Short communication

Inhibition of *Salmonella enterica* serotype Enteritidis on agar and raw chicken by carvacrol vapour

Sara A. Burt [a],[*], Mattijs J. Fledderman [b], Henk P. Haagsman [b],
Frans van Knapen [a], Edwin J.A. Veldhuizen [b]

[a] *Institute for Risk Assessment Sciences, Veterinary Public Health Division, Faculty of Veterinary Medicine,
Utrecht University, P.O. Box 80175, 3508 TD Utrecht, The Netherlands*
[b] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80165, 3508 TD Utrecht, The Netherlands*

Abstract

Carvacrol is known to inhibit a number of food borne pathogens. The activity of carvacrol vapour was evaluated against *S. enterica* serotype Enteritidis on tryptone soya agar and on pieces of raw chicken. On agar, the size of the inhibition zone increased with decreasing volume of agar, increasing vapour exposure period, increasing temperature, increasing volume of carvacrol used and with decreasing bacterial density. Inhibition was equally effective under aerobic and anaerobic conditions. On chicken pieces ($10 \times 10 \times 5$ mm, UV-sterilized and inoculated with approx. 5×10^3 cfu) carvacrol vapour significantly reduced viable numbers of salmonellae at 4, 20 and 37 °C and all viable cells were eliminated by a minimum of 3 h at 37 °C ($p < 0.05$). A minimum concentration of 20% carvacrol v/v in ethanol was required to achieve a significant reduction and from 40% v/v no viable cells were recovered ($p < 0.05$). In conclusion, carvacrol vapour is effective at inhibiting the growth of *S. enterica* serotype Enteritidis on agar and at inhibiting and eliminating these bacteria on the surface of raw chicken.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Salmonella enterica serotype Enteritidis; Carvacrol; Vapour; Chicken; Meat; Decontamination

1. Introduction

Salmonella enterica serotype Enteritidis is the most frequently reported zoonotic disease in many countries (World Health Organization, 2002) and has been reported as causing more deaths than any other species of bacteria in the United States of America (Olsen et al., 2000). Although over the last 40 years this serotype has been associated with the consumption of eggs (Rabsch et al., 2000), eating chicken prepared outside the home was recently identified as the greatest risk factor for infection in the USA (Kimura et al., 2004). Measures to reduce the numbers of cases of illness due to infection include training food handlers, educating the public and developing techniques to reduce contamination of poultry during slaughter and processing (Kimura et al., 2004; Mead and Mulder, 1997).

Bacteria can be transferred to the surface of the carcass during defeathering and evisceration, and hygiene measures do not prevent contamination entirely (Mead and Mulder, 1997). Post-processing decontamination of carcasses, in the countries where it is allowed, reduces contamination of the outside of the carcass and should reduce the chance of infection (Smulders, 1995; Yang et al., 1998). This is particularly relevant for food intended for the young and the elderly since these groups have high mortality rates due to *Salmonella* infection (Thorns, 2000).

The essential oils of certain herbs such as thyme and oregano are known to exhibit antimicrobial properties against certain food borne pathogens (Dorman and Deans, 2000; Skandamis et al., 2001). These properties are due principally to the major constituents carvacrol and thymol, which have possibilities as food preservatives (Burt et al., 2005; Ultee et al., 1999). One limitation on the use of essential oils or their constituent in foods is their herbal aroma; in fact carvacrol and thymol are permitted food flavourings in the U.S. and Europe (http://www.cfsan.fda.gov/~dms/eafus.html, date consulted: 10 August 2006)

(European Commission, 1999; European Parliament, 1996). A possible method of achieving the antibacterial effect whilst reducing the flavour impact may be to apply the substances in the form of a vapour. There is some evidence to indicate that this could be achieved; oregano oil in the vapour phase has been shown to have a small effect on the microbial population on the surface of beef under certain conditions (Skandamis and Nychas, 2002) and a more significant effect on the flora of sea bass (Harpaz et al., 2003). Carvacrol vapour showed antibacterial activity against a selection of food pathogens on agar (Ben Arfa et al., 2006) but did not produce a significant reduction of salmonellae on alfalfa seeds (Weissinger et al., 2001). No other studies on the effect of carvacrol vapour on food pathogens on the surface of food are known.

The aim of this study was to determine whether carvacrol vapour could significantly reduce viable numbers of *S. enterica* serotype Enteritidis on agar and on the surface of raw chicken.

2. Materials and methods

2.1. Chemicals

Carvacrol was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

2.2. Bacterial cultures

S. enterica serotype Enteritidis 857, a Dutch field isolate (Van Asten et al., 1995), was stored on agar slants at 4 °C. Overnight cultures were prepared freshly for each experiment by incubation at 37 °C for 16 h in tryptone soya broth (TSB, Oxoid, Basingstoke, UK). Before use, the optical density (OD) of the suspension was measured using a Pharmacia Ultraspec III spectrophotometer at 620 nm and the suspensions were diluted in TSB to the bacterial density required.

2.3. Carvacrol vapour inhibition assay

The standard experimental set-up was as follows: a 100 µl portion of a bacterial suspension containing approximately 1×10^5 cfu/ml was spread over the surface of a tryptone soya agar (TSA, Oxoid) plate and allowed to dry. A paper disc (diameter 45 mm, Schleicher and Schuell, Dassel, Germany) was laid on the inside surface of the lid and 10 µl carvacrol was placed on the disc. The plate inoculated with salmonella was immediately inverted on top of the lid and sealed with parafilm to prevent leakage of the vapour. Plates were incubated at 37 °C for 24 h and the diameter of the resulting inhibition zone in the bacterial lawn was measured.

Per experiment, one of the following factors was varied: volume of carvacrol placed on the paper disc (1, 5, 10, 15, 20, 30, 40 or 50 µl); duration of carvacrol vapour treatment prior to removal of the carvacrol disc (30 min, 1, 2, 3, 4, 5 or 16 h); and bacterial load used to inoculate the agar (10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 cfu). To determine whether carvacrol vapour had to diffuse into the agar to cause the antibacterial effect or whether

the vapour itself was active, an experiment was performed to compare the use of 12.5 ml or 25 ml TSA per plate. Doubling the agar volume would result in a smaller airspace in the Petri dish and a shorter distance from the source of carvacrol vapour to the bacteria. If carvacrol exerts its antibacterial action in the vapour phase, doubling the agar volume would therefore increase the inhibition zone obtained. However if carvacrol needs to dissolve into the agar before inhibiting growth of salmonellae, a smaller inhibition zone would be observed because the vapour would diffuse into a larger volume of agar and away from the salmonella at the surface. Furthermore, an experiment was carried out with different holding temperatures during overnight vapour treatment (4, 20, 25 or 37 °C) prior to the removal of the carvacrol disc and 24 h incubation at 37 °C. Aerobic and anaerobic conditions during vapour treatment and incubation were also compared; anaerobic conditions being achieved by use of GasPak Plus (Becton Dickinson and Co., Sparks, MD). Each experiment was carried out six times.

2.4. Chicken decontamination assay

Chicken breasts were bought from a local supermarket and frozen on day of purchase. The meat was defrosted on day of use and pieces of approximately 10×10 mm and 5 mm thick were cut using sterilized equipment. The pieces were exposed to the UV lamp in a laminar flow cabinet for 15 min on both sides so that the natural bacterial flora should interfere as little as possible. In a pilot experiment in which the chicken pieces were not sterilized, the natural flora was treated by the standard carvacrol vapour treatment method described below and no viable cells were recovered (data not shown). Nevertheless, the pieces were sterilized by UV as a precaution.

A 50 µl portion of bacterial suspension containing approximately 10^5 cfu/ml was dotted onto the chicken in a Petri dish. A paper disc with carvacrol was placed inside the lid of the Petri dish and secured by means of a small piece of laboratory tape on the back. The dish was sealed with parafilm. As standard, 10 µl carvacrol was placed on the paper disc and the plates were stored at 4 °C for 24 h followed by overnight incubation at 37 °C. Per experiment, one of these factors was varied: treatment temperature (4, 20 or 37 °C); duration held at 37 °C before refrigerated storage (1, 2, 3 or 24 h); and concentration of the carvacrol used (0, 20, 40, 60, 80% v/v in ethanol or 100% carvacrol); After vapour treatment, chicken pieces were transferred to a sterile plastic bag containing 10 ml minimal TSB (a 100-fold dilution of TSB) and massaged for 90 s in a stomacher machine (Interscience bagmixer, St. Nom, France). Colony counts were carried out by decimal dilution in physiological salt solution (0.85% w/v NaCl) and plating out on TSA. Each experiment was carried out four or six times.

2.5. Statistical analyses

SPSS version 12.0.1 software was used to compare group means by analysis of variance followed by post hoc tests of Least Significant Difference or Tukeys HSD test apart from the data

for agar volume and anaerobic conditions, which were compared by *T*-test. All probabilities are quoted as significant at the 5% level ($p \leq 0.05$).

3. Results and discussion

3.1. Carvacrol vapour inhibition assay

The inhibitory effect of carvacrol vapour on the growth of *S. enterica* serotype Enteritidis on agar is presented in Fig. 1A to D. The inhibitive effect of the vapour increased with increasing volume of carvacrol used from 1 to 20 µl and levelled off from 30 µl carvacrol (Fig. 1A). The serial increases in diameter of the inhibition zone were significantly different from each other up to 40 µl. Based on the results of this first experiment, the rest of the study was carried out using 10 µl carvacrol in order to make any changes in inhibition zone easily visible. No other studies on the effect of carvacrol vapour on salmonellae on agar could be found in the literature, however the minimum inhibitory dose of carvacrol vapour against a suspension of *Escherichia coli* dotted onto agar was determined as 5 mg per agar plate (Ben Arfa et al., 2006), which is approximately equivalent to 5 µl and in the same range as our results.

To determine the effect of changes in the duration of vapour exposure on growth of salmonellae, periods from 30 min up to 16 h were evaluated and the results are presented in Fig. 1B. A sharp and significant increase in the diameter of the inhibition zone was found during the first two hours of treatment and from 2 h to 16 h further small increases were seen. The difference between 5 h and 16 h was also significant. Longer periods

allowed evaporation of more carvacrol and more time for diffusion of vapour through the air and/or agar, enabling interaction with bacterial cells. This is in agreement with a study on the treatment of salmonellae on alfalfa seeds with carvacrol or thymol vapour. Greater reductions in numbers of viable salmonellae were achieved in the order 1 h < 3 h < 7 h (Weissinger et al., 2001).

The effect of increasing the bacterial density from 10^2 to 10^7 cfu per plate is shown in Fig. 1C. In this range, a total decrease in the average inhibition zone of 6 mm was measured. Although the difference was statistically significant from 10^5 cfu/plate, the total effect of increasing bacterial density was small. This indicates that the method of applying carvacrol vapour to inhibit bacterial growth is fairly insensitive to the magnitude of bacterial contamination.

The effect of temperature during exposure of salmonellae to carvacrol vapour on agar is shown in Fig. 1D. Growth was clearly inhibited at 4 °C and the efficacy of the vapour improved significantly at 20 °C and again at 25 °C, presumably due to the increase in volatility of carvacrol with increasing temperature. No further significant improvement in efficacy was seen at 37 °C.

Increasing the volume of agar used in the Petri dish from 12.5 ml to 25 ml caused a significant decrease in the inhibition zone from a mean of 62 ± 3 mm to 44 ± 2 mm. This suggests that it was the concentration of carvacrol vapour diffused into the agar rather than the concentration gradient of carvacrol vapour in the air that had the greatest influence on the inhibition zone. This is confirmed by the fact that fresh bacterial cultures inoculated within the inhibition zones and incubated without
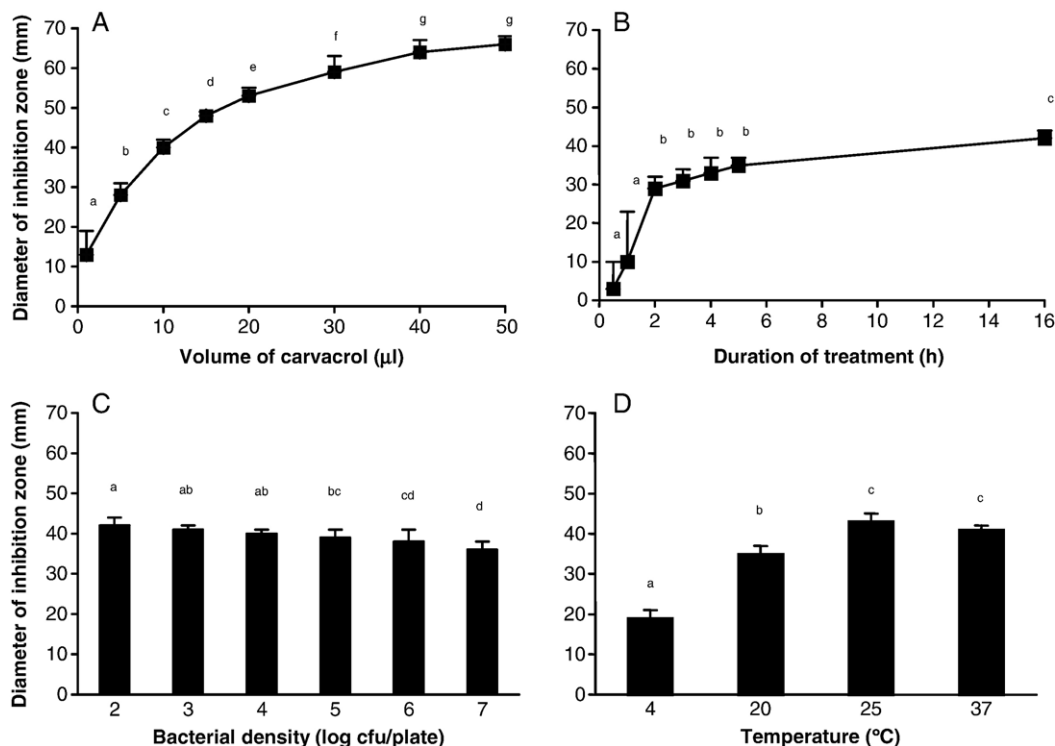

Fig. 1. Effect of various conditions on the inhibition of *S. enterica* serotype Enteritidis on TSA at 37 °C by carvacrol vapour. A) volume of carvacrol used; B) duration of vapour treatment; C) bacterial density; D) temperature. Data points with different letters are significantly different ($p \leq 0.05$).

carvacrol vapour for a further 24 h did not grow (data not shown).

In standard experiments under aerobic and anaerobic conditions, mean inhibition zones were 40±2 mm and 42±2 mm respectively. This difference was not significant. No other data for testing of carvacrol vapour under anaerobic conditions has been found in the literature. However, vapour of oregano oil, which generally contains a large proportion of carvacrol (Burt, 2004), had a limited effect on the numbers of *Enterobacteriaceae* on beef and the effect did not differ after packaging in 100% carbon dioxide (Skandamis and Nychas, 2002).

3.2. Chicken decontamination assay

The inhibitory effect of carvacrol vapour on the growth of *S. enterica* serotype Enteritidis on pieces of raw chicken is presented in Figs. 2–4. Treatment with carvacrol vapour significantly reduced viable numbers of salmonellae on chicken relative to the controls at 4 °C, 20 °C and 37 °C (Fig. 2). The possibility of significantly reducing numbers of viable salmonellae on chicken at refrigeration temperature offers perspectives for commercial applications. Other workers have also found that essential oil components can be effective bacterial inhibitors at refrigeration temperatures. A study of the inhibition of mesophilic aerobes on packaged grapes by thymol in air found it to be effective at 1 °C; total viable counts were markedly lower than in control packs (Valverde et al., 2005). Similarly, oregano or thyme essential oil vapour in the packaging for sea bass produced a marked inhibition of total viable count for up to 33 d at 2 °C (Harpaz et al., 2003). A study on the effect of oregano oil vapour on bacterial populations on pieces of beef showed no clear inhibition during 4 d at 15 °C and a marginal improvement compared to the control at 5 °C (Skandamis and Nychas, 2002).

A significant interaction was found between temperature and treatment group; the higher the temperature, the greater was the reduction relative to untreated controls. At 37 °C no viable salmonellae were recovered from treated chicken. In an experiment to determine how long a period at 37 °C was

Fig. 3. Effect of duration of the holding period at 37 °C on *S. enterica* serotype Enteritidis during carvacrol vapour treatment of chicken pieces. Data points with different letters are significantly different and those with asterisks are significantly different to the controls ($p \leq 0.05$).

necessary to achieve elimination of all viable bacteria it was found that a significant reduction was achieved after 1 h of vapour treatment and from 3 h no viable cells were recovered (Fig. 3). A holding temperature of 37 °C would not be an obvious choice for application in the poultry-processing sector since it would be generally conducive to the growth of pathogens and a number of spoilage organisms. However it is conceivable that a vapour treatment process could be developed whereby newly slaughtered chicken pieces are decontaminated by carvacrol vapour to eliminate *S. enterica* before refrigeration. Particularly the safety of chicken destined for consumption by immunodeficient individuals would be improved by such a treatment. Further testing on meat with the intact natural bacterial flora would be necessary first.

The minimum concentration of carvacrol necessary to achieve a reduction in salmonellae on raw chicken was determined and the results are presented in Fig. 4. Concentrations from 20% v/v carvacrol in ethanol were significantly effective and from 40% v/v no viable salmonellae were recovered. Since the standard volume of carvacrol solution used was 10 μl, these concentrations are equivalent to using 2
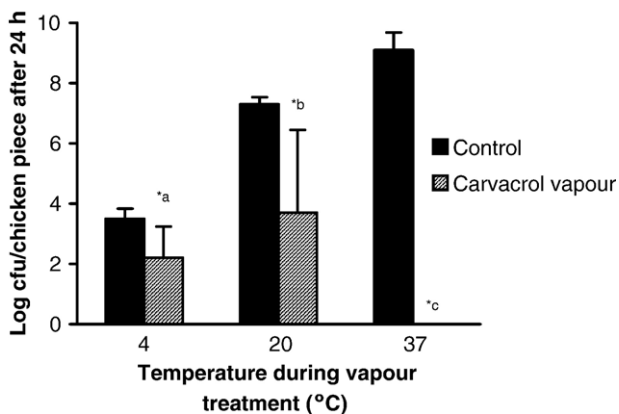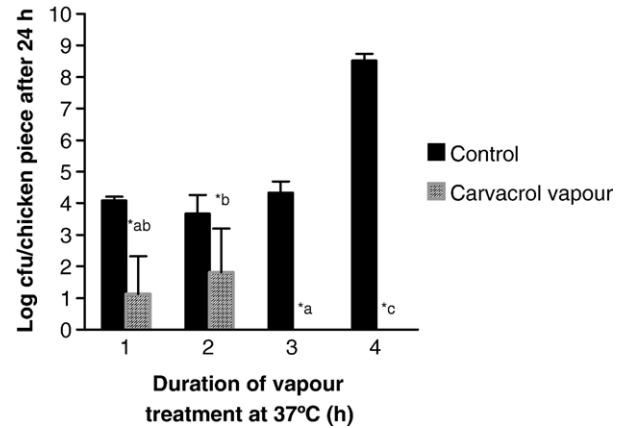
Fig. 2. Effect of temperature on *S. enterica* serotype Enteritidis during carvacrol vapour treatment of chicken pieces. Data points with different letters are significantly different and those with asterisks are significantly different to the controls ($p \leq 0.05$).
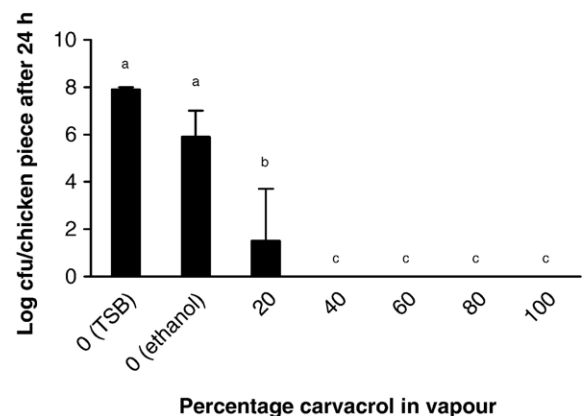
Fig. 4. Effect of concentration of carvacrol in the vapour on *S. enterica* serotype Enteritidis during vapour treatment of chicken pieces at 37 °C. Data points with different letters are significantly different ($p \leq 0.05$).

and 4 µl respectively of pure carvacrol. It is therefore possible that the cost of decontamination of chicken by means of carvacrol vapour could be reduced by the use of a smaller volume or a less concentrated vapour product.

Since it appears that carvacrol vapour diffused into the agar in the first set of experiments, it is likely that the vapour also diffused into the layer of moisture and/or cell membranes on the surface of the chicken and inhibited bacterial growth by that route. This could mean that the flavour and aroma of the meat may also be affected and this would limit commercial applications to products where a herbal flavour is desirable.

In conclusion, carvacrol vapour is effective at inhibiting the growth of *S. enterica* serotype Enteritidis on agar and at inhibiting and eliminating these bacteria on the surface of raw chicken under experimental conditions. The size of the inhibition zone on agar is dependent on time, temperature, amount of carvacrol and slightly on bacterial density, but is not dependent on aerobic conditions. On chicken breast meat, carvacrol vapour significantly reduced viable numbers of salmonellae in a temperature range from 4 to 37 °C and all viable cells (approx. 5×10^3 cfu) were eliminated by a minimum holding period of 3 h at 37 °C.

References

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., Chalier, P., 2006. Antimicrobial activity of carvacrol related to its chemical structure. Letters in Applied Microbiology 43, 149–154.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods — a review. International Journal of Food Microbiology 94 (3), 223–253.

Burt, S.A., Vlielander, R., Haagsman, H.P., Veldhuizen, E.J.A., 2005. Increase in activity of essential oil components carvacrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers. Journal of Food Protection 68 (5), 919–926.

Dorman, H.J.D., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88, 308–316.

European Commission, 1999. Commission decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of regulation (EC) No 2232/96 of the European Parliament and of the Council on 28 October 1996. 1999/217/EC. Official Journal L084, 1–137 (27/03/1999).

European Parliament, 1996. Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996 laying down a community procedure for flavouring substances used or intended for use in or on foodstuffs. Official Journal L299, 1–4 (23/11/1996).

Harpaz, S., Glatman, L., Drabkin, V., Gelman, A., 2003. Effects of herbal essential oils used to extend the shelf life of freshwater-reared Asian sea bass fish (*Lates calcarifer*). Journal of Food Protection 66 (3), 410–417.

Kimura, A.C., et al., 2004. Chicken consumption is a newly identified risk factor for sporadic *Salmonella enterica* serotype Enteritidis infections in the United States: a case-controlled study in FoodNet sites. Clinical Infectious Diseases 38 (suppl 3), S244–S252.

Mead, G.C., Mulder, R.W.A.W., 1997. Management in poultry processing before and after harvest, microbial control in the meat industry; 2. concerted action CT94-1456. University of Bristol Press, Bristol, p. 20.

Olsen, S.J., MacKinnon, L.C., Goulding, J.S., Bean, N.H., Slutsker, L., 2000. Surveillance for foodborne disease outbreaks — United States, 1993–1997. Morbidity and Mortality Weekly Report CDC Surveillance Summary 49, 1–62.

Rabsch, W., et al., 2000. Competitive exclusion of *Salmonella enteritidis* by *Salmonella gallinarum* in poultry. Emerging Infectious Diseases 6 (5), 443–448.

Skandamis, P., Nychas, G.-J.E., 2002. Preservation of fresh meat with active and modified atmosphere packaging conditions. International Journal of Food Microbiology 79, 35–45.

Skandamis, P., Koutsoumanis, K., Fasseas, K., Nychas, G.-J.E., 2001. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. Italian Journal of Food Science 13 (1), 65–75.

Smulders, F.J.M., 1995. Preservation by microbial decontamination, the surface treatment of meats by organic acids. In: Gould, G.W. (Ed.), New methods of food preservation. Blackie Academic and Professional, London, pp. 253–282.

Thorns, C.J., 2000. Bacterial food-borne zoonoses. Revues Scientifiques et Techniques 19 (1), 226–239.

Ultee, A., Kets, E.P.W., Smid, E.J., 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied and Environmental Microbiology 65 (10), 4606–4610.

Valverde, J.M., et al., 2005. Improvement of table grapes quality and safety by the combination of modified atmosphere packaging (MAP) and eugenol, menthol or thymol. Journal of Agricultural and Food Chemistry 53, 7458–7464.

Van Asten, A.J.A.M., et al., 1995. Identification of the domain which determines g,m-serotype of the flagellin of *Salmonella enteritidis*. Journal of Bacteriology 177, 1610–1613.

Weissinger, W.R., McWatters, K.H., Beuchat, L.R., 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. Journal of Food Protection 64 (4), 442–450.

World Health Organization, 2002. Future trends in veterinary public health. Technical Report. WHO study group on future trends in veterinary public health, Geneva, vol. 907.

Yang, Z., Li, Y., Slavik, M., 1998. Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. Journal of Food Protection 61 (7), 829–832.