Effect of interactions of plant phenolics with bovine meat proteins on their antibacterial activity

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Chemical compounds studied in this article:
Beef extract powder, dimethyl sulfoxide (PubChem CID: 679) (purity = 99.5 %)
and phenolics (gallocyanin (7-dimethylamino-4-hydroxy-3-oxo-phenoxazine-1-carboxylic acid) (PubChem CID: 73801)
isobutyl-4-hydroxybenzoate (PubChem CID: 20240)
resveratrol (3,4′,5-trihydroxy-trans-stilbene) (PubChem CID: 445154)
naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) (PubChem CID: 10141)
chrysin (5,7-dihydroxyflavone) (PubChem CID: 5281607)
and nisin (PubChem CID: 16219761)
(supplied as a 2.5 % (w/w) nisin commercial preparation (Nisaplin®, Danisco, DuPont Nutrition and Health) were purchased from Sigma Aldrich (St Quentin Fallavier, France)
Acetic acid (PubChem CID: 176)
sodium azide (NaN_3) (PubChem CID: 33557)
sodium hydroxide (NaOH) (PubChem CID: 14798)
sodium chloride (NaCl) (PubChem CID: 5234)
hydrochloric acid (HCl) (PubChem CID: 313)
acetonitrile (CH_3CN) (PubChem CID: 6342) (purity = 99.9 %)
chloroform ($CHCl_3$) (PubChem CID: 6212)

ABSTRACT

The activity of 5 phenolics totally inhibiting the growth of *Staphylococcus aureus* CNRZ3 at a 1 g L^{-1} concentration in Mueller-Hinton broth for 24 h incubation at 37 °C was reevaluated at 37 °C for 24 h, 15 °C for 6 days, or 6 °C for 8 days in the presence of up to 20% (w/w) bovine meat proteins to mimic the temperature of refrigerated storage of bovine meat and its protein content, respectively. These changes affected in a different way the antibacterial activity of the 5 phenolics. Isobutyl-4-hydroxybenzoate kept its bactericidal activity, while naphthazarin was bactericidal at 6 °C and 15 °C but not at 37 °C in the presence of bovine meat proteins. Gallocyanin was bactericidal at 37 °C up to a 5% (w/w) protein content in the medium but not at 15 °C or 6 °C. Resveratrol and chrysin always lost their bacteriostatic activity when bovine meat proteins were added. The partition coefficient at 6 °C of each phenolic between a 20% (w/w) bovine meat extract suspension with and without proteins was determined. The antibacterial activity reduction of phenolics in the presence of bovine meat proteins was correlated with their affinity for bovine meat proteins.

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(purity = 99.9 %) and hexadecane ($CH_3(CH_2)_{14}CH_3$) (PubChem CID: 11006) (purity = 99%) were from Sigma Chemical (Schnelldorf, Germany). The bacterial culture media Mueller Hinton Broth (MHB) and Agar (MHA) were from Biokar Diagnostics (Allonne, France)

1. Introduction

Increased interest for natural antimicrobial agents capable of controlling microbial contamination and spoilage in the food sector has been stimulated by concerns such as the wide use of antibiotics and biocides which favored the selection of resistant microorganisms and by the quest for safer alternatives to food preservatives such as sulfites and nitrites. In this context, plant extracts are promising sources of natural antimicrobial molecules such as some phenolics (Daglia, 2012) present in significant amounts in their composition. However, as many antimicrobial molecules, these compounds often have a far lower antibacterial activity in food matrices than *in vitro* in standard microbiological culture media used for their screening (Weiss, Loeffler, & Terjung, 2015). Real perishable food matrices such as raw meat generally have a far more complex composition and structure than microbiological culture media such as nutrient broth or agar. In food matrices such as raw minced meat, which contains up to 20% (w/w) protein and fat, phenolics can interact with either meat proteins or fat at the expense of their interaction with target bacterial cells thus decreasing their antibacterial activity (Weiss et al., 2015). Therefore, in order to investigate the impact of protein-phenolics interactions on the antibacterial activity of phenolics in meat, the antibacterial activity of 5 phenolics with different chemical structures inhibiting the growth of a *Staphylococcus aureus* strain was assayed in Mueller-Hinton broth supplemented with up to 20% (w/w) bovine meat proteins. These antibacterial activity assays were performed either at 37 °C (*S. aureus* optimal growth temperature), 15 °C, and 6 °C (to mimic temperature abuse conditions of refrigerated food products and refrigeration conditions, respectively).

S. aureus is a Gram-positive bacterium, it has an extraordinary ability to colonize and survive for long periods in a wide variety of environmental niches (Ou et al., 2017). Its presence in food is often attributed to insufficient hygiene when handling the product (Da Silva Malheiros, dos Passos, Casarin, Serraglio, & Tondo, 2010). The presence of enterotoxigenic strains of *S. aureus* is a food safety concern because of their ability to produce intoxication or poisoning of food products including meat (Blaiotta et al., 2004). The partition coefficient at 6 °C of phenolics between a phase with and a phase without bovine meat proteins was determined experimentally in order to assess whether antibacterial activity loss of phenolics in the presence of meat proteins at refrigeration temperature was correlated with their affinity for meat proteins. Membrane disruption is the most frequently reported mechanism of antibacterial action of plant phenolics (Rempe, Burris, Lenaghan, & Stewart, 2017). We considered thus the interactions between phenolics and the surface of *S. aureus* cells as a key parameter conditioning their antibacterial activity. Therefore, the surface properties of *S. aureus* CNRZ3 strain used in this study were estimated by microbial adhesion to solvents and zeta-potential measurements. The differences susceptibility to bovine meat proteins addition of antibacterial activity could thus be discussed considering the interactions between each phenolic, *S. aureus* cells surface, and bovine meat proteins.

2. Materials and methods

2.1. Bacterial strain and growth conditions

S. aureus CNRZ3 strain was stored at - 40 °C in MHB containing 15% (v/v) glycerol. Bacterial cells were sub-cultured and grown in MHB as follows: 9 mL of MHB were inoculated with 1 mL of the stock culture and incubated at 37 °C for 8 h. Then, 1 mL of this first culture was inoculated again with 9 mL of fresh MHB and incubated overnight. Finally, a third culture was prepared from the second one (same conditions) and used for bacterial cells surface characterization and antibacterial susceptibility testing.

2.2. Antibacterial activity assay

The antibacterial activity of each phenolic compound was assessed by monitoring the growth of *S. aureus* CNRZ3 at 37 °C for 24 h, 15 °C for 6 days, and 6 °C for 8 days. The antibacterial activity assays were conducted in the presence or absence (control) of each phenolic compound diluted in dimethyl sulfoxide (DMSO) 1% (v/v, distilled water) at a final 1 g L^{-1} concentration in MHB supplemented with 4 concentrations (5, 10, 15, or 20% w/v) of bovine meat proteins (by adding beef extract powder) at pH 7.0. MHB without any added beef proteins (0.15% of proteins initially present in the culture media) (control) was also considered.

The broth microdilution method was used for antibacterial susceptibility testing performed as outlined in the National Committee for Clinical Laboratory Standards (2004). Briefly, 270 μL of broth culture media (MHB with or without added proteins) alone (control) or supplemented with phenolics (final concentrations: 1 mg of powder *per* milliliter of culture medium) were mixed either with 30 μL of bacterial inocula (5.0 10^6 CFU (colony forming units) mL^{-1}) in the exponential phase or 30 μL of sterile broth culture media (control) in each well of the microplate and incubated at 37 °C for 24 h, at 15 °C for 6 days, or at 6 °C for 8 days in a Bioscreen C apparatus (Oy Growth Curves AB Ltd., Helsinki, Finland). The optical density of the culture was monitored every 15 min, in the 420 nm - 580 nm wavelength range ($OD_{420\text{-}580}$). Positive control wells containing 2000 IU mL^{-1} nisin were also monitored. Briefly, the $OD_{420\text{-}580}$ was determined just prior to incubation (t_0) and again after 24 h, 6 days or 8 days incubation at 37 °C, 15 °C, or 6 °C, respectively (t_{final}). The $OD_{420\text{-}580}$ for each replicate at t_0 was subtracted from the $OD_{420\text{-}580}$ for each replicate at t_{final}. The adjusted $OD_{420\text{-}580}$ of each control well was then assigned a value of 100% growth. The percent reduction of $OD_{420\text{-}580}$ after 24 h, 6 days or 8 days incubation at 37 °C, 15 °C, or 6 °C, respectively, was thus determined using the formula:

Percent reduction of $OD_{420\text{-}580}$ after 24 h, 6 or 8 days of incubation = (1 − $OD_{420\text{-}580}$ of test well/$OD_{420\text{-}580}$ of corresponding control well) × 100

All measurements were performed in triplicate.

After screening, *S. aureus* cells were enumerated, 10 μL of the suspension from each inoculated well were spotted on plates

containing MHA, and then incubated at 37 °C for 24 h. Data were expressed as log CFU *per* mL of suspension. If no growth occurred, this indicates that the compound was bactericidal. Serial dilutions of phenolics totally inhibiting the growth of *S. aureus* for 24 h at 37 °C or having killed all bacteria after 24 h incubation at 37 °C allowed to estimate their Minimal Inhibitory (MIC) and Bactericidal Concentrations (MBC), respectively.

2.3. Response surface design

The antibacterial susceptibility testing of the 5 phenolics has been designed by response surface hexagonal design (Design-Expert, version 6.0.4).

Response surface methodology is generally used to explore a quadratic relationship between explanatory variables and one or more response variables, and then an optimal response can be obtained from a sequence of designed experiments. Two numerical factors have been considered for this design: factor A (protein content had 5 levels (0.15, 5, 10, 15, and 20% (w/w))) and factor B (temperature) had 3 levels (6 °C, 15 °C, and, 37 °C). The design was duplicated for every combination of the categorical factor (C) level: *S. aureus* with each of the 5 phenolics at a 1 g L^{-1} concentration, negative (*S. aureus* without any phenolics) and positive controls (*S. aureus* with nisin). For each of these 7 categorical factors, 10 experiments were performed: 4 repetitions of the central point (10% (w/w) protein content and 15 °C) and 6 other combinations of protein contents and incubation temperatures except for positive controls (3 repetitions of the central point and 7 other combinations of protein contents and incubation temperatures). The quadratic model designed, thus based on 70 experiments, is considered as first order model:

$$Y = b_0 + \Sigma\, b_i\, X_i.$$

The count (log CFU mL^{-1}) was the response variable Y, b_0 was estimated at +7.21 in the final equation in terms of the coded factors X_i which correspond to the factors described above (A, B, C, A2, B2 and combination of these factors to each other; AC, AB, BC).

ANOVA for response surface quadratic model was evaluated. The model F-value of 13.43 implies the model is significant. Values of $P > F$ less than $0.01\% < 0.05$ indicate model terms are significant. The coefficients of determination R^2 and adjusted R^2 were found to be equal to 0.8704 and 0.8056, respectively. Based on these statistical indicators the model can be validated.

The optimal response corresponded to a minimal *S. aureus* population after 24 h, 6 days or 8 days incubation at 37 °C, 15 °C or 6 °C, respectively.

2.4. Partitioning of phenolics between a phase with bovine meat proteins and a phase without bovine meat proteins (ultrafiltrate (<3 kDa) of a bovine meat proteins suspension)

2.4.1. Dialysis

Bovine meat proteins suspension (20% (w/w)) was prepared by direct rehydration of beef extract powder with distilled water at pH 7.0. Proteins which have a molecular weight exceeding 3 kDa were removed from this suspension by ultrafiltration onto a 3 kDa cut-off membrane with a 3 kDa Microsep™ Advance Centrifugal Device (Pall Life Sciences, Port Washington, NY). Membranes with a 3 kDa cut-off were used since short polypeptides, containing less than 20–30 residues (i. e. having a less than 3 kDa molecular weight) are considered to be peptides and not proteins. In order to estimate the affinity of each phenolic for bovine meat proteins with a molecular weight exceeding 3 kDa, 1 mL of ultrafiltrate with 1 g L^{-1} of each phenolic was placed into a dialysis tube (Spectra/Por Float-A-Lyzer G2, G235029, cut-off threshold 3.5–5 kDa) which was surrounded by 20 mL of bovine meat proteins (20% (w/w)) suspension. The system was continuously stirred with a magnetic stirrer and placed in dark at 6 °C for 2 weeks. Sodium azide 0.05% (w/v) was added to prevent any microbial contamination.

2.4.2. Determination of partitioning coefficients of phenolic compounds between bovine meat proteins suspension (20% (w/w)) and its ultrafiltrate (<3 kDa) without bovine meat proteins

The partitioning coefficient between bovine meat proteins suspension (20% (w/w)) and its ultrafiltrate (<3 kDa) is defined as follows:

$$\log P_{\text{phase with bovine meat proteins/phase without bovine meat proteins (<3 kDa)}} = \log\left([\text{phenolic in the 20\% (w/w) bovine meat proteins suspension}]_{\text{at equilibrium}} / [\text{phenolic in the <3 kDa corresponding ultrafiltrate}]_{\text{at equilibrium}}\right)$$

The concentration of each phenolic was assayed by reversed-phase high performance liquid chromatography (RP-HPLC) in the phase without meat proteins (<3 kDa ultrafiltrate). Monitoring over time of phenolics concentration in this phase allowed to check that equilibrium was reached within 6 days at 6 °C. A Shimadzu RP-HPLC system (Kyoto, Japan) controlled by a LC solution software was used. Twenty microliters of each sample were directly injected into RP-HPLC system using an automatic sampling system. An Omnispher C_{18} Chromsep column, 110 Å (250 × 4.6 mm, 5 μm) (Agilent Technologies, Les Ulis, France) in an oven maintained at 30 °C was used. The mobile phase was water acidified with acetic acid (pH 2.65) (eluent A) and acetonitrile (eluent B) at a flow rate of 1.2 mL min^{-1}, according to a linear gradient given; % B (0.1% 0–35 min, 24% 35–40 min, 40% 40–45 min, 80% 45–60 min, and 0.1% 60–65 min). The phenolics were quantified at a wavelength of 190–600 nm depending on their absorbance spectra.

2.5. Cell surface properties of S. aureus CNRZ3

2.5.1. Microbial Adhesion To Solvents (MATS)

The hydrophobicity and the electron-donor/electron-acceptor character, i.e. Lewis acid base of cell surface of *S. aureus* was evaluated by Microbial Adhesion To Solvents (MATS) according to the method proposed by Bellon-Fontaine, Rault, and van Oss (1996).

This partitioning is based on the comparison between microbial cell affinity to a monopolar solvent and an apolar solvent (chloroform, an acidic solvent (electron accepting) or hexadecane (apolar *n*-alkane)).

Experimentally, *S. aureus* cells were harvested by centrifugation at 7000×*g* for 10 min at 4 °C, washed twice in physiological water (150 mM NaCl) and resuspended to $OD_{400nm} = 0.8$.

0.4 mL of the solvent under investigation was added to 2.4 mL of cell suspension. The two-phase system was mixed with a vortex for 2 min and allowed to separate for 15 min to ensure complete separation of the two phases before sampling. One milliliter was carefully removed with a Pasteur pipette from the aqueous phase and the OD_{400nm} was measured. The percentage of microbial adhesion to solvent was calculated as follows:

$$(1 - OD/OD_0) \times 100$$

where OD_0 and OD are the optical density measured at 400 nm of the bacterial suspension before and after mixing, respectively. Each

MATS experiment was performed in triplicate.

2.5.2. Assessment of bacterial cells surface charge by zeta potential measurements

The electrical properties of the cell surface of *S. aureus* CNRZ3 were assessed by microelectrophoresis. The zeta potential (ZP) was evaluated by the measurement of the electrophoretic mobility of *S. aureus* cells as described by Ly-Chatain, Le, Thanh, Belin, and Waché (2010). Briefly, bacterial cells in the stationary phase, were harvested by centrifugation at 7000×*g* for 5 min, washed twice in physiological water (150 mM NaCl) and resuspended at about 10^7 cells mL^{-1}. pHs were adjusted to pH 7.0 before measurements. The ZP was evaluated in triplicate at room temperature with a NanoZS90 Zetasizer (Malvern Instruments, Malvern, UK). It is expressed in mV and derived from the velocity of the bacteria in suspension under an applied electric field of 100 mV.

2.6. Statistical analysis

Results are presented as mean values and standard deviations (SD). The collected data were subjected to two-way analysis of variance (ANOVA) by Fisher's test (F) to compare the means using the Microsoft Excel Stat package. The level of significance was set at $P < 0.05$ for all comparisons.

3. Results and discussion

3.1. Antibacterial activity of phenolics in the absence of beef proteins

Five out of 36 phenolics inhibiting the growth of *S. aureus* at 37 °C in MHB for 24 h at a 1 g L^{-1} concentration were selected following a screening of 117 pure phenolics (data not shown). These 5 molecules were chosen since they belonged to various phenolics groups based on their number of carbons and since they had different physico-chemical properties: 2 benzoic acids (C6-C1) (one *para*-hydroxybenzoate ester (paraben) well known for its antibacterial properties (isobutyl-4-hydroxybenzoate) and one positively charged and thus far more hydrophilic molecule (gallocyanin)), 1 flavonoid (chrysin, C15), 1 naphtoquinone (naphthazarin, C10), and 1 stilbenoid (resveratrol, C6-C2-C6). Chrysin, naphthazarin, and resveratrol have been reported to be present in various medicinal plants, plant foods and herbal sources such as passion flower (*Passiflora incarnata* and other *Passiflora* species) (Shin, Kwon, Kim, Shin, & Kim, 2009), endemic Turkish *Alkanna* species (Pekin et al., 2007), and grapes, peanut, soy, wine and Itadori tea (Burns, Yokota, Ashihara, Lean, & Crozier, 2002), respectively. Their MIC or MBC values are stated in Table 1.

The criterion for their selection means that these compounds all had a MIC ≤ 1 g L^{-1}. At a 1 g L^{-1} concentration, 3 out of 5 phenolics were bactericidal: gallocyanin, isobutyl-4-hydroxybenzoate, and naphthazarin, while chrysin and resveratrol were bacteriostatic. It is also noteworthy that naphthazarin had a far lower MBC (0.05 g L^{-1}) than gallocyanin and iso-butyl-4-hydroxybenzoate (MBC = 1 g L^{-1}).

3.2. Antibacterial activity of phenolics in the presence of bovine meat proteins

The growth curves of *S. aureus* CNRZ3 cells for 24 h incubation at 37 °C in MHB and in MHB supplemented with 20% (w/w) bovine meat proteins are presented in Fig. 1A and B, respectively.

No *S. aureus* growth was observed over 24 h incubation at 37 °C in the presence of 1 g L^{-1} of each phenolic in MHB (Fig. 1A): this is consistent with their lower than 1 g L^{-1} MIC or MBC reported in Table 1. While nisin (positive control, 2000 IU mL^{-1}), gallocyanin and isobutyl-4-hydroxybenzoate (at 1 g L^{-1}) kept their bacteriostatic action over 24 h incubation in MHB supplemented with 20% (w/w) bovine meat protein, chrysin, resveratrol and to a lesser extent naphthazarin had no more bacteriostatic action (Fig. 1B). This means that in presence of the same bovine meat proteins content than in raw meat, some phenolics kept their antibacterial activity while others did not.

In order to study more in depth this difference of susceptibility of the antibacterial activity of the 5 phenolics to bovine meat proteins addition, a 5.0 10^5 CFU mL^{-1} *S. aureus* CNRZ3 cells inoculum was incubated in MHB supplemented or not with up to 20% (w/w) bovine meat proteins in the presence of 1 g L^{-1} of each phenolic at 37 °C, 15 °C or 6 °C for 24 h, 6 days or 8 days, respectively. Culturable *S. aureus* CNRZ3 cells were then enumerated (Table 2).

The observation that no *S. aureus* CNRZ3 cells (i. e. population <1 CFU mL^{-1}) could be enumerated in the presence of 1 g L^{-1} of gallocyanin, isobutyl-4-hydroxybenzoate or naphthazarin after 24 h incubation at 37 °C in MHB without added bovine meat proteins is consistent with the fact that these 3 phenolics were bactericidal unlike resveratrol and chrysin which were bacteriostatic (Table 1). After 24 h incubation at 37 °C, only isobutyl-4-hydroxybenzoate was still bactericidal in MHB supplemented with 20% (w/w) bovine meat proteins, while gallocyanin and naphthazarin were still bactericidal when 5% (w/w) bovine meat proteins were added but not 10% (w/w).

Most of *in vitro* screening experiments of antibacterial compounds are performed at the optimal temperature of growth of the target micro-organisms (e. g. 37 °C for *S. aureus* CNRZ3), while their final application mainly concerns refrigerated perishable foods. Therefore, the antibacterial activity of phenolics was also assayed after 8 days at 6 °C or 6 days at 15 °C to mimic refrigeration temperatures and temperature abuse conditions, respectively.

The bactericidal action of nisin (positive control) and isobutyl-4-hydroxybenzoate was also preserved after 6 days at 15 °C or 8 days at 6 °C, while chrysin and resveratrol had no significant bactericidal action whatever the temperature of incubation. Interestingly unlike following a 24 h incubation at 37 °C, naphthazarin kept its bactericidal activity after 6 days incubation at 15 °C or 8 days incubation at 6 °C even in MHB supplemented with 20% (w/w) bovine meat proteins.

The set of data obtained with the enumeration of culturable *S. aureus* cells after 24 h, 6 days or 8 days incubation at 37 °C, 15 °C, or 6 °C, respectively in MHB with varying protein concentrations was exploited to build a response surface design model as a function of incubation temperature and protein content in MHB. A

Table 1
Minimal Inhibitory (MIC) or Bactericidal (MBC) Concentrations of phenolics against *S. aureus* CNRZ3 strain determined after 24 h incubation at 37 °C in Mueller Hinton broth (n = 3).

phenolic compound	gallocyanin	isobutyl-4-hydroxybenzoate	resveratrol	naphthazarin	chrysin
bacteriostatic (BS) or bactericidal (BC) at a 1 g L^{-1} concentration	BC	BC	BS	BC	BS
corresponding MIC or MBC	MBC = 1 g L^{-1}	MBC = 1 g L^{-1}	MIC = 1 g L^{-1}	MBC = 0.05 g L^{-1}	MIC = 1 g L^{-1}

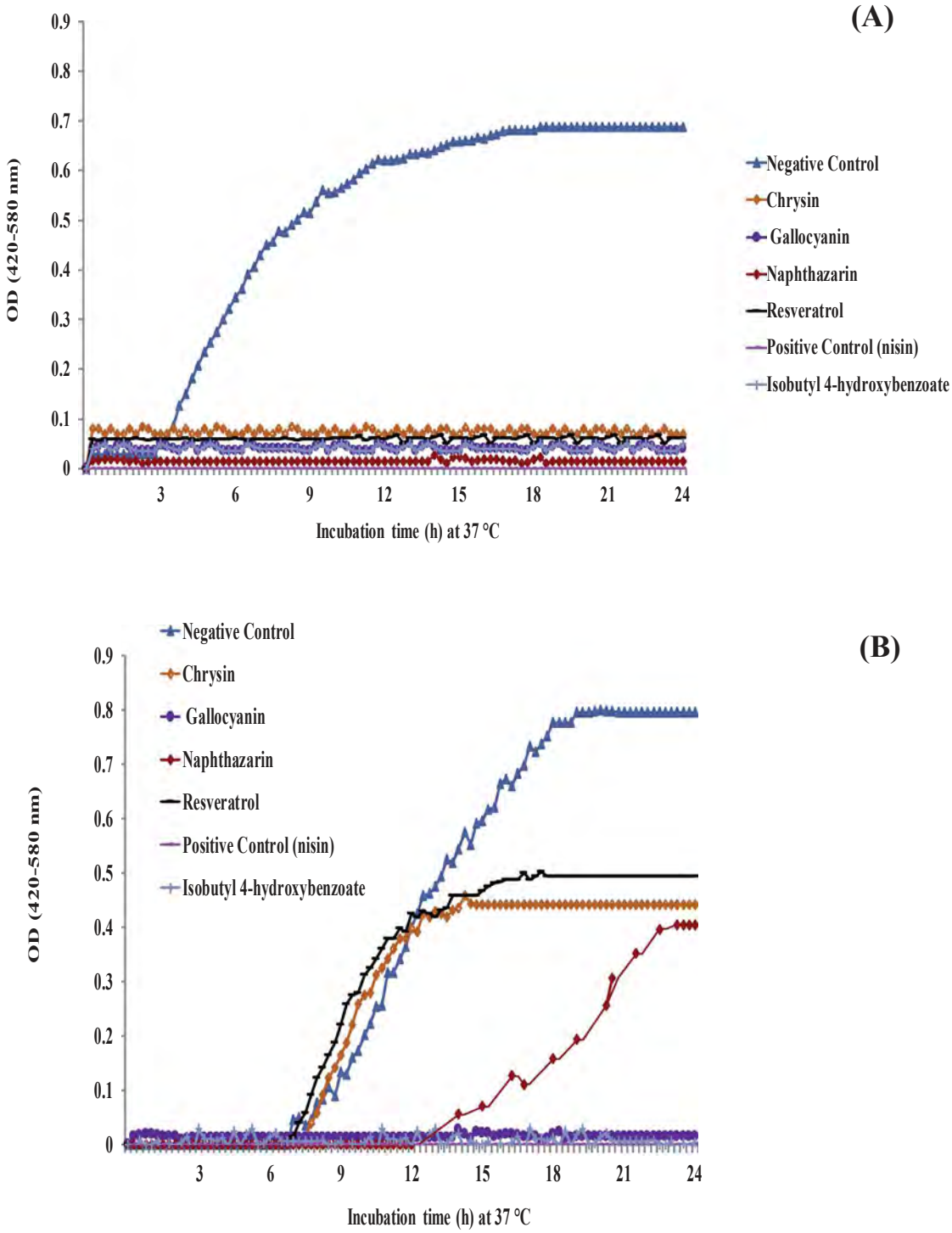


Fig. 1. Effect of phenolic compounds addition (1 g L^{-1}) on the growth of *S. aureus* CNRZ3 at 37 °C in the absence (A) and presence (B) of bovine meat extract (20% (w/v) of proteins).

response of *S. aureus* (expressed as microbial counts in CFU mL^{-1}) to the 5 phenolics was thus obtained for every combination of temperature associated to an incubation time and protein content (Fig. 2).

The shapes of response surfaces were similar after contact with nisin (2000 IU mL^{-1} i. e. 50 mg L^{-1}) or isobutyl-4-hydroxybenzoate (1 g L^{-1}) (Fig. 2B and C). These 2 compounds were the only ones to exert a bactericidal effect whatever the incubation temperature and the protein content added to MHB. In the case of naphthazarin (Fig. 2D), a minimal *S. aureus* population (i. e. < 1 log CFU mL^{-1}) was obtained after incubation at 15 °C and 6 °C whatever the protein content, while this was only observed in the presence of less than 5% (w/v) protein following an incubation at 37 °C.

A less than 1 log CFU mL^{-1} *S. aureus* population was only obtained following 24 h incubation at 37 °C in the presence of less than 5% (w/w) protein with gallocyanin (Fig. 2E). It can also be

Table 2
Enumeration of *S. aureus* CNRZ3 following incubation (at 6, 15, and 37 °C, for 8, 6, and 1 days, respectively) in the presence or absence (negative control) of 1 g L^{-1} of phenolics or 50 mg L^{-1} of nisin (positive control) and 5 different bovine meat proteins concentrations (0.15, 5, 10, 15, and 20% (w/w)). (*S. aureus* CNRZ 3 cells were enumerated on Mueller-Hinton agar following 24 h incubation at 37 °C).

Incubation temperature (°C)	Bovine meat proteins concentration (% (w/w))	*S. aureus* CNRZ3 N (log CFU mL^{-1} expressed as mean ± SD (n = 3))						
		Negative control	Positive control (Nisin)	Isobutyl-4-hydroxybenzoate	Resveratrol	Chrysin	Gallocyanin	Naphthazarin
6	0.15	4.3 ± 0.3^{ab}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	4.0 ± 0.0^{cd}	4.5 ± 0.3^{a}	4.2 ± 0.3^{bc}	$<1.0 \pm 0.0^{g}$
	5	4.5 ± 0.4^{a}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	3.5 ± 0.1^{e}	3.2 ± 0.4^{f}	3.5 ± 0.4^{e}	$<1.0 \pm 0.0^{g}$
	10	4.2 ± 0.2^{bc}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	3.5 ± 0.2^{e}	4.2 ± 0.1^{bc}	3.9 ± 0.2^{d}	$<1.0 \pm 0.0^{g}$
	15	3.3 ± 0.2^{ef}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	3.5 ± 0.2^{e}	3.3 ± 0.1^{ef}	4.2 ± 0.2^{bc}	$<1.0 \pm 0.0^{g}$
	20	4.3 ± 0.1^{ab}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	3.5 ± 0.1^{e}	3.3 ± 0.2^{ef}	3.5 ± 0.1^{e}	$<1.0 \pm 0.0^{g}$
15	0.15	11.9 ± 0.2^{bc}	$<1.0 \pm 0.0^{m}$	$<1.0 \pm 0.0^{m}$	11.7 ± 0.2^{cd}	11.3 ± 0.1^{f}	5.7 ± 0.2^{j}	$<1.0 \pm 0.0^{m}$
	5	11.6 ± 0.1^{de}	$<1.0 \pm 0.0^{m}$	$<1.0 \pm 0.0^{m}$	9.9 ± 0.1^{g}	10.0 ± 0.2^{g}	3.7 ± 0.1^{l}	$<1.0 \pm 0.0^{m}$
	10	11.7 ± 0.3^{cd}	$<1.0 \pm 0.0^{m}$	$<1.0 \pm 0.0^{m}$	11.9 ± 0.0^{bc}	11.4 ± 0.3^{ef}	7.6 ± 0.3^{h}	$<1.0 \pm 0.0^{m}$
	15	12.0 ± 0.4^{b}	$<1.0 \pm 0.0^{m}$	$<1.0 \pm 0.0^{m}$	7.8 ± 0.1^{h}	12.0 ± 0.4^{b}	7.6 ± 0.4^{h}	$<1.0 \pm 0.0^{m}$
	20	11.7 ± 0.2^{cd}	$<1.0 \pm 0.0^{m}$	$<1.0 \pm 0.0^{m}$	7.0 ± 0.2^{i}	12.3 ± 0.1^{a}	4.0 ± 0.2^{k}	$<1.0 \pm 0.0^{m}$
37	0.15	7.0 ± 0.3^{d}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	7.0 ± 0.0^{d}	7.3 ± 0.3^{c}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$
	5	8.6 ± 0.4^{a}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	7.0 ± 0.1^{d}	8.6 ± 0.4^{a}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$
	10	8.6 ± 0.2^{a}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	8.6 ± 0.2^{a}	8.6 ± 0.1^{a}	6.0 ± 0.2^{e}	7.5 ± 0.1^{c}
	15	8.6 ± 0.2^{a}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	8.6 ± 0.2^{a}	8.6 ± 0.1^{a}	6.0 ± 0.2^{e}	8.6 ± 0.1^{a}
	20	8.0 ± 0.1^{b}	$<1.0 \pm 0.0^{g}$	$<1.0 \pm 0.0^{g}$	8.6 ± 0.1^{a}	8.6 ± 0.2^{a}	5.0 ± 0.1^{f}	8.6 ± 0.2^{a}

[a–m] Means both within rows and columns by incubation temperature with different superscript letters are significantly different ($P < 0.05$).

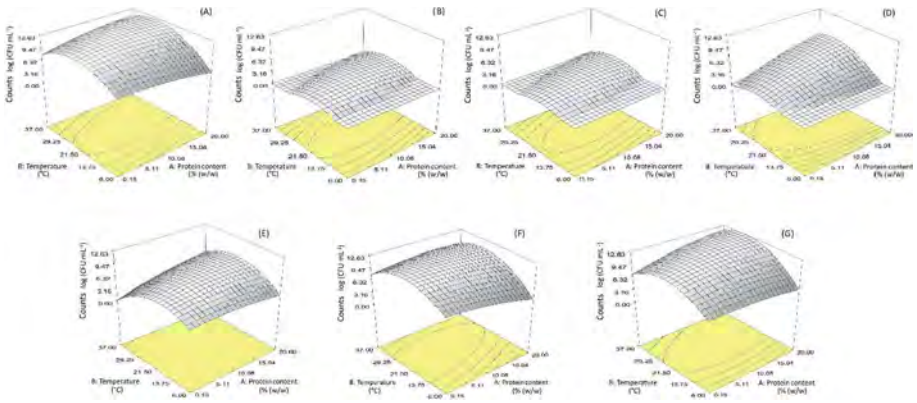


Fig. 2. Influence of temperature of incubation and bovine meat proteins content in Mueller-Hinton broth on *S. aureus* culturable counts enumerated after 24 h at 37 °C, 6 days at 15 °C or 8 days at 6 °C. Response surface plot in the absence of any antibacterial molecule (negative control **A**), with nisin (positive control **B**), with isobutyl-4-hydroxybenzoate (**C**), with naphthazarin (**D**), with gallocyanin (**E**), with resveratrol (**F**), and with chrysin (**G**).

observed that while *S. aureus* population is significantly reduced by the presence of 1 g L^{-1} gallocyanin whatever the protein concentration following incubation at 37 °C for 24 h or at 15 °C for 6 days, this is not any more the case following incubation at 6 °C for 8 days. It is thus noteworthy that while decreasing incubation temperature from 37 °C to 6 °C decreased the antibacterial activity of gallocyanin, it increased that of naphthazarin.

As previously mentioned, chrysin and resveratrol (Fig. 2F and G, respectively) did not significantly reduce *S. aureus* population whatever the temperature of incubation and protein content in the medium, since these compounds were not bactericidal but only had a bacteriostatic action at a 1 g L^{-1} concentration in the absence of bovine meat proteins.

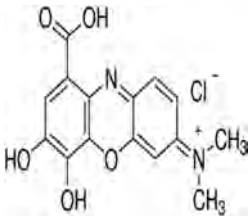
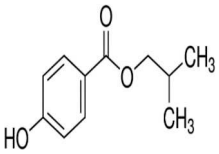
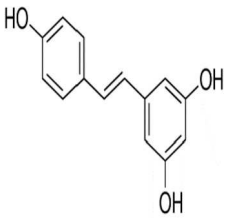
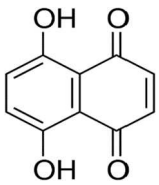
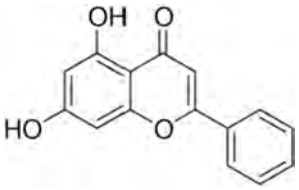
3.3. Affinity of phenolics for bovine meat proteins

Partitioning coefficients of phenolics between a 20% (w/w) bovine meat proteins suspension and its ultrafiltrate (<3 kDa) without bovine meat proteins were determined in order to estimate the affinity for bovine meat proteins of each phenolic. The results are presented in Table 3 together with other physico-chemical parameters which were calculated based on their chemical structure.

Based on these experimental partition coefficients, the 5 phenolics can be ranked in decreasing order regarding their affinity at 6 °C for bovine meat proteins (with a molecular weight exceeding 3 kDa) as follows: chrysin > naphthazarin > resveratrol > isobutyl-4-hydroxybenzoate > gallocyanin. The partition coefficients varied from 0.06 for gallocyanin to more than 2 for chrysin. Since this is a logarithmic scale, this indicates (i) that the phenolics concentration was always higher in the phase with whole bovine meat extract than in the phase without bovine meat proteins and (ii) that this preferential interaction with these proteins was negligible for phenolics like gallocyanin (a log P value of 0.06 corresponds to an only 1.14-fold higher gallocyanin concentration in the phase with proteins) and marked for phenolics like chrysin (its concentration in the phase with proteins was more than 100-fold higher than in the phase without these proteins). These observations are consistent with numerous studies on protein-polyphenol interactions. For instance, Acharya, Sanguansri, and Augustin (2013) reported that resveratrol had strong affinity for sodium caseinate. Protein-phenolics interactions are namely due to hydrophobic or dipole-dipole interactions and to hydrogen-bonding (Zhang et al., 2014).

The bovine meat extract used in this study contains intra- and

Table 3
Physico-chemical properties of phenolics.
Total surface area, polar surface area and log Po/w (octanol-water partition coefficient) are topologic chemometric descriptors calculated with the DataWarrior software. Polarizabilities of non-charged phenolics were calculated with Gaussian 09 (Revision D.01) (Frisch et al., 2013), using the PBE functional (Perdew, Burke, & Ernzerhof, 1996) and the 6-311G (d, p) basis set.

Phenolic compound	Chemical structure	Molecular weight (g mol^{-1})	Total surface area ($Å^2$)	Polar surface area ($Å^2$)	Polar surface area/Total surface area ratio (%)	Polarizability (Bohr^3)	Log $P_{o/w}$	Log P bovine meat proteins
Gallocyanin		336.73	n. d.	n. d.	n. d.	n. d.	0.87	0.06
Isobutyl-4-hydroxybenzoate		194.23	193	47	24	141	2.31	0.31
Resveratrol		228.24	207	61	29	208	2.83	0.90
Naphthazarin		190.15	163	74	46	131	0.75	1.36
Chrysin		254.24	217	67	31	197	2.68	>2

n. d.: not determined.

inter-muscle proteins estimated to be 60% contractile proteins (actin, myosin), 30% sarcoplasmic proteins and 10% connective tissue proteins (Swatland, 1984). Some of these proteins (actin, myoglobin, fibrinogen) possess the properties of globular proteins, representing hydrophobic core protein models that can trap the most hydrophobic phenolics, but phenolic-protein interactions can also occur in the periphery with the participation of the C = O and N-H groups of bovine meat proteins. The C = O and N-H groups of peptide bonds generally do not form hydrogen bonds between themselves but rather with the molecules of the solvent (water) (Branden & Tooze, 1996). The loop regions exposed to the solvent are rich in hydrophilic, charged or polar amino acids, this can be favorable to hydrogen bonding with the phenolics in direct contact with bovine meat proteins.

Indeed, while the 5 phenolics were far more soluble in octanol than in water (from 5.6 to 676 times, as calculated from their log $P_{octanol/water}$ values) and can thus be considered as hydrophobic compounds which is also consistent with the fact that their relative polar surface area is less than 50% (i. e. comprised between 24% and 46%), their interactions with bovine meat proteins likely not only result from hydrophobic interactions but also from dipole-dipole interactions and hydrogen-bonding (the chemical structures of the 5 phenolics contain from 1 to 3 hydroxyl groups). However, while the presence of hydroxyl groups may favor interaction with *S. aureus* cells surface and thus the antimicrobial activity of phenolics, this could also contribute to interactions with proteins. Moreover in the case of compounds such as chrysin, naphthazarin (Gomez, Gonzalez, & Gonzalez, 2005), and resveratrol, the formation of intramolecular hydrogen bonds should also be considered: intramolecular hydrogen bonds could prevent the formation of intermolecular hydrogen bonds with food matrices and thus contribute to the preservation of their antimicrobial activity. The contribution of not only hydrophobic interactions but also dipole-dipole interactions and hydrogen bonding likely explains why the

partitioning coefficient between bovine meat extract with and without proteins was not correlated with that between octanol and water.

Among the 5 phenolics, gallocyanin was the only one bearing a charge. The positive charge of gallocyanin likely explains its water solubility and thus its far lower interaction with proteins of meat extract. However, its log $P_{octanol/water}$ value (0.87) was close to that of naphthazarin (0.75) which has a far higher affinity for bovine meat proteins. Isobutyl-4-hydoxybenzoate and naphthazarin log $P_{octanol\text{-}water}$ values were 2.31 and 0.75, respectively, while their log $P_{bovine\ meat\ proteins}$ values were 0.31 and 1.36, respectively. The ranking of the affinity of the 5 phenolics for bovine meat proteins (chrysin > naphthazarin > resveratrol > isobutyl-4-hydroxybenzoate > gallocyanin) was not correlated with the ranking of their log $P_{octanol\text{-}water}$ values (resveratrol > chrysin > isobutyl-4-hydroxybenzoate > gallocyanin > naphthazarin): this fully justifies the necessity to measure the affinity for proteins of phenolics which depends on the physico-chemical properties of both phenolics and proteins. Here, the interaction of pure phenolics with the array of proteins present in bovine meat extracts was investigated. Other authors already studied the interaction of resveratrol with bovine β-casein and suggested a high affinity of resveratrol for β-casein via hydrophobic interactions (Ghorbani Gorji et al., 2015). However, β-casein represents a model of supramolecular association of proteins with a hydrophobic core which could better trap hydrophobic compounds than bovine meat proteins (a mixture of proteins with different structures).

3.4. Surface properties of S. aureus CNRZ3 cells

The most commonly reported mechanism of action of phenolics against bacteria is based on their accumulation at the surface of bacteria (Negi, 2012). This accumulation depends on interactions between phenolics and the cell wall of bacteria. Therefore, the surface properties of *S. aureus* CNRZ 3 cells were investigated: the microbial adhesion to different water-solvent interfaces of *S. aureus* CNRZ3 cells was determined (Table 4).

The adhesion of bacterial cells to solvents can be explained in terms of acid-base and van der Waals interactions. *S. aureus* CNRZ3 had a high affinity for chloroform (85.0 ± 4.0%), an acidic solvent with electron-acceptor properties: it can thus be proposed that it results from a basic or electron-donating property of bacterial cells. Microbial adhesion to hexadecane (33.4 ± 0.4%), the n-alkane investigated in this study was far lower. Surface of *S. aureus* CNRZ3 cells can thus be considered hydrophilic since affinity for hexadecane is less than 40%.

Zeta potential (ZP) was also measured to characterize bacterial surface charge. This parameter can play a key role in phenomena such as self-aggregation, cluster formation and adhesion to surfaces. In our case, bacterial surface charge can have a role in adhesion of bacteria to food constituents like bovine meat proteins on one hand and the uptake of phenolics on the other hand. The results indicated that the surface of *S. aureus* CNRZ3 cells is negatively charged (ZP = −14.9 ± 0.8 mV). This value is consistent with ZP values of −12.2 mV for methicillin-resistant *S. aureus* MRSA USA100 (Birkenhauer & Neethirajan, 2014) and of −9 mV for *S. aureus* BCRC 10451, 10780, and 10781 strains (Chen et al., 2012). Negative ZP of *S. aureus* was expected due to its membrane mainly constituted by anionic lipids (phosphatidylglycerol (57%) and lysylphosphatidylglycerol (38%)) (Teixeira, Feio, & Bastos, 2012). The presence of lipoteichoic acids in the membrane structure of *S. aureus* also contributes to the reported negative charge (Fischer, 1994).

Table 4
Adhesion of *S. aureus* CNRZ3 cells to solvent-water interfaces.

Solvent	Percentage (%) adhesion of *S. aureus* CNRZ3 cells (Mean ± SD (n = 3))
Chloroform	85.0 ± 4.0[a]
Hexadecane	33.4 ± 0.4[b]

[a–b] Means within rows with different superscript letters are significantly different ($P < 0.05$).

4. Discussion

Addition of bovine meat extract to MHB up to a 20% (w/w) protein content corresponding to the protein content of bovine meat differentially affected growth inhibition of *S. aureus* CNRZ3 by 5 phenolics totally inhibiting its growth at 37 °C for 24 h in MHB. *S. aureus* CNRZ3 growth inhibition by phenolics likely results from their accumulation at the surface of these bacterial cells. In this context, our main hypothesis is that when bovine meat proteins interacting with phenolics are added to MHB, this limits the quantity of "free" phenolics which could accumulate at the surface of *S. aureus* CNRZ3 cells. This hypothesis is consistent with the observation that gallocyanin and isobutyl-4-hydroxybenzoate which were the only phenolics keeping their bacteriostatic activity at 37 °C for 24 h in the presence of 20% (w/w) protein had the lowest affinity for bovine meat proteins. The 3 other plant phenolics had a far higher affinity for bovine meat proteins and their bacteriostatic effect was not preserved for 24 h at 37 °C. However a difference could be observed between naphthazarin which delayed the growth of *S. aureus* CNRZ 3 in the presence of 20% (w/w) bovine meat proteins in the medium while chrysin and resveratrol did not. Indeed, chrysin and resveratrol were not bactericidal at a 1 g L^{-1} concentration which corresponds to their MIC in the absence of bovine meat proteins. Therefore, any significant reduction of their "free" concentration in MHB may have led to the loss of their antibacterial activity: indeed, resveratrol and chrysin concentrations in the phase with bovine meat proteins were about 8 times and more than 100 times higher than in bovine meat extract without these proteins, respectively. Similarly, naphthazarin concentration in the phase with bovine meat proteins was about 20 times higher than in the phase without these proteins. However, naphthazarin has a 0.05 g L^{-1} MBC. This means thus that even in the presence of 20% (w/w) bovine meat proteins, its "free" concentration was close to its MBC. The fact that its bactericidal effect was restored when *S. aureus* CNRZ3 cells were incubated at 15 °C or 6 °C may thus result from the fact that when temperature decreased hydrophobic or van der Waals interactions between naphthazarin and bovine meat proteins would be reduced while hydrogen bonding between naphthazarin and the surface of *S. aureus* CNRZ3 cells might be promoted.

For these interpretations, *S. aureus* CNRZ3 cells are considered as inert particles. However, it should also be considered that *S. aureus* CNRZ3 cells metabolism might also be affected by bovine meat extracts addition. Indeed, it cannot be ruled out that proline present in bovine meat extract might stimulate the growth of *S. aureus* CNRZ3. Previous studies on *Helicobacter pylori* (Lin, Kwon, Labbe, & Shetty, 2005) and *S. aureus* (Kwon, Apostolidis, Labbe, & Shetty, 2007) reported that the addition of proline may decrease the inhibitory effect of phenolics. Proline or proline precursors such as glutamate and arginine, present in beef extract at levels of 1.2%, 4% and 1.5%, respectively (Calorie-Count.com, 2016) may be sufficient to stimulate the growth of *S. aureus*. Nevertheless, some phenolics such as gallic acid owe their antibacterial activity to their analogy with proline and disturb the bacterial metabolism (Apostolidis, Kwon, & Shetty, 2008; Kwon et al., 2007).

Moreover, decreasing *S. aureus* CNRZ3 temperature of incubation from 37 °C to 6 °C, besides having opposite effects on polar and hydrophobic interactions, likely also modifies the surface properties of *S. aureus* CNRZ3 cells. Indeed, the saturated and unsaturated fatty acid composition of the membrane phospholipids of the bacteria evolves as a function of the culture temperature: this is one of the physiological responses of the bacteria to adapt their membrane fluidity to their environment. It was reported that when *S. aureus* was abruptly shifted from 37 to 25 °C in exponentially growing phase, there was a 15% increase in total phospholipids (Joyce, Hammond, & White, 1970). This would result in more negatively charged membranes for cells cultivated at 15 °C or 6 °C, attracting more polar phenolics, especially since the low temperature is more favorable to dipole-dipole interactions (Keesom interactions) and less favorable to Debye (between polar and apolar species) and London (between two apolar species) interactions, leading to a lower affinity of bacterial cells to beef proteins.

5. Conclusion

The antibacterial activity of 5 phenolics which had a bacteriostatic or bactericidal action against *S. aureus* CNRZ3 cells when added at a 1 g. L^{-1} concentration for 24 h at 37 °C in Mueller-Hinton broth was re-examined at 15 °C or 6 °C (to mimic refrigeration temperatures) in Mueller-Hinton broth supplemented with up to 20% (w/w) bovine meat proteins (to mimic the protein content of bovine meat). Only isobutyl-4-hydroxybenzoate kept its bactericidal activity whatever the incubation temperature and bovine meat proteins content in the medium. Interestingly, naphthazarin was bactericidal in the presence of bovine meat proteins at 6 °C and 15 °C unlike at 37 °C. However, gallocyanin lost its bactericidal activity and resveratrol and chrysin lost their bacteriostatic activity when bovine meat proteins were added. These observations support the necessity to perform the screening of the antibacterial activity in complexified media mimicking the composition of perishable foods and at refrigeration temperatures in order to identify compounds of interest for the preservation of refrigerated perishable foods. Based on an estimation of the affinity of each phenolic for bovine meat proteins at 6 °C, it can be proposed that the antibacterial activity loss of some phenolics might result from their complexation by bovine meat proteins thereby reducing their concentration available to interact with *S. aureus* cells below their MIC. However, the contribution of other factors such as the modifications of the surface properties of *S. aureus* cells when cultured at 6 °C instead of 37 °C or the effects of compounds such as proline present in bovine meat extract which would stimulate *S. aureus* growth and induce their resistance to phenolics cannot be excluded.

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Declarations of interest

All authors have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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