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Activity of wine against *Campylobacter jejuni*

Anabela Carneiro [a,b], José António Couto [a,*], Cristina Mena [a], Jorge Queiroz [b], Tim Hogg [a]

[a] *Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[b] *Faculdade de Ciências da Universidade do Porto, Secção Autónoma de Engenharia das Ciências Agrárias, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal*

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Abstract

This study focuses on the activity of wine against the important food-borne pathogen *Campylobacter jejuni*. The kinetics of inactivation of two strains of *C. jejuni* (one food-borne and one clinical) were characterised in various scenarios of exposure to wine and wine components. Undiluted wine was found to rapidly inactivate *C. jejuni* (>6D inactivation within 30 s); further inactivation data were obtained from experiments performed in wine diluted with water (1:2 and 1:4). Experiments with isolated antimicrobial fractions of wine (ethanol and certain organic acids) suggest that these two components act synergistically, demonstrating an inactivation capacity similar to wine itself. The results indicate that the exposure of contaminated food to wine, as in marinade conditions, significantly reduces the number of viable cells of *C. jejuni*. A model stomach, containing a food matrix and a synthetic gastric fluid, was used to infer the effect of wine against *C. jejuni* in a consumption-like scenario. Wine was found to potentiate the anti-*Campylobacter* effect of gastric fluid. The results strongly suggest that the ingestion of wine during a meal may greatly diminish the quantity of *C. jejuni* persisting further in the alimentary tract, thus lowering the risk of infection.

Keywords: *Campylobacter jejuni*; Inactivation; Wine; Model stomach

1. Introduction

Several relatively recent studies describe in detail the antimicrobial properties of wine against a number of relevant, food-borne, pathogenic bacteria (Just & Daeschel, 2003; Moretro & Daeschel, 2004; Sugita-Konishi, Hara-Kudo, Iwamoto, & Kondo, 2001). Such studies reaffirm the anecdotal and historical evidence of the protective role wine can play in this respect, both as a food additive (in marinades and similar treatments) and as a component of the eating process itself. *In vitro* studies indicate that, for a given ethanol concentration, wine has a more potent antibacterial activity than other alcoholic beverages. This potency has been partly attributed to the combination of ethanol and organic acids (tartaric, malic, lactic and acetic) (Just & Daeschel, 2003; Weisse, Eberly, & Person, 1995). Malic and tartaric acids are the most abundant organic acids in wine, and their antimicrobial effects are well-known, especially in low pH conditions, such as those found in wines (Hsiao & Siebert, 1999; Ricke, 2003). The importance of ethanol to this overall antimicrobial activity is illustrated by the findings of Just and Daeschel (2003) who showed that a grape juice had a very little antimicrobial activity against *Escherichia coli* O157:H7 and *Salmonella* spp., whereas wine made from the same juice demonstrated considerable activity in this respect. The bactericidal effect of ethanol alone, in concentrations generally encountered in wine (between 10 and 13% v/v) is low, when compared with the bactericidal effect of wine itself (Just & Daeschel, 2003; Marimon, Bujanda, Gutierrez-Stampa, Cosme, & Arenas, 1998; Weisse et al., 1995). Moretro and Daeschel (2004), when testing different combinations of ethanol, organic acids and acidity, found that within the ranges tested, a mixture of 0.15% of malic acid, 0.6% of tartaric acid, 15% of ethanol and pH 3.0 had the

* Corresponding author.
E-mail address: jacouto@esb.ucp.pt (J.A. Couto).

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strongest bactericidal effect. This same study suggest that these compounds act synergistically and represent the major components responsible for the bactericidal effect of wine.

Gastric juice is recognised as a bactericidal barrier, being one of the first lines of defence against ingested pathogens (Just & Daeschel, 2003). Although a complex fluid, some authors suggest that the bactericidal activity of the stomach is predominantly pH dependent (Gianella, Broitman, & Zamcheck, 1973; Peterson, Mackowiak, Barnett, Marling-Cason, & Haley, 1989). On the other hand, Alm (1983) reported that human gastric juice (pH 1.7–1.8) was more efficient in the inhibition of *Salmonella* spp. and *Shigella* spp. than a physiological saline solution adjusted to the same pH, supporting the antibacterial role of other components of this fluid. The antibacterial effect of wine in model stomach systems, simulating the consumption of wine during a meal, has been demonstrated for *E. coli* O157:H7, *Salmonella typhimurium* (Just & Daeschel, 2003) and *Listeria innocua* (Fernandes, Gomes, Couto, & Hogg, 2007).

In humans, *Campylobacter* spp. causes campylobacteriosis, a gastro-intestinal tract infection (Carter, Chengappa, & Roberts, 1995; Reilly & Gilliland, 2003; Roberts, Baird-Parker, & Tompkin, 1998) characterised by severe diarrhoea, fever, abdominal pains, nausea and vomiting that usually lasts for five to seven days (Koenig, 2005; Tortora, Funke, & Case, 2002). The principal species responsible for the majority of infections is *Campylobacter jejuni* (Roberts et al., 1998; Tortora et al., 2002). Although any person can be affected (Beumer, de Vries, & Rombouts, 1992; Roberts et al., 1998), the symptoms are normally more serious in children, elderly people and persons with underlying health problems (Roberts et al., 1998; (www.health.vic.gov.av, 2000)) *Campylobacter* spp. is part of the intestinal flora of several animals (Carter et al., 1995; Reilly & Gilliland, 2003; Tortora et al., 2002). The initial source in human infections is largely caused by direct or indirect contamination from faecal or intestinal material of food animals. The main initial source of contamination is poultry products (Hirsh, 1999a; Koenig, 2005), the infection occurring after food has been improperly cooked or contaminated (or re-contaminated) after any cooking stage (Koenig, 2005; Reilly & Gilliland, 2003). However, some occurrences are associated with raw milk (Hirsh, 1999b; Reilly & Gilliland, 2003) and contaminated water supplies (Hirsh, 1999b; Koenig, 2005; Reilly & Gilliland, 2003). Contamination by domestic animals (Hirsh, 1999a; Koenig, 2005) or person-to-person, are also sources of dissemination of the illness (www.health.vic.gov.av, 2000). Despite these alternative routes of infection, *Campylobacter* spp. is essentially a food-borne pathogen and has recently overtaken *Salmonella* spp. as the major reported source of food-borne bacterial disease (Zhao et al., 2001).

The objective of this work was to characterise the effect of exposure to wine on the survival of *C. jejuni*. This characterisation aimed to describe the effects of this exposure in both direct-immersion, marinade conditions and in simultaneous-consumption (wine/food/bacteria) scenarios. Thus, the kinetics of inactivation of *C. jejuni* are described, both in wine itself and in various combinations of the major components of wine (alcohol and organic acids). A simple model stomach was also used to study the influence of wine on the survival of this organism in simulated gastric fluid, with and without the addition of wine. The results of both approaches are interpreted in a risk assessment context. This is, to our knowledge, the first study about the effect of wine on *Campylobacter* spp.

2. Materials and methods

2.1. Bacterial strains and culture media

Two strains of *C. jejuni* were studied, one a food isolate (CIN55c) and the other isolated from an infected patient (CIN59c). The cultures were preserved in Columbia agar with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) in petri dishes at 4 °C and in cryopreservation tubes (Cryobanks, Mast, Merseyside-Liverpool, UK) at −80 °C.

2.2. Inactivation test solutions

Inactivation assays were carried out in the following solutions:

- Red wine 2001, from the Douro demarcated region (Portugal) with the following parameters: 5 mg/l of free sulphur dioxide, 54 mg/l of total sulphur dioxide, pH 3.6, titrable acidity of 5.5 g/l (expressed as tartaric acid equivalents), 0.48 g/l of volatile acidity (expressed as acetic acid equivalents) and 12.5% of ethanol (v/v). The wine was filter sterilised using 0.45 μm cellulose acetate membranes, (Orange Scientific, Braine L' Alleud, Belgium) and was kept at 4 °C in sterile, 200 mL, bottles until use.
- Ringer's solution, used as control and, also, as base to the preparation and dilution of the other solutions used in the assayed treatments.
- Acids solution: the following organic acids were added to Ringer's solution to give final concentrations of 5.5 g/l of tartaric acid (Sigma CO, St. Louis, MO), 0.5 g/l of acetic acid (Merck, Darmstad, Germany), 2 g/l of lactic acid (Sigma CO, St. Louis, MO) and 0.5 g/l of citric acid (Merck, Darmstad, Germany) in the test solutions, with pH adjusted to pH 3.6 with HCl 1 M (Pronalab, Lisbon, Portugal).
- Ethanol Solution 12.5% (v/v): obtained by the addition of 12.5 ml of Ethanol (Aga, Prior Velho, Portugal) to 87.5 ml of Ringer's solution.
- Solution of acids with ethanol: the acids solution described above was supplemented with 12.5% (v/v) of ethanol.

The acids and the ethanol solutions were filter sterilised using 0.2 μm cellulose acetate membranes (Orange Scien-

tific, Braine L' Alleud, Belgium). The Ringer's solutions were sterilised in the autoclave (121 °C, 15 min).

2.3. Survival of bacteria in wine and in organic acids and ethanol solutions

Cell suspensions were prepared in 5 mL of Ringer's solution by scraping the colonies grown on the surface of Columbia agar with 5% sheep blood, at 42 °C for 48 h in a microaerophilic generated atmosphere (Genbox microaer, bioMérieux, Marcy l'Etoile, France) in 2.5 L anaerobic jars (Oxoid, Basingstoke, England). 0.5 ml of the resulting cell suspensions were used to inoculate 9.5 ml of the experimental solutions in test tubes immersed and thermally stabilised in a thermostatted water bath at 37 °C. At selected times, 1 mL samples were collected and serially diluted in Ringer's solution. Then, 20 μL of each tenfold dilutions were plated onto Columbia agar with 5% sheep blood by the drop count technique of Miles and Misra (1938). After incubation at 37 °C for 48 h in a microaerophilic atmosphere as described above, the CFU/mL numbers were determined. All the experiments were performed at least in duplicate. Survival at any given time point was determined as the ratio CFU/mL after each treatment to the CFU/mL at the zero time point.

2.4. Survival of bacteria in the model stomach system

The synthetic stomach mixture was prepared in sterile stomacher bags (VWR, Buffalo Grove, USA). Fifty millilitres of sterile wine and 150 mL of sterile synthetic gastric fluid (SGF) were added to 150 g of solid food matrix – commercially available sterile homogeneous chicken baby meals (Blédina, Danone, Villefranche-Sur-Saône, France), obtaining a final volume of approximately 350 mL. In proportion to the amount of food in the stomach model, the volume of wine used corresponds, approximately, to a glass of wine ingested in a regular meal (100–150 mL). Two-hundred millilitres of sterile water or 50 mL of sterile water and 150 mL of SGF were used as controls. The composition of the synthetic gastric fluid (SGF) was adapted from Just and Daeschel (2003): 2.05 g of NaCl, 0.6 g of KH_2PO_4, 0.1 g of $CaCl_2$, 0.3 g of KCl and 13.3 mg of pepsin in 1 L of distilled water, with the pH adjusted to 1.5 with HCl (1 M). All reagents were from Merck (Darmstad, Germany). The SGF was made fresh daily and sterilised by filtration using 0.2 μm cellulose acetate membranes.

The experimental stomacher bags with the food matrix, to which each solution to be tested was added, were immersed in a thermostatted water bath at 37 °C. After homogenisation and stabilisation of the temperature, 1 mL of the cell suspension was transferred to the stomacher bags reaching an initial concentration of *C. jejuni* of approximately 10^6 CFU/mL. The bags were energetically mixed during the assays. Tenfold serial dilutions were performed and 20 μL aliquots were plated onto Columbia agar with 5% of sheep blood. Plates were incubated at 37 °C for 48 h in a microaerophilic atmosphere for bacterial counts. All the experiments were performed at least in duplicate. Points shown on graphs are means with error bars representing ± standard errors of these means.

2.5. Statistical analysis

Statisitical analysis (ANOVA, significance level: $P < 0.05$) was conducted with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

3.1. Inactivation of C. jejuni by wine and wine compounds

The results presented here show that wine exerts a strong effect on the survival of the tested strains of *C. jejuni*. Bacterial populations of 10^6–10^7 CFU/mL exposed to wine were dramatically inactivated to undetectable numbers (detection limit of 500 CFU/mL) within 30 s (data not shown). Further characterisation of inactivation was performed in diluted wine (1:2 and 1:4). As can be seen in Fig. 1, cells of strain CIN55c exposed to diluted wine 1:2 suffered a reduction of almost 6 log cycles in 30 s. Viable cells were not detected (<500 CFU/mL) in the subsequent sampling times. When the 1:4 dilution was used, a reduction of 2 log cycles was noticed after 1 min, and of 3 log cycles after 3 min. The concentration of live cells in the control assay (Ringer's solution) remained constant until the end of the experiment. *C. jejuni* CIN59c was found to be more resistant to the wine treatments than *C. jejuni* CIN55c (Fig. 2). A notable difference was found between the 2 strains in wine dilution 1:2 in the first 30 s ($P < 0.05$). While, as mentioned above, strain CIN55c suffered a reduction of almost 6 log cycles in this period of time, the decrease in the cell survival of strain CIN59c was only 2.5 log cycles. No viable cells were detected (<500 CFU/mL) in the following sampling times. At the end of the experimental time, only a 2 log cycles reduction was obtained in the wine dilution 1:4.

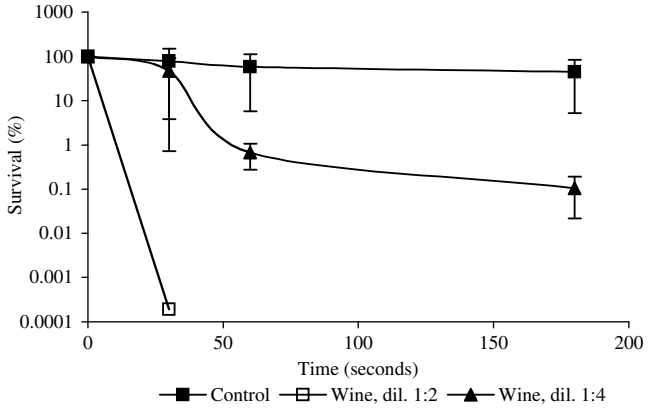

Fig. 1. The effect of wine, dilutions 1:2 and 1:4, on the survival of *Campylobacter jejuni* CIN55c.

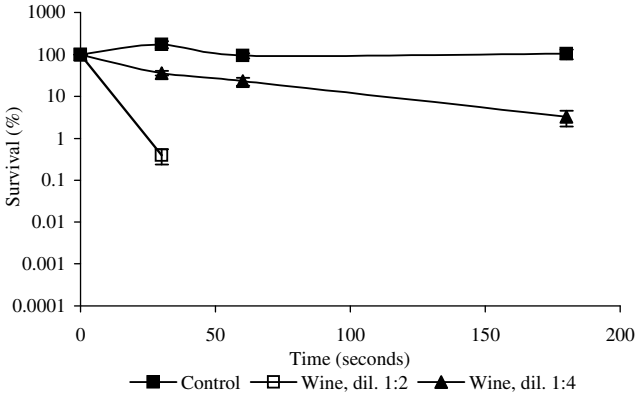

Fig. 2. The effect of wine, dilutions 1:2 and 1:4, on the survival of *Campylobacter jejuni* CIN59c.

Comparison with published data suggests that *C. jejuni* is more sensitive to wine than other food-borne bacteria. Weisse et al. (1995) showed that wine reduced the viable number of *Salmonella enteritidis*, *Shigella sonnei* and *E. coli*, 5–6 log cycles, in 20 min of exposure. Other works obtained the same extent of inactivation of *Salmonella* spp. and *E. coli* in 5–30 min and of 20–60 min, respectively (Harding & Maidment, 1996 cit. Just & Daeschel, 2003; Marimon et al., 1998; Moretro & Daeschel, 2004). Moretro and Daeschel (2004) studied the bactericidal effect of the wine on *E. coli* O157:H7, *Listeria monocytogenes*, *S. typhimurium* and *Staphylococcus aureus* and concluded that *S. typhimurium* was the most sensitive species, with a reduction of 6 log cycles after 10 min of exposure. *S. aureus* was the most resistant. Bacterial inactivation experiments are notoriously sensitive to medium and conditions variations (ex: variability of wine composition) and direct comparison in this case must also contemplate the special cultivation conditions of *Campylobacter* spp. Nevertheless, the results presented here suggest that *Campylobacter* spp is, amongst the vegetative food-borne pathogens, at the lower end of the range of resistances to exposure to wine.

Since ethanol and organic acids are among the main bactericidal elements in wine, the activity of these compounds was tested in this respect. The main organic acids (except malic acid since the wine used had undergone a malolactic fermentation) and a mixture of the acids with ethanol (at similar concentrations to those present in wines) were used. When the mixture of wine organic acids was used alone, only a slight decrease in cell survival (1 log cycle after 30 s) was observed for both strains, the CFU/mL remaining constant from this time on (data not shown). The effect of ethanol was studied at the concentrations of 3%, 6.25% and 12.5% (v/v) which correspond to the ethanol content of the wine diluted 1:4, 1:2 and with no dilution, respectively. Inactivation under these conditions was negligible over the time scale employed here, for both strains (data not shown). This finding is in accordance with those of Just and Daeschel (2003), Marimon et al. (1998) and Weisse et al. (1995) which showed that the bactericidal effect of ethanol in concentrations commonly found in wine (10–13% v/v) was substantially less than the wine itself.

The combination of the acids with ethanol showed a much higher bactericidal effect than the mixture of acids and ethanol in separate. An inactivation of greater than 6 log cycles viable cells was detected after 30 s of exposure to a mixture of acids, at the concentrations described above, in ethanol at 12.5% (v/v) (data not shown). An identical result was obtained when this solution was diluted 1:2 (data not shown). Fig. 3 shows the cell inactivation of *C. jejuni* CIN55c caused by the dilution 1:4. It can be observed that the cell inactivation attained at the end of the assay was identical to that obtained with wine diluted 1:4. Fig. 4 shows the results obtained for *C. jejuni* CIN59c. Viable cells were detected at the end of the experiment time (3 min) in the 1:2 solution dilution and a reduction of viable cells of only 2 log cycles was attained in the 1:4 dilution, similar to what was obtained in wine dilution 1:4.

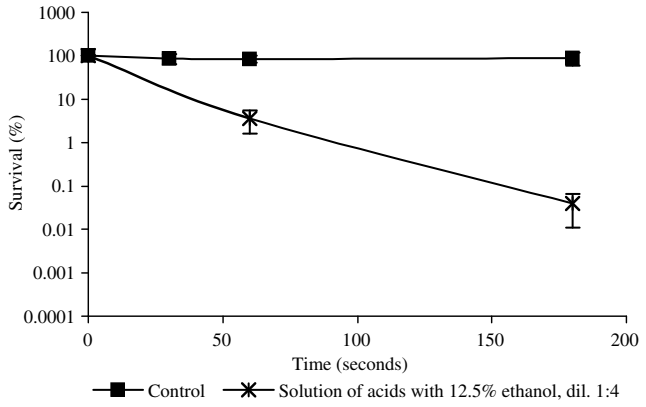

Fig. 3. The effect of the acids solution (5.5 g/l tartaric acid, 0.5 g/l acetic acid, 2 g/l lactic acid and 0.5 g/l citric acid) supplemented with 12.5% (v/v) ethanol, diluted 1:4, on the survival of *Campylobacter jejuni* CIN55c.

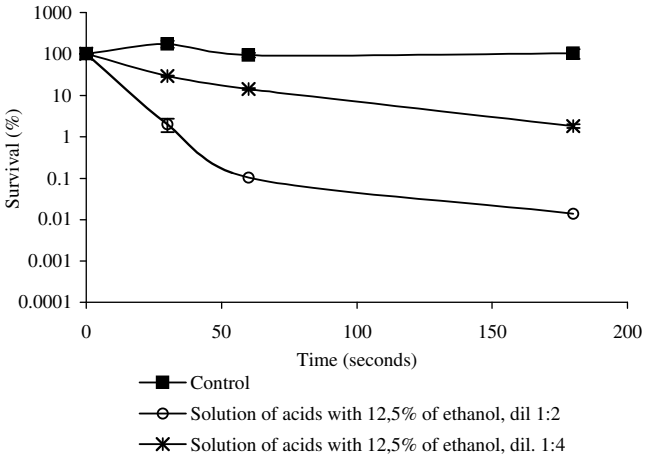

Fig. 4. The effect of the acids solution (5.5 g/l tartaric acid, 0.5 g/l acetic acid, 2 g/l lactic acid and 0.5 g/l citric acid) supplemented with 12.5% (v/v) ethanol, diluted 1:4, on the survival of *Campylobacter jejuni* CIN59c.

3.2. Inactivation C. jejuni in a model stomach system

The role that wine, consumed at a meal, might have on the survival of *C. jejuni* in the human stomach, was studied using the model stomach described in the materials and methods. Fig. 5 shows the results obtained for *C. jejuni* CIN55c. In the assay used as control (food + water), the cells of *C. jejuni* remained constant throughout the 20 min period of the experiment. The treatment food + SGF (synthetic gastric fluid) + water caused a reduction in the number of viable cells of 1 log cycle in 5 min and 2 log cycles in 10 min. When this strain was submitted to the treatment food + SGF + wine, the inactivation effect was faster ($P < 0.05$, for 5 min of exposure), with a reduction of viable cells of 2 log cycles in 5 min. No viable cells were detected (<500 CFU/mL) in the following sampling times. In comparison to the SGF, recognised as a bactericidal barrier against the ingested pathogens, the presence of wine, in an equivalent amount of a glass of wine in a meal (proportionally to the food in the model stomach), led to an additional cell inactivation effect.

C. jejuni CIN59c was also tested in the stomach model (Fig. 6). The treatment food + SGF + water caused a similar behaviour as the control (food + water), with a survival rate of about 100% until the end of the experiment. When wine was added to the system (food + SGF + 50 mL of wine), a significant decrease ($P < 0.05$) of almost 2 log cycles in the cell survival was noticed after 20 min. It was also possible to observe a higher decrease in the concentration of viable cells, an almost 3 log cycles reduction, when the quantity of wine was doubled (100 mL, with the total volume of the stomach model adjusted to 400 mL). It is clear in this experiment that *C. jejuni* CIN59c exhibits a higher resistance to the SGF than *C. jejuni* CIN55c ($P < 0.05$, for 10 min of exposure).

The importance of the gastric juice as a bactericidal barrier has been known for almost a century, being one of the first lines of defence (host unspecific immunity) against ingested pathogenic organisms (Tortora et al., 2002). It has been already described, in some previous works, that the bactericidal activity of stomach is predominantly pH (chloridric acid) dependent (Just & Daeschel, 2003). But the acidification of the ingested food is not the only physiological mechanism responsible for this activity. For example, salivary nitrite, under the acid conditions of the stomach is converted to nitrous acid and other unidentified nitrogenous metabolites that have considerable antibacterial activity (Xu, Xu, & Verstraete, 2001).

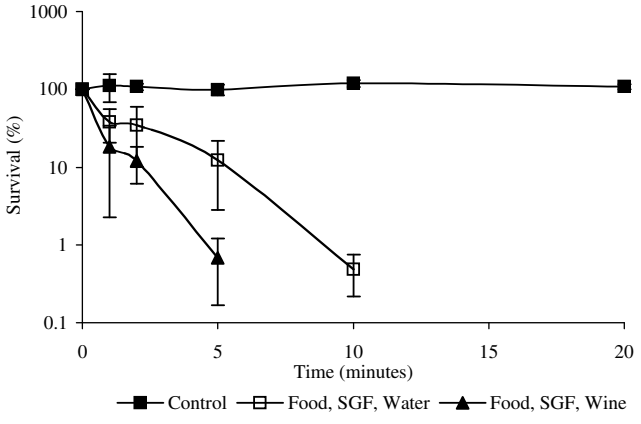

Fig. 5. Inactivation of *Campylobacter jejuni* CIN55c, in a model stomach system (350 mL total volume).

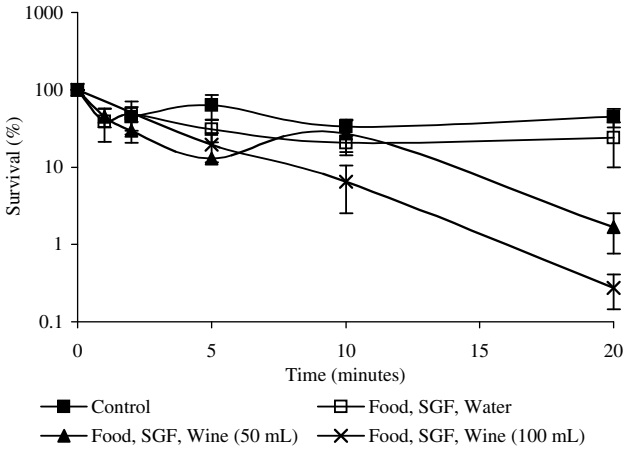

Fig. 6. Inactivation of *Campylobacter jejuni* CIN59c, in a model stomach system (350 or 400 mL total volume).

4. Conclusions

This is, to our knowledge, the first study that shows the antibacterial effect of wine on *Campylobacter* spp. This work clearly demonstrates that wine exerts a strong bactericidal effect over *C. jejuni*. The variability in resistance of *C. jejuni* will need to be more fully described before any concrete orientations can be made as to safety practices.

Ethanol or a solution of wine organic acids, when used in separate, had a negligible influence in the survival of *C. jejuni* over the timescale studied. However, when used in a combined solution, these compounds had a similar inactivation effect as that of wine. This apparent synergistic effect is consistent with the findings of other authors for other food pathogens and these components of wine (Moretro & Daeschel, 2004). The results obtained in simulated consumption scenarios in a model stomach, suggest that concurrent ingestion of wine with food significantly decreases the number of *C. jejuni* persisting further in the alimentary tract. The infective dose of *Campylobacter* species is not known precisely, but is normally considered to be small. Human feeding studies suggest that about 400–500 bacteria may cause illness in some individuals, while in others, greater numbers are required, certainly depending on the virulence of the strain and on the susceptibility of the individual (Kothary & Babu, 2001). This work suggests that the immersion of food (ex: meat and poultry) in wine, in marinade conditions, leads to a reduction of the number of viable *C. jejuni* cells eventually present, thus

lowering the risk of cross contamination of cooked foods. As shown here, a substantial reduction of viable cells is attained even with diluted wine, 1:2 (6 log cycles reduction in 30 s) or 1:4 (3 log cycles reduction in 3 min), therefore in similar wine proportions to those normally used in marinades. The antimicrobial effect of wine in marinades can be expected to be higher than this, due to the relatively large periods of exposition time (one to several hours), and to the synergistic effect with other substances (spices, vinegar, etc) commonly used in these preparations. Several studies have reported on the prevalence of *Campylobacter* in food samples. Wong et al. (2007) found a high incidence in chicken (89.1%), in comparison with pork (9.1%), lamb (6.9%) and beef (3.5%). 83%, 70.7% and 64% of the chicken meat samples analysed by Jørgensen et al. (2002), Sallam (2007) and Zhao et al. (2001), respectively, were positive for *Campylobacter*. The number of cells present in positive samples is variable. The overall estimated mean count obtained by Manfreda, De Cesare, Bondioli, Stern, and Franchini (2006) was 5.16 $\log_{10}$ CFU per carcass of chicken. Wong et al. (2007) had counts from <0.3–110 MPN/g and Dufrenne, Ritmeester, Delfgou-van Asch, van Leusden, and de Jonge (2001) from <10 to more than 5500 CFU per fresh carcass. Wine, used as a beverage or as a marinade, may be expected to diminish the incidence of campylo- bacteriosis.

It would be important to extend the study of the inactivation effect of wine not only to a larger number of strains of *C. jejuni*, but also to *Campylobacter coli* which is responsible for 3–5% of the human cases of campylobacteriosis.

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