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Research Article

Effect of interaction with food constituents on plant extracts antibacterial activity

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Abstract

The Gaillac red wine powder and *Cinnamon cassia* essential oil were selected for their *in vitro* antibacterial activity against *Staphylococcus aureus* CNRZ3 and *Listeria innocua* LRGIA 01, respectively. In order to assess the potential application of Gaillac wine powder to the preservation of raw meat, its antibacterial activity was assayed in Mueller Hinton broth supplemented with up to 20% (w/w) bovine meat proteins (bovine meat protein content): Gaillac wine powder as well as resveratrol, a stilbene polyphenol present in red wine, lost their antibacterial activity, likely as a result of interactions of Gaillac wine antibacterial molecules with bovine meat proteins at the expense of their interactions with *Staphylococcus aureus* CNRZ3 cells. *Cinnamon cassia* essential oil antibacterial activity assays in Tryptone Soya broth, skimmed, semi-skimmed, and whole milk showed that its antibacterial activity was significantly reduced by milk fat globules but not by milk proteins: it could thus be used for the preservation of skimmed milk. The developed methodology based on the use of microbiological media mimicking the composition of perishable foods or of liquid foods such as sterilized milk with various milk fat contents could be used for the rapid screening of antibacterial plant extracts of interest for perishable foods preservation.

Keywords: plant extracts, red wine powder, *Cinnamon cassia* essential oil, antibacterial activity, interactions with proteins, interactions with milk fat globules

Abbreviations:

EO – essential oil, MHB – Mueller-Hinton broth, TSB – Tryptone Soya broth, MIC – minimal inhibitory concentration, MBC – minimal bactericidal concentration

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Introduction

There is a growing interest in antimicrobial plant extracts as alternatives to some synthetic food preservatives for sanitary, environmental, regulatory, and marketing reasons (Oulahal et al. 2017). Plant extracts for food preservation containing non-volatile (e.g. polyphenols) and volatile (e.g. essential oils) antimicrobial molecules can be distinguished. In previous studies, Gaillac red wine powder (Bouarab-Chibane et al. 2017) and *Cinnamon cassia* essential oil (Trinh et al. 2015) were selected based on their *in vitro* antibacterial activity against several pathogenic or food-spoiling bacterial strains.

Gaillac red wine powder is rich in phenolics, while trans-cinnamaldehyde is the main component of *C. cassia* essential oil (EO). The fact that these plant extracts had major compounds containing either a phenyl structure and/or aldehyde groups is consistent with Dorman and Deans (2000) observation that antibacterial activity of plant extracts is namely due to the presence of hydrophilic functional groups, such as hydroxyl groups of phenolic components and aldehyde groups.

Due to their respective organoleptic properties (odour, taste, colour), Gaillac red wine extract and *C. cassia* EO could be added in meat and milk, respectively. However, *in vitro* screening of antibacterial activity of plant extracts is usually performed in microbiological media (Mueller-Hinton (MHB) or Tryptone Soya (TSB) broth in the present case), while perishable foods such as raw beef patties or cow's milk have a far more complex composition and structure. This difference of composition and structure may result in interactions of antibacterial plant molecules with food components such as proteins or fat. Moreover, the structure of most of perishable foods is hetero-geneous: this could also result in a heterogeneous distribution of antibacterial molecules in such foods. As a consequence, target bacterial cells might be exposed to a reduced concentration of plant antimicrobial molecules due to their binding to other food components or to the distribution of target bacterial cells in another phase of heterogeneous foods than antimicrobial molecules. This has been suggested by Weiss et al. (2015) as the main cause of the loss of antibacterial activity of most of food preservatives added to foods in comparison to their activity in model media.

This observation led us to propose that *in vitro* screening of antimicrobial plant extracts intended for food preservation should not only be performed in microbiological media but also in more complex media supplemented with food components and/or mimicking the heteregoneous structure of many foods. Therefore, increasing concentrations of bovine meat extract were added in MHB in order to mimick the protein content of bovine meat (20% (w/w)). This allowed to study the effect of proteins present in meat on the antibacterial activity of Gaillac red wine and of resveratrol. Resveratrol is a stilbene polyphenol present in red wines known for its antioxidant and antibacterial activities. In order to check the effect of the presence of casein micelles and dispersed fat in cow's milk on *C. cassia* EO antibacterial activity, its minimal inhibitory (MIC) and bactericidal (MBC) concentrations in TSB, skimmed milk, semi-skimmed milk, and whole milk were compared.

Materials and Methods

Materials

Chemicals and reagents. Resveratrol, trans-cinnamadehyde, sodium azide, Nisaplin® (a 2.5% (w/w) nisin commercial preparation), beef extract powder prepared from beef muscle (75% (w/w) protein) and all reagents unless otherwise stated were purchased from Sigma (St Quentin Fallavier, France). Skimmed, semi-skimmed and whole milk powder were purchased from Régilait (St Martin Belleroche, France). Their respective compositions once resuspended in distilled water are stated in Table 2.

Plant extracts preparation and characterization

Gaillac red wine powder. Dry extract from Gaillac red wine was directly obtained by vacuum evaporation of ethanol and water. Total phenolic content and reducing sugars content of Gaillac red wine powder were 81.2±4.2mg gallic acid

equivalent per g and 80.2±3.3mg glucose equivalent per g, respectively (Bouarab-Chibane et al. 2017).

***C. cassia* essential oil.** *C. cassia* EO was extracted by hydrodistillation from leaves harvested at Yen Bai (Vietnam) by the laboratory of Chemical Analysis, Institute of Natural Products Chemistry (Vietnamese Academy of Science and Technology). Its composition was determined by gas chromatography-mass spectrometry analysis performed in the same laboratory. Trans-cinnamaldehyde was the major component: it represented approximately 90% (w/w) of the EO (Trinh et al. 2015).

Bacterial strains. The bacterial strains used as test microorganisms were *Staphylococcus aureus* CNRZ3 and *Listeria innocua* LRGIA01 (isolated in a dairy, collection of BioDyMIA laboratory (Université Claude Bernard Lyon 1, Bourg en Bresse, France)) for Gaillac red wine powder or resveratrol and *C. cassia* EO antibacterial activity assays, respectively. *S. aureus* and *L. innocua* strains were stored at -40°C in MHB or TSB (Biokar, Beauvais, France) with 15 % (v/v) of glycerol, respectively. One milliliter of the stock culture was transferred to 9 mL of MHB or TSB and incubated for 8h at 37°C for *S. aureus* or 30°C for *L. innocua*. Then, 1mL of this first culture was inoculated again with 9mL of fresh MHB or TSB and incubated overnight at 37°C or 30°C. Finally, a third culture was made from the second one (same conditions) and used for antibacterial susceptibility testing.

Methods

Antibacterial activity assays of Gaillac red wine powder and of resveratrol. *In vitro* antibacterial activity was assessed by monitoring the growth of *S. aureus* in the presence or absence (control) of Gaillac red wine powder or resveratrol at a final 1g.L^{-1} concentration in MHB supplemen-ted or not with beef extract at 37°C for 24h. Gaillac red wine powder and resveratrol were pre-diluted at a 10g.L^{1} concentration in a 10% (w/w) DMSO solution. Beef extract was added to MHB to reach a 5, 10, 15, or 20% (w/w) final protein concentration. Briefly, 270µL of MHB with or without beef extract alone (control) or supplemented with Gaillac red wine powder or resveratrol (final concen-trations: 1g.L^{-1}) were mixed either with 30 µL of bacterial inocula (5.0 x 105 cfu.mL^{-1} (Colony Forming Units)) or 30µL of sterile MHB with or without beef extract (control) in each well of the microplate and incubated at 37°C for 24h in a Bioscreen C® plate reader (Oy Growth Curves AB Ltd., Helsinki, Finland). The optical density of the culture was monitored every 15min, in the 420nm - 580nm wavelength range (OD420-580). Negative control wells containing 2000 IUmL^{-1} nisin were also monitored. All measurements were performed in triplicate. After screening, *S. aureus* cells were enumerated, 10µL of the suspension from each inoculated well were spotted on plates containing Mueller Hinton Agar, and then incubated at 37°C for 24h. Data were expressed as log cfu per mL.

Determination of resveratrol partitioning coefficient between a phase with and a phase without beef extract proteins. A 20% (w/w) bovine meat proteins suspension was prepared by resuspending 26.66% (w/w) of beef extract powder (75% (w/w) protein) in distilled water at pH 7.0. A part of this suspension was ultrafiltrated through 3 kDa cut-off ultrafiltration membranes (3kDa Microsep™ Advance Centrifugal Device, Pall Life Sciences, USA) in order to remove bovine meat proteins with a molecular weight exceeding 3kDa. In order to estimate the "affinity" of resveratrol for bovine meat proteins with a molecular weight exceeding 3kDa, 1 mL of the ultrafiltrate of the 20% (w/w) bovine meat proteins suspension was placed in a 3.5-5kDa cut-off dialysis tube (Spectra/ Por Float-A-Lyzer G2, G235029, cut-off threshold 3.5-5 kDa). This 1 mL tube was dialyzed against 20 mL of the 20% (w/w) bovine meat proteins suspension supplemented with 1g.L^{-1} of resveratrol for 2 weeks at 6°C in order to reach the equilibrium of distribution of resveratrol between the phase with bovine meat proteins and the phase without bovine meat proteins. The system was continuously stirred in dark. After 2 weeks, the resveratrol concentration in dialysis tubes (initial concentration: 0g.L^{-1}) was assayed by Reversed-Phase High Performance Liquid Chromatography

(RP-HPLC). A Shimadzu RP-HPLC system (Kyoto, Japan) controlled by LC solution software was used. Samples (20µL) were directly injected into RP-HPLC column using an automatic sample changer. An Omnispher C_{18} Chromsep column, 110 Å (250 x 4.6mm, 5µm) (Agilent Technologies, France) in an oven maintained at 30°C was used. The mobile phase was acidified water (pH 2.65) with acetic acid (eluent A) and acetonitrile (eluent B), at a flow rate of 1.2mL min^{-1}, according to a gradient given; % B (0.1% 0-35min, 24% 35-40min, 40% 40-45min, 80% 45-60min, and 0.1% 60-65min). Resveratrol elution was detected at a 304nm wavelength. An external standard resveratrol calibration curve was used to determine resveratrol concentration. A partitioning coefficient of resveratrol between the phase with bovine meat proteins (with a molecular weight exceeding 3kDa) and the phase without these proteins was thus calculated as follows:

$$\log P_{\text{bovine meat proteins > 3 kDa / ultrafiltrate (< 3 kDa)}} = [\text{resveratrol}]_{\text{in 20\% (w/w) bovine meat proteins suspension}} / [\text{resveratrol}]_{\text{in ultrafiltrate (<3 kDa) of 20\%(w/w) bovine meat proteins suspension}}$$

Determination of Minimal Inhibitory (MIC) and Bactericidal (MBC) concentrations of *Cinnamon cassia* EO. Skimmed, semi-skimmed or whole milk powder were suspended in distilled water before being sterilized for 15 min at 110°C. They were then supplemented with serial dilutions of *C. cassia* EO and inoculated with *L. innocua* LRGIA 01 strain to determine corresponding MIC and MBC values. First, an overnight *L. innocua* LRGIA01 culture was diluted in TSB to a final concentration of 105cfu.mL^{-1}. One hundred microliters of this bacterial suspension were used to inoculate 10 mL of either TSB (1% (w/w)), skimmed, semi-skimmed, or whole milk supplemented or not (control) with serial dilutions of *C. cassia* EO. After 24h incubation at 30°C, the MICs of *C. cassia* EO were determined using micro-dilution broth method. The lowest concentration of the EO resulting in no growth of the strain was taken as the MIC. 10µL from each medium were streaked on solid agar medium and incubated for 24h at 30°C. The lowest concentration at which no visible growth on solid agar medium was obtained was considered as the MBC.

Results and Discussion

Effect of bovine meat proteins addition on the antibacterial activity of Gaillac red wine powder and resveratrol. *S. aureus* growth in MHB supplemented with up to 20% (w/w) bovine meat proteins was monitored in the presence or absence of Gaillac red wine powder or of resveratrol (Table 1). In MHB, both Gaillac red wine powder and resveratrol at 1g.L^{-1} were bacteriostatic but not bactericidal: this means that no growth occurred over 24h incubation at 30°C, while regrowth of *S. aureus* cells was observed after 24h incubation in fresh MHB without these antibacterial agents. The antibacterial activity of phenolics present in red wines has already been reported by several authors (Rodriguez-Vaquero et al. 2007, 2013; Friedman 2014). The addition of 20% (w/w) bovine meat proteins in MHB annihilated the bacteriostatic effect against *S. aureus* of 1g.L^{-1} of Gaillac red wine powder or resveratrol. However, while *S. aureus* growth was no more inhibited by Gaillac red wine powder, a 38% reduction of the maximal optical density (OD420-580nm) over 24h incubation was still observed in the presence of 1g.L^{-1} of resveratrol. This means that in the presence of the same concentration of proteins than in bovine meat (20% (w/w)), a 1g.L^{-1} resveratrol concentration still significantly inhibited *S. aureus* growth unlike Gaillac red wine powder at the same concentration. This observation is consistent with the fact that in the presence of 5% (w/w) bovine meat proteins in MHB, the bacteriostatic effect of resveratrol is preserved unlike that of Gaillac wine powder. The higher the bovine meat protein concentration, the lower the *S. aureus* growth inhibitory activity of Gaillac wine powder and to a lesser extent of resveratrol.

The antimicrobial activity of Gaillac wine powder is likely due to phenolic compounds. Vaquero et al. (2007) reported that the antibacterial activity of red

wines against pathogenic bacteria such as *S. aureus* was mainly due to phenolics. Gaillac red wine has a high total phenolics content (2800 mg gallic acid equivalent.L^{-1}).

Since it contained 29 g dry matter.L^{-1}, this corresponds to a 81.2±4.2mg gallic acid equivalent per g of Gaillac red wine powder. The mean concentration of resveratrol in Gaillac red wine is about 5 mg.L^{-1} corresponding thus to about 0.17 mg.g^{-1} of powder. Resveratrol is thus likely not a significant antibacterial compound since it represents only a 0.17mg.L^{-1} concentration in MHB supplemented with 1g. L^{-1} of Gaillac wine powder. Resveratrol is a hydrophobic molecule with a log $P_{\text{octanol-water (o/w)}}$ value of 3.4. The decrease of antibacterial activity of resveratrol when increasing beef extract concentration might thus result from hydrophobic interactions with bovine meat proteins. Other phenolic antibacterial compounds present in red wine such as gallic acid (log $P_{o/w}$ = 3.72), catechin (log $P_{o/w}$ = 1.5), quercetin-3-β-glucoside (log $P_{o/w}$ = -0.34), and rutin (log $P_{o/w}$ = -1.25) have different physico-chemical properties which might modulate their interactions with bovine meat proteins and thus the susceptibility of their antibacterial activity to beef extract proteins. In the present case, both Gaillac red wine powder and resveratrol antimicrobial activity decreased in the presence of bovine meat proteins. This is likely due to electrostatic and hydrophobic interactions of antimicrobial plant phenolics with bovine meat proteins as proposed by Weiss et al. (2015). Interactions between proteins and polyphenols have been extensively reviewed (Papadopoulou and Frazier 2004; Ozdan et al. 2013).

In order to investigate the interactions between bovine meat proteins and resveratrol, we determined the partitioning coefficient at 6°C of resveratrol between the phase without proteins with a molecular weight exceeding 3kDa (ultrafiltrate of a 20% (w/w) bovine meat proteins suspension) and a 20% (w/w) bovine meat proteins suspension. At equilibrium, the resveratrol concentration in the 20% (w/w) bovine meat proteins suspension was 7.95-fold higher than in the phase without bovine meat proteins with a molecular weight exceeding 3 kDa. This corresponds to a log $P_{\text{bovine meat proteins > 3 kDa/ultrafiltrate (< 3 kDa)}}$ value of 0.9. This clearly indicates the affinity of resveratrol for bovine meat proteins with a molecular weight exceeding 3kDa. The interaction of resveratrol (a relatively hydrophobic stilbene polyphenol with a log $P_{o/w}$ value of 3.4) with bovine meat proteins is likely both due to electrostatic (ionic, hydrogen bonding) and hydrophobic interactions. The affinity of resveratrol for meat proteins substantiates the hypothesis that resveratrol antibacterial activity reduction in their presence likely results from a lower quantity of "free" resveratrol which could interact with target bacterial cells and inhibit their growth. However, the partition coefficient determined to estimate the affinity of resveratrol for bovine meat proteins was determined at 6°C while antibacterial activity assays in the presence of bovine meat proteins were performed at 37°C. At 37°C, electrostatic interactions are weaker, while hydrophobic interactions are stronger than at 6°C. Therefore, the affinity of resveratrol for proteins should also be determined at 37°C.

Effect of milk proteins and milk fat on the anti-*L. innocua* activity of *C. cassia* essential oil. In order to investigate the effect of milk proteins and milk fat on the anti-*L. innocua* activity of *C. cassia* EO, a 103cfu.mL^{-1} initial *L. innocua* LRGIA 01 population was incubated for 24 h at 30°C in TSB (1% w/w), skimmed, semi-skimmed or whole milk with or without (control) serial dilutions of *C. cassia* EO. Although TSB 1% (w/w), skimmed, semi-skimmed, and whole milk had very different compositions (Table 2), the *L. innocua* population in control cultures without *C. cassia* EO reached 8.8, 8.5, 8.5, and 8.4 log cfu.mL^{-1} after 24 h incubation, respectively. The MIC and MBC values of *C. cassia* EO in these 4 media are presented in Table 3. When TSB 1% (w/w) medium was replaced by skimmed milk, the MIC and MBC values of *C. cassia* EO increased from 300 to 400mg.L^{-1} and from 700 to 1000mg.$L^{-1,}$ respectively. This reduction of antibacterial activity was far more limited than in semi-skimmed (MIC and MBC values of 1000 and 5000 mg.L^{-1}, respectively) and whole milk (MIC and MBC values of 2000 and 5000mg.L^{-1},

respectively). MIC value of *C. cassia* bark EO against *L. innocua* LRGIA 01 strain in semi-skimmed milk is consistent with the 1000mg.L^{-1} MIC value against a *L. monocytogenes* strain of cinnamon bark EO in pasteurized semi-skimmed milk incubated at 35°C for 24h reported by Cava et al. (2007).

Table 1. Effect of proteins (bovine meat extract) addition to Mueller-Hinton broth on the antibacterial activity of Gaillac red wine powder and of resveratrol against *S. aureus* CNRZ3 strain (initial population: 5.0 x 10^5cfu.mL^{-1}).

	Gaillac red wine powder (1g.L^{-1})		**Resveratrol (1 g.L^{-1})**	
Protein content in Mueller-Hinton broth (% (w/w))	**Variation of maximal $OD_{420\text{-}580\ nm}$ compared to control culture without Gaillac red wine powder (%)**	***S. aureus* population after 24h incubation at 37°C (cfu.mL^{-1})**	**Variation of maximal $OD_{420\text{-}580\ nm}$ compared to control culture without resveratrol (%)**	***S. aureus* population after 24h incubation at 37°C (cfu.mL^{-1})**
0.15	100±0	$7.1x10^5$	100±0	$1.0x10^7$
5	27±1	$1.7x\ 10^7$	100±0	$1.0x10^7$
10	9±1	$1.7x\ 10^7$	76±0	$4.0x10^8$
15	7±1	$6.6x10^8$	45±1	$4.0x10^8$
20	-7±1	$3.9x10^9$	38±0	$4.0x10^8$

Table 2. Composition of Tryptone Soya broth (TSB (1% (w/w)), skimmed, semi-skimmed and whole milk (once milk powder was resuspended in distilled water).

	TSB (1% (w/w))	**Skimmed milk**	**Semi-skimmed milk**	**Whole milk**
Total carbohydrate (glucose in TSB, lactose in milk) (% (w/w))	0.67	5.2	4.9	5.5
Protein (% (w/w))	0.08	3.6	3.4	3.6
Fat (% (w/w))	0	0.08	1.6	3.6

Table 3. Minimal inhibitory (MIC) and bactericidal (MBC) concentrations in Tryptone Soya broth at 30°C of *C. cassia* essential oil against *L. innocua* LRGIA01 strain (initial population: 10^3 cfu.mL^{-1}).

Medium	**TSB (1% w/w)**	**Skimmed milk**	**Semi-skimmed milk**	**Whole milk**
MIC of *C. cassia* essential oil (mg.L^{-1})	300	400	1000	2000
MBC of *C. cassia* essential oil (mg.L^{-1})	700	1000	5000	5000

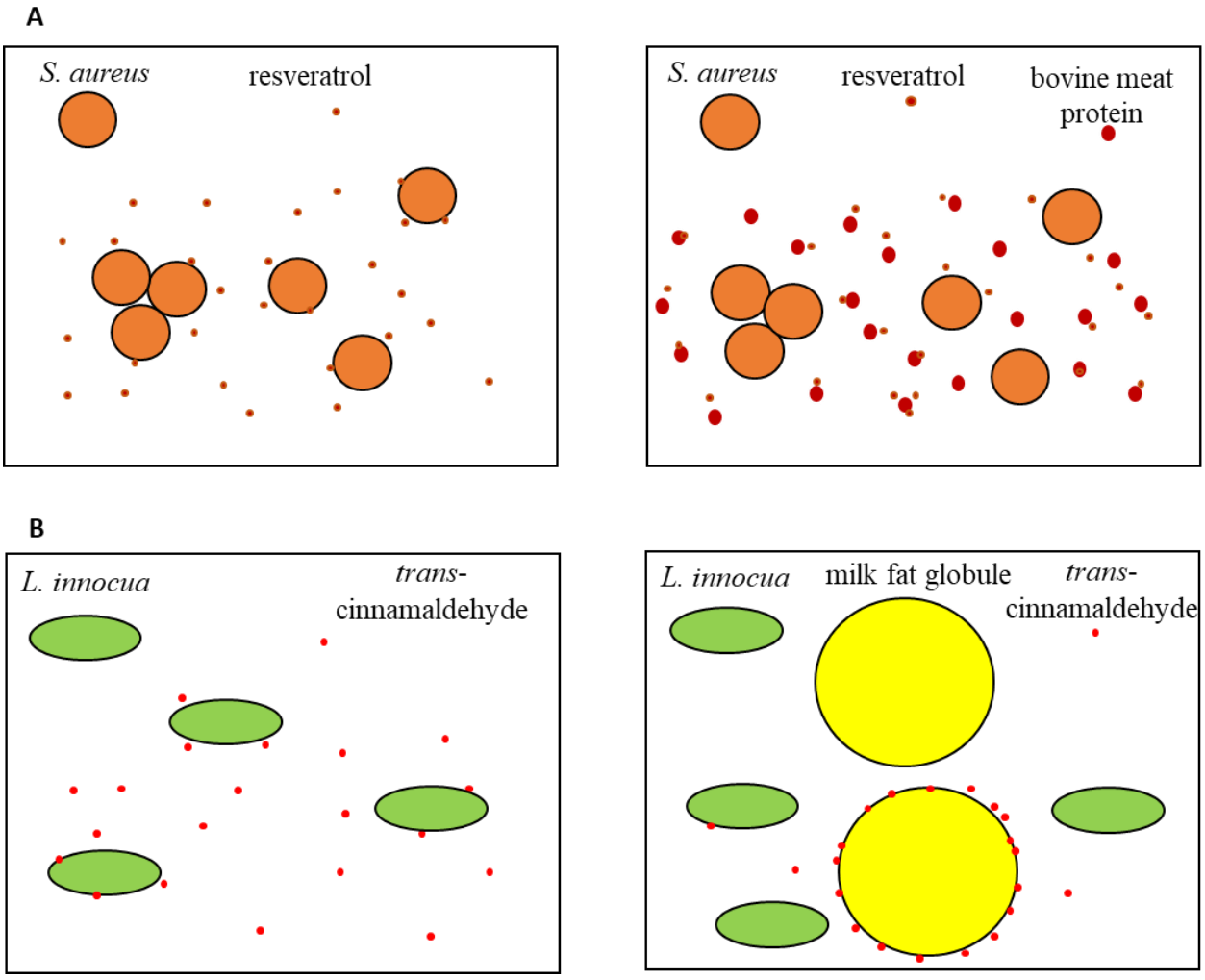


Figure 1. Scheme presenting interactions between resveratrol (as an example of polyphenol) and meat proteins (**A**) or between *trans*-cinnamaldehyde and milk fat globules (**B**) at the expense of interactions with *S. aureus* (**A**) or *L. innocua* (**B**) cells, respectively.

Taken together, the results indicate that while MIC and MBC values are quite similar in TSB 1% (w/w) and skimmed milk, respective 2.5-fold and 5-fold increases of MIC and MBC were observed between skimmed and semi-skimmed milk. This suggests that *C. cassia* EO antibacterial activity is more affected by the presence of milk fat globules than by the increase of proteins and carbohydrate concentrations between TSB 1% (w/w) and skimmed milk. The reduction of *C. cassia* EO anti-*Listeria* activity in the presence of milk fat globules is substantiated by the fact that a further 2-fold decrease of its MIC was observed in whole milk. The increase of MIC observed in whole milk containing 3.6% (w/w) milk fat in comparison to TSB 1% (w/w) is similar to the increase of MIC in emulsified TSB 1% (w/w) containing 2.5% (w/w) sunflower oil and 1.5% (w/w) soya lecithin reported by Trinh et al. (2013).

Since *C. cassia* EO anti-*Listeria* activity is mainly due to *trans*-cinnamaldehyde which represents 90% (w/w) of its components (Trinh et al. 2015), physico-chemical characteristics of *trans*-cinnamaldehyde such as its log $P_{o/w}$ value (2.12) might explain why its antibacterial activity is far more affected by the presence of milk fat globules

than by the presence of proteins like casein micelles and β-lactoglobulin which represent 80% (w/w) and 10% (w/w) of cow's milk proteins, respectively. Compounds with the highest log Po/w values are likely more susceptible to bind to hydrophobic particles like milk fat globules. As illustrated by these results concerning *C. cassia* EO, several authors have recently studied or reviewed the effect of the interaction with food components of EOs on their antimicrobial activity (Gutierrez et al. 2008, 2009; Perricone et al. 2015).

Conclusions

Gaillac red wine powder antibacterial activity was annihilated when bovine meat proteins were added. This antibacterial activity probably results from interactions of bovine meat proteins with antibacterial phenolics present in red wines such as Gaillac (as examplified with resveratrol). *C. cassia* EO antibacterial activity only slightly decreased in skimmed milk while it strongly decreased in whole milk and to a lesser extent in semi-skimmed milk. This is likely due to interactions between trans-cinnamaldehyde, the main component of *C. cassia* EO, and milk fat globules.

A putative explanatory scheme presenting interactions between resveratrol (as an example of phenolics) and meat proteins or between *trans*-cinnamaldehyde and milk fat globules at the expense of interactions with *S. aureus* or *L. innocua* cells, respectively is presented in Figure 1. *In vitro* screening of plant extracts antibacterial activity in complexified media should both allow not to consider plant extracts which lose their activity in real foods and provide a better understanding of the interactions between antimicrobial plant molecules such as phenolics and aldehydes and dispersed fat or proteins.

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