberry cultivars against *Salmonella* Typhimurium and *Listeria monocytogenes* cocktails, 3) to investigate the possible antibacterial mechanisms of action of phenolic fractions and 4) investigate *Salmonella* Typhimurium and *Listeria monocytogenes* survival in strawberry juice as food model system.

MATERIALS AND METHODS

Extraction, identification and quantification of low molecular weight phenolic fractions (LMPFs) of strawberries.

Camarosa and *Albion* strawberries were selected from plantations located in northwestern Argentina, in September and November 2016, respectively. The fruits (1 kg) were washed and the LMPF *Camarosa* (LMPF-C) and LMPF *Albion* (LMPF-A) of their juice was extracted using ethyl acetate as solvent according to the technique described by Ghiselli et al.[10]. Total phenolic compounds in LMPFs were measured using the Folin Ciocalteu method [11] and individual phenolic compounds were identified and quantified by HPLC analysis coupled to a diode array detector according to the technique described by Fanzone et al.[12].

Antibacterial assay

Bacterial strain

The antibacterial activity was determined using 3-strain *Salmonella* Typhimurium cocktail (Typhimurium FBQF, Typhimurium H3, and Typhimurium FBQF Q5) and 3-strain *Listeria monocytogenes* cocktail (*L. monocytogenes* FBQF, *monocytogenes* H26 and *monocytogenes* FBQF A7). The bacterial strains were isolated from foods (*Salmonella* Typhimurium FBQF, FBQF Q5, and *L. monocytogenes* FBQF, FBQF A7) and obtained from culture collection of the Faculty of Biochemistry, Chemistry and Pharmacy (FBQF) of National university of Tucumán. *Salmonella* Typhimurium H3 and *L. monocytogenes* H26 were isolated from human infection and identified in The

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Public Hospital of Tucumán, Argentina. Selection of these strains and the use of bacterial cocktails are carried out to ensure that the antibacterial effect was at least against three different strains of each bacterium. The bacterial strains were grown by two successive loop transfers of individual strains incubated at 37 °C for 24 h in 5 mL BHI medium (Sigma-Aldrich). A final transfer of 0.2 mL was made into 50 mL BHI medium with incubation at 37 °C for 18 h. The bacterial cells were harvested by centrifugation (4000×g, 10min) at 4 °C. Resulting cell pellets were washed twice in 0.1% (w/v) peptone water (PW) and finally suspended in PW. Equal volumes of individual cultures were then mixed to obtain a 3-strain cocktail of *Salmonella* and *Listeria* of cell population of about 10^8 cfu mL^{-1} in the inoculums.

Antibacterial activity of strawberry LMPFs and individual phenolic compounds

The antibacterial activity of strawberry LMPFs and different concentrations of pure individual phenolic compounds (10, 50 and 100 µg mL^{-1}) (Sigma-Aldrich) detected in LMPFs were determined using the agar diffusion test, carried out according to Rodríguez-Vaquero, et al. [7] against selected bacteria. Chloramphenicol (1000 µg mL^{-1}) was used as a positive control and sterile water or ethanol as negative control. After 24 h incubation, inhibition zones were measured to an accuracy of 0.5 mm. For each strawberry LMPF, the minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) were determined following the CLSI guidelines [13]. Briefly, Mueller-Hinton broth supplemented with serial dilutions of LMPF-C and LMPF-A was inoculated individually with bacteria 3-strain cocktail and then cultures were incubated at 37 ºC during 24 h.

Determination of antibacterial mechanism of strawberry LMPF-C and LMPF-A

Listeria monocytogenes H26 and *S.* Typhimurium H3 (isolated from human infections) were selected to investigate the antibacterial mechanism of LMPFs and individual phenolic compounds.

Bacterial survival and cell membrane permeabilization. L. monocytogenes H26 and *S.* Typhimurium H3 cultures were inoculated to obtain approximately 10^8 cfu mL^{-1} in HEPES buffer (Sigma-Aldrich) added with strawberry LMPFs (MBC concentration) and individual phenolic compounds at concentration 10, 50 and 100 µg mL^{-1}. Then, cells were incubated at 37 ºC during 3 hours and viability was assessed using the method of serial dilutions. Bacterial suspensions without ethanol or polyphenols

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were taken as controls. After 3 h incubation, the quantification of the phosphate and potassium released from bacteria was evaluated in those samples of Na-HEPES buffer containing LMPFs or individual phenolic compounds in which a reduction of around 1 log cycle was observed, using Ames´s method [14] and flame photometry Berry et al. [15], respectively.

Measurement of the transmembrane electrical potential

The dissipation of the transmembrane electrical potential ($\Delta\Psi$) was studied only in microorganisms in which intracellular ions were detected in supernatants, following the protocol described by Bennik et al. [16]. Briefly, cells were resuspended at $1.5x10^8$ cells mL^{-1} in 50 mM HEPES-K buffer pH 7.4 containing 10 mM glucose and 0.5 µM of 3,3´-dipropylthiadicarbocyanine iodide ($DiSC_3[5]$). Changes in the fluorescence were measured upon addition of LMPFs or individual phenolic compounds. An ISS PC1 spectrofluorometer was used, adjusting at 30 °C, whereas the excitation and emission wavelengths were set at 622 and 674 nm respectively. A complete dissipation of $\Delta\Psi$ was achieved with 1 µM of valinomycin. ΔpH was dissipated with nigericin.

Electron Microscopy

Transmission electron microscopy was used for evaluating the *L. monocytogenes* H26 membrane integrity after 3 h incubation with strawberry LMPFs. Cell suspensions were concentrated by centrifugation (5000 rpm, 20 min), and fixed with Karnovsky fixative containing 8% (v/v) paraformaldehyde, Na_3PO_4 (0.1M), pH 7.40 and 16% (w/v) glutaraldehyde. Subsequently, samples were cuts using ultra-microtome and mounted on copper grids and contrasted with uranyl acetate solution and with lead citrate. The pellet was analyzed by transmission electron microscopy using a Zeiss EM 109 electron microscope (Carl Zeiss, Germany).

Determination of oxygen consumption and NADH oxidase activity

LMPF-C and LMPF-A (1 µg mL^{-1}) were added in BHI medium the culture media was inoculated individually with *L. monocytogenes* H26 and *S.* Typhimurium H3 (10^8 cfu mL^{-1}); then cultures were incubated at 37 °C for 30 min and the rate of cellular respiration was polarographically measured with an oxygraph-type Clark electrode and normalized to the DO_{600}.

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The NADH oxidase activity in presence of LMPFs was determined in membrane preparations of *L. monocytogenes* H26 and *S.* Typhimurium H3 that were obtained as described by Evans et al. [17]. Measurements were carried out in the presence and the absence of 6 mM potassium cyanide, in a total volume of 0.5 mL of 50 mM phosphate buffer (pH 7.5) containing 0.5 mM NADH as substrate. LMPFs were pre-incubated with membranes (10 µl mL^{-1} proteins) for 10 min at 37 °C. The NADH oxidase activity was measured at 570 nm. The oxygen consumption and NADH oxidase activities of *L. monocytogenes* H26 and *S.* Typhimurium H3 in presence of individual phenolic compounds (100 µg mL^{-1}) was only determined in those samples in which the LMPFs showed an inhibitory effect.

Preparation of strawberry juice and treatments

Strawberry juice was prepared following the protocol described by a regional company. The strawberry was provided by the National Institute of Agricultural Technology (INTA). The fruits were washed and processed until obtaining the fresh juice, then centrifuged (8000 g, 10 min, 5 °C, in Heal Force centrifuge) to separate the pulp. Strawberry juice (15 ± 1 °Brix, pH = 4.0 ± 0.2), was pasteurized using the LTLT (low temperature-long time) method, exposing the juice at 62 °C for 30 min, then it was cooled and maintained at 4 °C. Strawberry juice was bottled under sterility conditions in 200 mL sterile bottles and sealed with caps. Different batches of strawberry juice (1 liter) were prepared with different treatments: a) strawberry juice without bacterial inoculation; b) strawberry juice inoculated individually with *L. monocytogenes* or *S.* Typhimurium cocktails (10^4 cfu mL^{-1}); c) Strawberry juice inoculated with bacteria and added with 1 mg of LMPF-C or LMPF-A obtained from 1 kg of each fruit (1X), with 2 mg of LMPF-C or LMPF-A (2X) and with 3 mg of LMPF-C or LMPF-A (3X). Then, all samples were storage at different temperatures (4, 20 and 37 °C). The survivors of *L. monocytogenes* or *S.* Typhimurium were enumerated at 0 and 7 d. The samples were serially diluted with isotonic solution and spread on Palcam agar or SSA agar. Plates were incubated for 24 h before enumeration.

All assays were carried out by triplicate in two independent experimental runs.

Sensory evaluation of strawberry juice enriched with LMPFs

Quantitative descriptive analysis was used to evaluate if the addition of LMPFs to strawberry juice produce negative effect in the sensory attributes of natural strawberry juice. A teach storage time,

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treated and untreated strawberry juices with and without phenolic extracts addition were subjected to a panel of testers to evaluate the sensory quality of the beverages. Ten judges, aged 25-50 years, with sensory evaluation experience were trained in descriptive evaluation of strawberry juices. The attributes evaluated were: color, odor, flavor and turbidity taste of the beverages. Evaluations were performed immediately after juices removal from storage conditions. The intensity of the attributes evaluated was quantified on an unstructured scale from 0 to 4. Color was rated from 0 (deteriorated color) to 4 (typical color), odor from 0 (intense off-odors) to 4 (fresh) and flavor taste from0 (extremely dislike) to 4 (extremely like) and turbidity was rated from 0 (extremely dislike) to 4 (extremely like). The limit of acceptance was 2.5, indicating that score below 2.5 for any of the attributes evaluated was deemed to indicate end of shelf-life.

Statistical analysis

All experiments were repeated three times with duplicate samples. Data were analyzed by ANOVA using Minitab (Minitab Inc., PA, USA). Multiple means comparison was carried out by Duncan's multiple range tests ($p < 0.05$).

RESULTS AND DISCUSSION

The total phenolic content, the profile and quantification of individual phenolic compounds in strawberry LMPFs are shown in Table 1. The major compound found in strawberries LMPFs was quercetin. Gouveia-Figueira and Castilho [18] reported that the most common flavonols in fruits and vegetables were quercetin and kaempferol.

Table 2 shows the antibacterial activities of strawberry LMPFs and pure phenolic compounds against *L. monocytogenes* and *S.* Typhimurium cocktails. Both strawberries LMPF-C and LMPF-A were effective as antibacterial agent, and quercetin and kaempferol were the most effective individual compounds. Some studies have reported the antimicrobial activity of quercetin and kaempferol rich extracts from different sources against *Listeria monocytogenes* and *Salmonella* Typhimurium [4,19,20,21]. The use of the phenolic fractions of strawberry as a new natural preservative for limiting strawberry juice bacterial contamination without altering sensory or nutritional values of strawberry juice, could

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set the basis for the development of a natural antibacterial agent which could be used in pharmaceutical industry. Hence, the present study would enrich the existing knowledge regarding antibacterial activity of phenolic extracts, indicating that phenolic fractions of strawberry could be an effective natural bactericidal agent against *Listeria monocytogenes* and *Salmonella* Typhimurium to be used in food or alimentary industry". The MIC and MBC of LMPF-C and LMPF-A against *S.* Typhimurium and *L. monocytogenes* cocktails are shown in Table 3. Coincident results were reported by Lima et al. [20] who reported similar values of MIC and MBC on blueberry and grape phenolic extracts rich in quercetin, ρ-coumaric and protocatechic acids, on antibiotic-resistant *Escherichia coli* and *Staphylococcus aureus*. Coman et al. [22] reported the inhibitory effect of phenolic extracts of plums and grapes with MIC values between 7.81 to 15.63 mg L^{-1} for *Bacillus cereus*, *E. coli*, *L. monocytogenes* and *S. aureus*.

The bacterial survival after 3 h incubation in buffer added with strawberry LMPFs and individual phenolic compounds at concentrations of 10, 50 and 100 µg mL^{-1} was determined. With the addition of 10 µg mL^{-1} of LMPFs or individual phenolic compounds there was not observed significantly difference between the numbers of viable cells observed with the control. The addition of 50 µg mL^{-1} of gallic, protocatechuic, ρ-coumaric, ferulic, caffeic, chlorogenic, ellagic acids, catechin, quercetin and kaempferol reduced 0.17; 0.18; 0.32; 0.27; 0.36; 0.27; 0.28; 0.30; 0.42 and 0.39 log cycle the number of viable cells of *L. monocytogenes* H26 and 0.14; 0.25; 0.54; 0.47; 0.55; 0.34; 0.43; 0.41; 0.62; and 0.64 of *S.* Typhimurium H3, respective (Data not shown). The bacterial survival after 3 h incubation in buffer added with strawberry LMPFs and individual phenolic compounds at the concentration of 100 µg mL^{-1}, the quantification of the phosphate and potassium ions released in culture supernatants, the effect on NADH oxidase activity and O_2 consumption by *L. monocytogenes* H26 and *S.* Typhimurium H3 are shown in Table 4. All individual phenolic compounds were effective in inhibiting microbial growth against *L. monocytogenes* H26 and *S.* Typhimurium H3, reducing less than one logarithmic cycle with respect to control without treatment at the concentration of 100 µg mL^{-1}. The most effective phenolic compounds on *L. monocytogenes* H26 and *S.* Typhimurium H3 were ρ-coumaric, caffeic, chlorogenic acids, quercetin and kaempferol. *S.* Typhimurium H3 was more sensitive to phenolic compounds and LMPFs. LMPFs from strawberries failed to induce any release of

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these ions from *S.* Typhimurium H3 cells. The LMPFs from strawberries produce a low inhibition of NADH oxidase activity in *L. monocytogenes* H26 with respect to the control. However, strawberry LMPFs produced NADH oxidase inhibition in *S.* Typhimurium H3 and it was correlated with a decrease in oxygen consumption. Thus, strawberry LMPFs may prevent the growth of *S.* Typhimurium H3 by blocking oxygen consumption and thus inhibiting the activity of NADH oxidase, while ferulic acid, quercetin and kaempferol seem to be responsible for the strongest effect. Some studies have also shown that phenolic compounds available in berries probably play their role by increasing the efflux of ATP from pathogens and impede respiratory metabolism [23].

The changes in the ΔΨ in *L. monocytogenes* H26 and *S.* Typhimurium H3 in presence of both LMPFs were observed in Figure 1 (a). The ΔΨ was dissipated upon addition of both LMPFs only in *L. monocytogenes* H26. The changes in the ΔΨ in *L. monocytogenes* H26 in presence of individual phenolic compounds were observed in Figure 1 (b). The ΔΨ was dissipated upon addition of both LMPFs and individual phenolic compounds to the cell suspension. Quercetin, ρ-coumaric acid and chlorogenic acid, produced the greatest increase of the fluorescence in *L. monocytogenes* H26 cell suspensions. *L. monocytogenes* H26 cell morphology after 3 h contact with LMPFs analyzed by transmission electron microscopy was observed in Figure 2. A disruption in the plasma membrane was observed in presence of strawberry LMPFs. These results suggest that the possible antibacterial mechanism of strawberry LMPFs on *L. monocytogenes* H26 is related to the damage of the cellular envelope. Similar results were reported by Yuan et al. [24], both crude extract and eucalyptol induced membrane damage in *L. monocytogenes*. Our results are coincident with those reported by Cui et al. [25] who demonstrated that phenolic fraction of clove oil affect the cell structure of *L. monocytogenes* and cause irreversible damage to the cell membrane. Other authors reported that crude phenolic extracts show better antimicrobial activity than individual compounds [27, 23, 8, 21]. The differences between the effects caused by LMPF-C and individual phenolic compounds could be due with a synergetic effect between all phenol compounds present in the phenolic fraction. The synergic effect of individual phenolic compounds was reported by Rodríguez-Vaquero et al. [27] in a previous work. Moreover, the

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antimicrobial effect of individual phenolic compounds and phenolic extracts from other sources against other pathogenic microorganisms was also reported [28, 29, 8].

The results of LMPF-C and LMPF-A treatment of strawberry juice inoculated with *L. monocytogenes* and *S.* Typhimurium cocktails are shown in Figure 3a and 3b, respectively. The phenolic fractions (LMPF-C and LMPF-A) treatment in strawberry juice had a lethal effect reducing both microbial prevalence and level. The efficacy of phenolic fractions treatment varied as a function of concentration and temperature for both bacterial pathogens. A steady decrease in the surviving population of *S.* Typhimurium and *L. monocytogenes* cocktails was observed at increasing concentration (1X and 2X). Initial inoculation population (4 log cfu mL^{-1}) in the strawberry juice sample was reduced significantly ($\rho < 0.05$) due to treatment at 3X for 7 days at 4 °C for *Salmonella* and *Listeria* cocktails. Results indicated *Listeria* to be more resistant to phenolic fractions treatment compared to *Salmonell*a in strawberry juice. Similarly, Buzrul et al. [30] reported 2.5 and 3.5 logs reductions for *E. coli* and *L. innocua*, respectively in pineapple juice but the inactivation's were further increased >1 log during storage at 4 °C for 24 h for both bacteria. Flessa et al. [31] studied survival of *Listeria monocytogenes* on intact and cut surface of strawberries at 4 °C and 24 °C for 7 days and reported that populations on cut surfaces remained constant at both temperatures irrespective of initial inoculum a levels throughout the storage period and concluded that *L. monocytogenes* is capable of survival but not growth on the surface of fresh intact or cut strawberries throughout the expected shelf life of the fresh fruit. Golden et al. [32] studied growth of *Salmonella* multiserotypes in fresh cut cantaloupe, honeydew and water melons and observed no growth of *Salmonella* population and little or no decrease in viable populations during 24 h incubation at 5 °C. Reports also indicated that *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 can survive during cold storage and could be recovered from apple, orange, pineapple and white grape juice concentrates and banana puree through 12 weeks of storage at -23 °C [33].

Hemolysis after addition of strawberry PMPF-C and LMPF-A was similar to that of the negative control ($\rho > 0.05$), demonstrating that strawberry LMPFs are not toxic and could be safely applied to

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food. In addition, strawberry juice enriched with LMPF-C and LMPF-A (1X, 2X or 3X) do not modify the sensory attributes of untreated juice (Data not shown).

CONCLUSION

The present work reported the phenolic profile of two strawberry cultivars and their antibacterial effect against *S.* Typhimurium and *L. monocytogenes in vitro* and in juice. Moreover, this study is the first to elucidate their antibacterial mechanism of action against *S.* Typhimurium and *L. monocytogenes*, demonstrating that the antibacterial mechanism was different in both bacteria. Phenolic extracts produce disruption on membrane integrity of *L. monocytogenes*; whereas the same extracts inhibit NADH oxidase activity, avoiding the consumption of oxygen by *S.* Typhimurium. The big finding of this work was that the use of these natural extracts produces a complete inactivation of *Listeria* and *Salmonella* cocktails in strawberry juice, without sensorial attributes modifications. This study could set the basis for the development of natural antibacterial agents, important for food industry.

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Table 1. Total phenolic compounds and phenolic profile in LMPF-A and LMPF-C.

	LMPF-A	LMPF-C
Total phenolic content (μg GAE mg^{-1})	551.93±38.63[a]	589.90±41.29[a]
Phenolic profile (μg mg^{-1})		
Gallic acid	0.06 ± 0.01[a]	0.03 ± 0.01[b]
Protocatechuic acid	0.38 ± 0.03 [a]	0.36 ± 0.02[b]
Trans-caffeic acid	12.14 ± 0.95[a]	2.56 ± 0.18[b]
Ester of ρ-coumaric acid	1.44 ± 0.10[a]	0.68 ± 0.05[b]
Trans-ρ-coumaric acid	2.76 ± 0.19[a]	1.67 ± 0.12[b]
Ferulic acid	0.99 ± 0.01[a]	0.70 ± 0.05[b]
Ellagic acid	10.64 ± 0.74[a]	4.51 ± 0.31[b]
(+)-catechic	11.90 ± 0.83[a]	9.63 ± 0.67[b]
(-)-epicatechic	4.03 ± 0.28[a]	3.37 ± 0.23[b]
Procyanidin	10.44 ± 0.73[a]	3.78 ± 0.26[b]
Kaempferol-3-galactoside	5.84 ± 0.41[a]	8.74 ± 0.61[b]
Kaempferol-3-glucoside	5.44 ± 0.38[a]	6.30 ± 0.44[a]
Quercetin-3-glucuronide	10.24 ± 0.72[a]	6.75 ± 0.47[a]
Quercetin-3-galactoside	10.12 ± 0.71[a]	3.82 ± 0.27[b]
Quercetin-3-glucoside	20.44 ± 1.43[a]	23.07 ± 1.61[a]
Laricitrin-3-galactoside	2.09 ± 0.15[a]	2.29 ± 0.16[a]
Laricitrin-3-glucoside	3.92 ± 0.20[a]	4.60 ± 0.32[b]

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Isoramnetine-3-glucoside	3.61 ± 0.18[a]	3.74 ± 0.26[a]

Different letters in the same row show significant differences ($p < 0.05$).

Table 2. Antibacterial activities of strawberry LMPFs and individual phenolic compounds.

	***L. monocytogenes* cocktail**			***S.* Typhimurium cocktail**		
LMPF-C	++			++		
LMPF-A	++			++		
	Phenolic compound concentration (µg mL^{-1})					
	10	***50***	***100***	***10***	***50***	***100***
Gallic acid	w	+	+	w	+	++
Protocatechuic acid	w	+	+	w	+	++
ρ-coumaric acid	w	+	+	+	++	+++
Ferulic acid	w	+	+	w	+	++
Caffeic acid	w	+	++	+	++	+++
Chlorogenic acid	w	+	++	+	++	+++
Ellagic acid	w	+	+	w	+	++
Catechin	w	+	+	w	+	++
Quercetin	+	++	+++	+	++	+++
Kaempferol	+	++	+++	+	++	+++
Control (+)	+++			+++		
Control (-)	-			-		

Antibacterial activity: Inhibition zone <1 mm, nil (-); Inhibition zone 1-5 mm, weak (w); Inhibition zone 6-11 mm, moderate (+); Inhibition zone 12-19 mm, high (++); inhibition zone >19 mm, strong (+++).

Table 3. MIC and MBC of LMPF-C and LMPF-A against *L. monocytogenes* and *S.* Typhimurium cocktails

	LMPF-C		**LMPF-A**	
	MIC*****	MBC*****	MIC*****	MBC*****
L. monocytogenes cocktail	650 ± 20.00[a]	850 ± 40.00[a]	700 ± 25.00[a]	950 ± 45.00[a]
S. Typhimurium cocktail	600 ± 18.00[b]	750 ± 32.00[b]	660 ± 20.00[b]	800 ± 30.00[b]

*****µg mL^{-1} of phenolic extract. Different letters in the same column show significant differences ($p < 0.05$)

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Table 4. Phosphate and potassium concentration released in culture supernatants, inhibition of NADH oxidase activity and oxygen consumption by *L. monocytogenes* H26 and *S.* Typhimurium H3 after 3 h of incubation in media supplemented with LMPFs and individual phenolic compounds.

	***L. monocytogenes* H26**					***S.* Typhimurium H3**				
Samples	**Log cfu mL^{-1}**	**Potassium release (µg mL^{-1})**	**Phosphate release (µg mL^{-1})**	**NADH inhibition (%)**	**O_2 consumption (%)**	**Log cfu mL^{-1}**	**Potassium release (µg mL^{-1})**	**Phosphate release (µg mL^{-1})**	**NADH inhibition (%)**	**O_2 consumption (%)**
Control	7.17±0.36^{a}					7.04±0.35^{a}				
LMPF-C	6.63±0.33^{a,b}	17.10±0.85^{a}	12.98±0.65^{a}	10.00±0.49^{a}	75.00±3.80^{a}	6.19±0.30^{b}	*Nd*	*Nd*	80.00±3.98^{a}	6.00±0.29^{a}
LMPF-A	6.52±0.32^{b,c}	15.70±0.76^{a,b}	15.87 ±0.79^{b}	13.00±0.65^{b}	70.00±3.50^{a}	6.12±0.31^{b}	*Nd*	*Nd*	85.00±4.22^{a}	5.00±0.16^{b}
Gallic acid	6.69±0.33^{c,a}	6.50±0.32^{c}	5.77±0.29^{c}	*nd*	*Nd*	6.40±0.32^{a,b}	*Nd*	*Nd*	51.67±2.58^{b}	50.35±2.52^{c}
Protocatechuic acid	6.56±0.32^{b,c}	7.10±0.35^{c,d}	6.55±0.33^{d}	*Nd*	*Nd*	6.37±0.32^{a,b}	*Nd*	*Nd*	50.31±2.52^{b}	24.00±1.23^{d}
ρ-coumaric acid	6.51±0.32^{c}	7.30±0.36^{d,e}	6.45±0.19^{d}	*Nd*	*Nd*	6.08±0.30^{b}	*Nd*	*Nd*	32.76±1.64^{c}	31.09±1.57^{e}
Ferulic acid	6.49±0.31^{c}	7.55±0.38^{d,f}	7.09±0.35^{d,e}	*Nd*	*Nd*	6.03±0.29^{b}	*Nd*	*Nd*	56.67±2.83^{b}	40.73±2.05^{f}
Caffeic acid	6.37±0.30^{c,d}	7.23±0.36^{d,e,f}	6.98±0.35^{e}	*Nd*	*Nd*	5.93±0.28^{b}	*Nd*	*Nd*	36.21±1.81^{c,d}	25.76±1.29^{d}
Chlorogenic acid	6.48±0.32^{a,b,c}	7.11±0.35^{d}	7.01±0.37^{d,e}	*Nd*	*Nd*	5.89±0.27^{b}	*Nd*	*Nd*	50.00±2.48^{b}	45.70±2.29^{c}
Ellagic acid	6.51±0.33^{c}	7.24±0.36^{d}	6.99±0.36^{d,e}	*Nd*	*Nd*	6.10±0.30^{b}	*Nd*	*Nd*	48.00±2.33^{b,e}	42.00±2.13^{f}
Catechin	6.53±0.34^{a,c,d}	7.97±0.40^{d,g}	6.59±0.33^{d,e}	*Nd*	*Nd*	6.11±0.31^{b}	*Nd*	*Nd*	53.00±2.76^{b}	39.00±1.97^{f}
Quercetin	6.36±0.31^{b,d}	9.26±0.46^{h}	8.60±0.43^{f}	*Nd*	*Nd*	5.75±0.28^{b}	*Nd*	*Nd*	63.33±3.17^{f}	48.12±0.42^{c}
Kaempferol	6.42±0.32^{c,d}	9.31±0.46^{h}	9.08±0.45^{f,g}	*Nd*	*Nd*	5.81±0.29^{b}	*Nd*	*Nd*	58.00±2.99^{b}	45.11±2.27^{c}
Positive control	100.00±6.21^{e}	100.00±6.21^{i}	100.00±6.21 ^{h}	100.00±6.21^{c}	*Nd*	100.00±6.21^{c}	+	+	100.00±6.21^{g}	*Nd*
Negative control	*Nd*	*Nd*	*Nd*	*Nd*	100.00±6.21^{b}	*Nd*	*Nd*	*Nd*	*Nd*	100.00±6.21^{g}

Different letters in the same column show significant differences ($p < 0.05$).
nd: no determine.
Nd: No detected

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Figure legends

Figure 1. Membrane potential of glucose-energized of *L. monocytogenes* H26 and *S.* Typhimurium H3 after addition of LMPF-A (■) (□) and LMPF-C (●) (○) strawberry cultivars, respectively **(a).** Effect of individual phenolic compounds: gallic (▣), chlorogenic (●) ρ-coumaric (◇), protocatechuic (●), ferulic (✱), caffeic (×), ellagic acid (◈), catechin (○) and kaempferol (□) and quercetin (▲) on the membrane potential of glucose-energized *L. monocytogenes* H26 **(b)**. Cells treated with $DiSC_3[5]$ and suspended in 10 mM glucose were treated for indicated times with 1 µM nigericin, 1 µM valinomycin (△) and negative control (▲). Each value in the figure is representative of at least 4 independent experiments.

Figure 2. Electron microscopy analysis of effect of LMPFs on *L. monocytogenes* H26 with a magnification of 12800x of in control buffer (a) and buffer added with LMPF-A (b) and LMPF-C (c) strawberry cultivars, after 3h of contact.

Figure 3. Survival of *L. monocytogenes* (a) and *S.* Typhimurium (b) cocktails in strawberry juice control (□) and strawberry juice supplemented with LMPF-C and LMPF-A 1x (▣), 2x (▣) or 3x (■), after 7 days incubation at different temperatures (37, 20 and 4° C).

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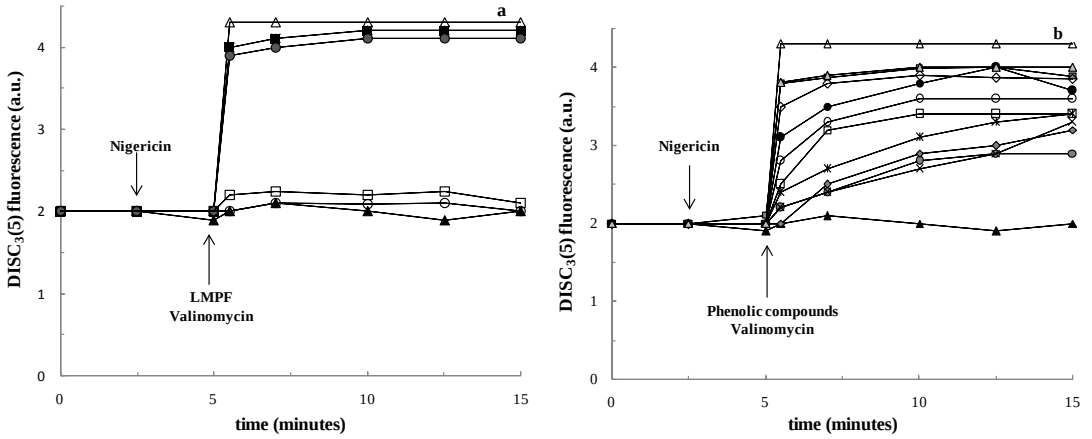

Figure 1

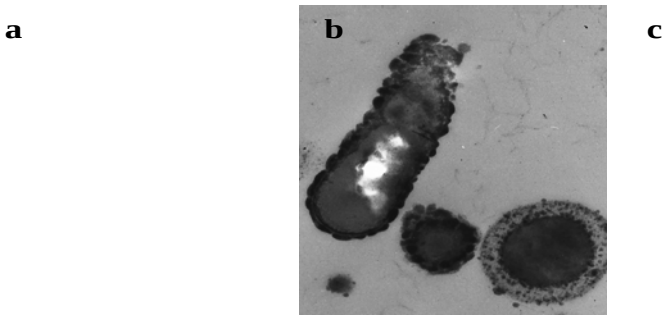

Figure 2

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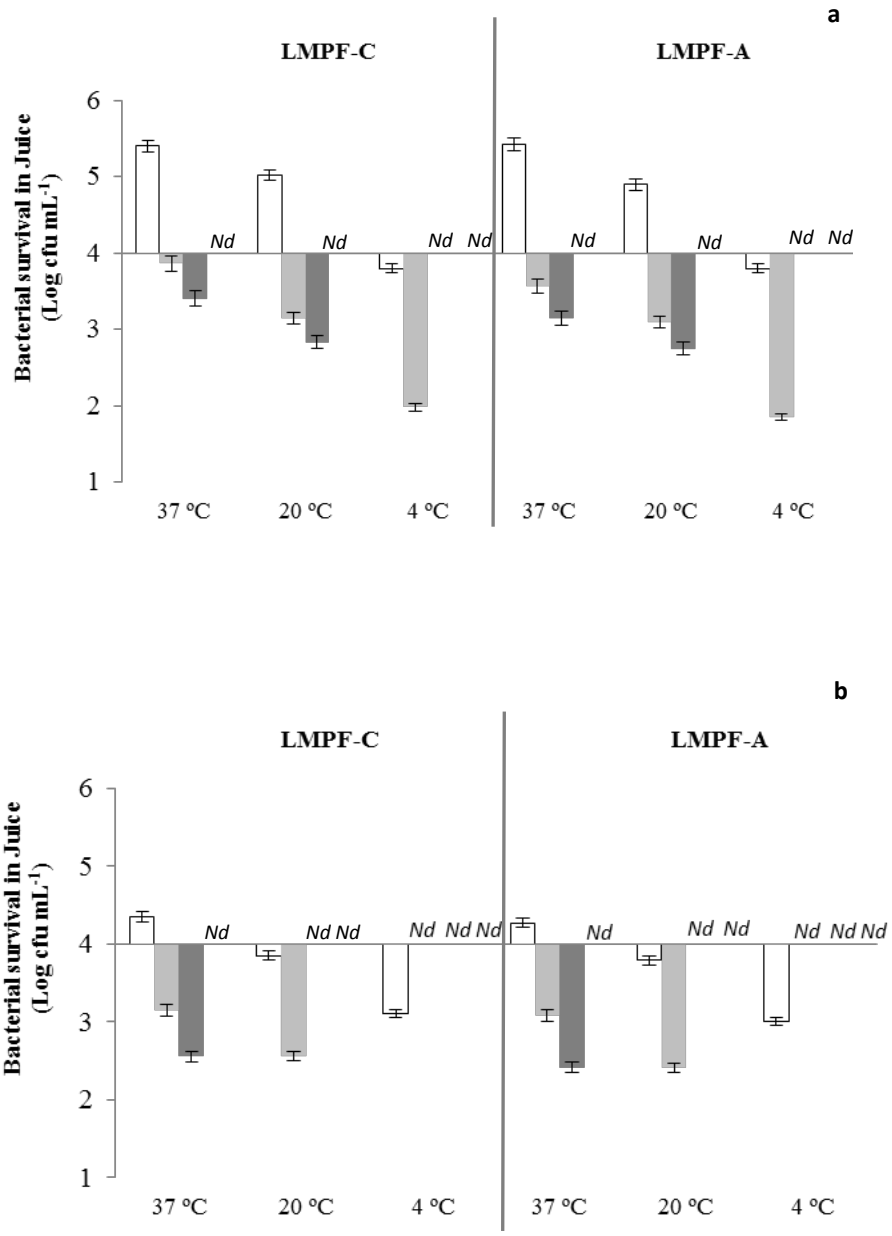

Figure 3

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