ANTIMICROBIAL ACTIVITY OF ENCAPSULATED ESSENTIAL OILS IN EMULSION AND MICROEMULSION AGAINST FOODBORNE ORGANISMS IN GROUDED PORK

Sarisa Suriyarak[1], Myriam Loeffler[1], Kurt Herrmann[1], and Jochen Weiss[1*]

[1]Department of Food Science and Biotechnology, University of Hohenheim, 70599 Stuttgart, Germany

[*]Corresponding author (phone: +49-0711-459-24415; fax: +49-0711-459-24446; e-mail: j.weiss@uni-hohenheim.de)

Abstract— **The antimicrobial activities of encapsulated thymol and cinnamaldehyde (CA) in oil-in-water-emulsions and microemulsions were examined against a Gram-negative (*Escherichia coli K12*) and a Gram-positive (*Staphylococcus carnosus*) bacteria. Thymol and CA were successful incorporated in oil-in-water emulsions stabilized by Tween 20 by formulating a mixed carrier oil – thymol or CA base. Similarly, thymol and CA were successfully solubilized in Tween 80 surfactant micelles i.e. 0.8 and 1.2% of thymol and CA could be solubilitzed in 9.1% Tween 80. The stability of both the antimicrobial emulsion and microemulsion carrier system were assessed by Dynamic Light Scattering (DLS). The minimum inhibitory concentration (MIC) was determined using a Microtiter Growth assay at inoculation levels of 10^6 CFU/ml. The MIC of both compounds in emulsions varied between 300 and 400 ppm. The same compounds formulated in microemulsions were more active than in emulsions. When the inhibitory effect of the emulsion and microemulsion-based antimicrobial system as assessed in ground pork during storage of ten days storage at 6°C using total and selective plate counting, activity was markedly lower. The only significant growth reduction was found in CA-encapsulated emulsions at as much as 5000 ppm. The studies demonstrated the importance of ingredient interactions and matrix structure on the activity of encapsulated naturally-occurring antimicrobials.**

***Index Terms*—Naturally-occurring antimicrobials, emulsion, microemulsion, ground meat**

I. INTRODUCTION

Microbial growth may causes changes in the quality of the product (e.g. appearance, texture and flavor), and lead to the generation of toxins of colonization of the intestines by pathogens that in turn cause foodborne diseases. Ground meat is an especially nutrient-rich medium, and due to its dispersed structure with associated high surface areas offers ideal conditions for the growth of pathogens and spoilage organisms (Zhang, Kong et al. 2009). For example, in 2006, 3314 case of food poisoning caused by *Escherichia coli* VTEC were reported to the European Food Safety Authority, and these were largely associated with the consumption of freshly ground beef. While these incidents appear to not have reduced consumer demand for meat products, it illustrates the need to find new methods to extend shelf life and prevent growth of pathogens, particularly in those products that are not thermally treated (Aymerich, Picouet et al.). Currently much research is being conducted on the use of naturally-occurring antimicrobials such as essential oils as preservatives rather than the use of traditional synthetic preservatives. Presently, essential oils and their compounds are only used to a limited extent in meat products, and in the case where they are used, they are typically added to meat and meat products in the form of herbs and spices with the intent to add a particular flavor to the product. An exception to the direct addition is the use of marinates, in which herb and spice extracts are used in the form of emulsions that are applied on the surface of products to extends shelf life and to impart a characteristic flavor. To date, direct addition of essential oil dispersions to the bulk of meat and meat products has not yet been extensively investigated. However, some natural antimicrobials, such as thymol, are only effective as preservatives in foods at concentrations which adversely affect the sensory quality (Lemay, Choquette et al. 2002; Oussalah, Caillet et al. 2006). The interaction of natural antimicrobials with food constituents, as well as the difficulty of homogeneous mixing, may reduce the activity of essential oils against spoilage organisms when the oils are added directly to food. One potential solution to these problems might be to formulate a carrier system that may be better suited to allow am incorporation in complex food matrices. For example, essential oils can be encapsulated in micelles (so called microemulsions) or in emulsion droplets (Weiss, Gaysinsky et al. 2009). In this study, we therefore investigated the potential of emulsions and microemulsions to inhibit model spoilage organisms (*E. coli* and *Staphylococcus carnosus*) in ground meat using microemulsions and emulsions composed of cinnamonaldehyde and thymol and compared it to the direct addition of compounds. .

II. MATERIALS AND METHODS

Materials

Hydrophobic essential oil components (EOC): cinnamaldehyde (>93%), and thymol (99.5%),); Tween 20 (polyoxyethylen(20)-sorbitan-monolaurate), Tween80 (Polyoxyethylenesorbitan monooleate), 10 mM citrate buffer

(citric acid and sodium citrate monohydrate), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Steinheim, Germany). Miglyol 812 (Caprylic/Capric Triglyceride) was supplied by Sasol Germany GmbH (Hamburg, Germany). Standard I-broth and Standard I-agar, Trypton-Galle-Agar (TBA), Baird-Parker-Agar (Basis), and Peptone was purchased from Carl Roth GmbH (Karlsruhe, Germany). All solutions were prepared with ultra pure water.

Preparation of thymol and cinnamaldehyde emulsions and microemulsions

Emulsions: A stock solution of a nonionic emulsifier (1% Tween 20 [all concentrations are on a weight per volume basis]) was prepared by dissolving the surfactant in 10 mM citrate buffer (pH 6). A lipid phase composed of a model triacylglyceride food oil (Miglyol 812) containing different concentrations of EOCs was prepared by mixing the essential oils at different concentrations overnight with Miglyol. 95% of the surfactant solution was coarsely homogenized with 5% of the lipid phase using a high shear mixer (Ultraturrax) for 2 minutes at maximum speed (24,000 min^{-1}) to prepare an emulsion premix. The premix was then repeatedly (3x) passed through a high pressure homogenization (Microfluics M110P) at a homogenization pressure of 10,000 psi to reduce the droplet size. Finally, the emulsions' pH was adjusted by hydrochloric acid (HCl) or sodium hydroxide (NaOH) to 6.0.

Microemulsions: Aqueous surfactant solutions were prepared by dispersing 9.1% non ionic surfactant Tween 80 in 10 mM citrate buffer pH6 at room temperature. Thymol and CA were added to surfactant solutions at concentration ranging from 0.16 to 1.2%. The solutions were stirred until either the absorbance at 630 nm remained constant or no visible oil droplets were left in the solution indicating that solubilization had been complete.

The stability of the produced emulsions and microemulsion were determined by measuring changes in droplet size using a Dynamic Light Scattering (DLS) technique (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, UK). Prior to all antimicrobial assays, solutions were filtered sterilized using a 0.20 μm polyethersulfone membrane filters (Pradusc^{TM} 25, Whatman® Inc.).

Antimicrobial activity assays

Bacterial strains. Two strains of bacteria; *Escherichia coli K12* and *Staphylococcus carnosus ssp. carnosus* were stored at -75ºC in Standard I-broth with 20% glycerol. Working cultures were stored on Standard I-Agar plates at 4°C. Prior to each experiment, a single colony of culture from the plate was activated twice by incubation in Standard I-broth medium overnight and incubated at 37°C for 24 hours.

Microtiter Growth Assay: Emulsion and microemulsions at varying droplet or micelle concentrations were dispensed in a 96-well microtiter plate containing 120 μl of a 10 mM citrate buffer (pH 6,0) per well. 120μl of inoculated double-strength bacteria suspension (approximately $1*10^6$ cfu/ml) was added to the each of the wells. The microtiter plates were then incubated at 37°C for 24. The optical density was measured after 0, 1, 3, 6, 12 and 24 hours at 630 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Germany).

Microbiological analysis in ground pork:

Fresh pork meat was standardized, and ground through a 5 mm perforated plate prior to testing. Preliminary tests showed that a maximum volume of 20 ml solution could be added to 200 g of ground meat and mixed with a blender for no longer than 3 mins without causing unacceptable changes in appearance. *E. coli K12* and *Staphylococcus carnosus* suspension were added to ground pork to obtain an inoculation level of approximately 10^4 cfu/g and 10^6 cfu/g for incubation at 14°C and 6°C, respectively. Thymol or CA encapsulated in emulsion (0– 6000 ppm), or microemulsion (0-751 ppm thymol and 0-1321 ppm CA), and unencapsulated thymol and CA (0– 6000 ppm) were gently mixed into the ground meat for 2 mins. Meat samples (25g) of each treatment including control without antimicrobial components or/and culture were transferred to sterile stomacher bags, which were closed and stored at the above mentioned temperatures. Samples assessed on day 0, 2, 4, 6, 8, 10, and 12.

Total plate count: Stored meat samples (25g per bag) were mixed with 225 ml of sterile 0.1% peptone water and pummeled for 90 s in a stomacher. Serial tenfold dilutions were prepared in 0.1% peptone in water, and a 0.1 ml sample from each tube was plated in duplicate. Tryptone-Bile Agar (TBA) for *E. coli K12* counts, Baird Parker Agar for *Staphylococcus carnosus* counts, and Standard 1 agar for total bacteria counts were used. Plates were incubated at 37°C for 24 h and the number of colonies on each plate determined using a plate reader.

III. RESULTS AND DISCUSSION

Formulation of Emulsions and Microemulsions. Stable thymol- and cinnamaldehyde-loaded emulsions could able to be formulated with a carrier oil (Miglyol812) using high pressure homogenization. At a fixed lipid phase concentration (5%), the maximum loading percentage of Thymol and CA was 30% and 95%, respectively. At loadings above these maximum loading percentages, emulsions immediately "broke" that is they phase separated into a lipid and an aqueous phase. Consequently, emulsions were prepared at the maximum loading percentages and assessed for long term emulsion storage stability studies. Thymol and CA emulsions that were maximally loaded had z-average droplet diameters of 200 and 60 nm respectively. Both emulsions were stable during a two week storage test at 28 degree, that is the droplet size remained constant and no visible phase separation occurred. Results may be attributed to Oswald

ripening, a key destabilizing process in emulsion that contains low molecular weight lipids that have an appreciable water solubility in the aqueous phase. Ostwald ripening is the growth of larger oil droplets in the emulsion at the expense of smaller ones. It is caused by a molecular mass transport between droplets of different sizes of small molecular weight lipids through the aqueous phase of the emulsion. This mass transport is driven by the differences in concentration of lipids above the water-oil interface that is due to the different curvatures of oil droplets of different sizes (Binks, Clint et al. 1999; Meinders and van Vliet 2004). Because of that, emulsions that contained 100% EOCs could not be formulated. In contrast, addition of a carrier oil obstructed this destabilizing process and resulted in the production of stable, loaded emulsions (Gaysinksy, Suriyarak et al. In preparation).

Microemulsions are thermodynamic stable and optically isotropic dispersions that consist of surfactant aggregates and one or more solubilized substances that are incorporated into the surfactant aggregates (Lawrence and Rees 2000). Microemulsions have shown to exhibit better oxidation stability and allow for a more precisely controlled released (due to the more narrow particle size distribution) than emulsions (Park and Kim 1999). Antimicrobial microemulsions were formulated in our studies by titrating thymol and CA in 9.1% Tween 80. A transparent solution containing the solubilized antimicrobial in surfactant micelles was obtained as a result. The size of the Tween 80 micelle increased from 11.47 +/- 0.17 nm to 23.12 +/-1.85 nm and 24.0 +/- 0.21 nm after uptake of 0.8% Thymol and 1.2% CA respectively. At antimicrobial concentrations above 0.8 and 1.2% of thymol and CA, the solutions' appearance changed from clear to turbid indicating that excess antimicrobial remained in the aqueous phase and could no longer be solubilized in the surfactant aggregates. Results may be attributed to the fact that the loading concentration in microemulsions is limited by the available volume in the micelle, the type of surfactant used, of lipid solubilized, and the temperature of the solution.

Antimicrobial activity. Many studies have shown the antimicrobial effect of essential oils thymol and cinnamaldehyde, as well as oils from cloves and oregano in microbial model systems. Their activities have been attributed to their functional groups, and there is a well-known synergistic interactions between the oil components that may enhance their individual activity (Gutierrez, Barry-Ryan et al. 2009). Generally, lipophilic phenol groups of essential oils appear to play a major role as inhibitors of bacterial growth. One of the proposed mechanisms is that they enter the lipid bilayer membrane of the microbial cell to disrupt the activity of membrane-based protein complexes that generate ATP. Unlike thymol, non-phenolic essential oils, such as cinnamaldehyde, have shown to posses antibacterial properties by inhibiting amino acid decarboxylase activity (Ouattara, Simard et al. 1997). Essential oils are generally less active against Gram-negative bacteria, due to the fact that the outer membrane of Gram-negative bacteria consists of lipopolysaccharide chains that may restrict permeation of hydrophobic compounds into the cell (Burt 2004). Similar results were found in this study. In the Microtiter Growth assays, *Staphylococcus carnosus* was more sensitive than *E. coli* to both encapsulated antimicrobials. Concentrations of 350 ppm of thymol-loaded and 275 ppm of CA-loaded emulsions were sufficient to significantly reduce the growth of *Staphylococcus carnosus*. However, even at concentrations of as much as 500 ppm of thymol in emulsion, little effect on the growth of *E. coli* K12 was observed. With 300 ppm of CA-loaded emulsions, the lag phase of *E. coli* K12 was significant longer, a phenomena that was also observed in CA-loaded microemulsion (**Fig. 1**). However, the microemulsions at similar effective compound concentrations appeared to be more antimicrobially active than the emulsion system, that is their minimum inhibitory concentration was lower. This might because of differences in mass transport processes in the respective systems that may cause concentrations of antimicrobial that reach the microbial cell to be lower or higher.

The concentrations of essential oil components in ground pork required for inhibition were dramatically higher in than in the microtiter growth assay. The concentrations of active compounds that could be tested were limited by both the loading percentage of the respective application system (emulsion or microemulsion) as well as changes in the appearance of the meat. The 6000 ppm thymol of loaded emulsion was the highest concentration that could be added to ground meat. Interestingly, there was no antimicrobial inhibition of the growth of *E. coli K12* and *Staphylococcus carnosus* in both inoculums at temperatures of 6°C and 14°C. In contrast, 5000 ppm of CA reduced the bacterial growth by approximately 3 logs compared to the control sample after 10 days of at 14°C (**Fig. 2**). Conversely, no effect was found when antimicrobials were directly mixed with the ground meat in the absence of a carrier system. This suggests that the emulsion-based carrier system provided some protection from ingredient interaction and partitioning processes in other lipid phases that may have reduced their activity. None of the microemulsion systems was able to inhibit the growth of microorganisms in ground pork system, likely due to the limitation in the maximal achievable concentration of active compounds.

Several explanations may be found as to why such high concentrations are needed to obtain an antimicrobial effect in ground meat. Meat products are complex nutrient-dense mediums and it has been reported that some constituents may protect microbial cells. For instance the sensitivity of bacteria in meat varied with the fat content in the meat. On the one hand fat in food could form a protective coat around bacteria, thereby protecting them from antimicrobial agents (Zhang, Kong et al. 2009). On the other hand, the fat in meat may have sequestered the antimicrobials. Finally, thymol could have been enzymatically inactivated by oxidation of the phenolic group (Ouattara, Simard et al. 1997). Clearly, more studies will be needed to elucidate the specific structure-functional relationships that govern effectivity of antimicrobials in meat and meat products.

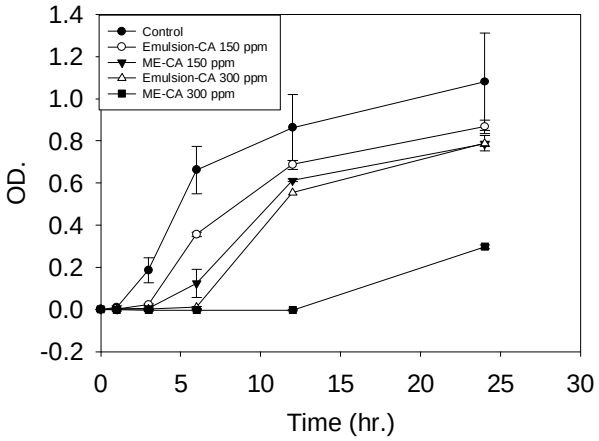

Fig 1. Antimicrobial activity of CA in emulsion and microemulsion against *E. coli* K12 with inoculums of 10^6 CFU/ml in a Microtiter Growth assay.

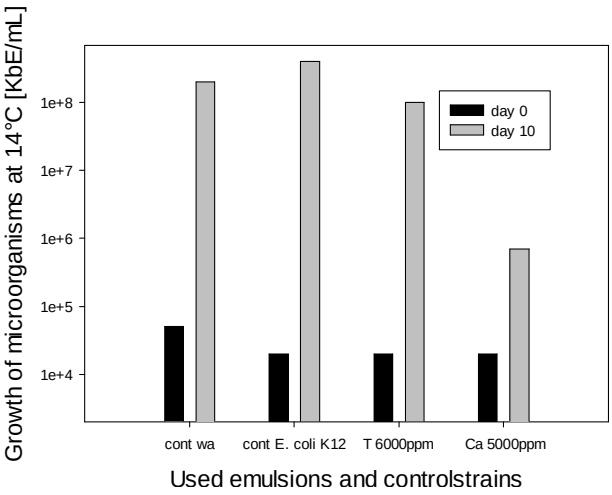

Fig 2. Antimicrobial activity of CA against *E. coli* K12 in ground pork

IV. CONCLUSION

Results demonstrate that the formulation of antimicrobial active emulsions and microemulsion with cinnamaldehyde, and thymol is feasible. The stability of EOC-loaded emulsions was improved when a carrier oil (Miglyol) was added. The concentration and type of EOC influenced the stability of emulsion (Taylor 2003; Gaysinksy, Suriyarak et al. In preparation). Using emulsions and microemulsions loaded with natural occurring antimicrobials caused significant reduction of bacterial counts in microbiological model assays. In ground pork, activity was markedly lower to the point where no microorganisms were inhibited. Limits in terms of payload in combination with the presence of lipid phases and antimicrobial degrading enzymes may be responsible for the observed discrepancy between model and real product. More systematic studies as to the impact of encapsulation system properties, choice of antimicrobials and composition and structure of the food in which antimicrobials are to be used, are required. Nevertheless, results with emulsions show some initial promising results and activity may be improved by moving from single antimicrobial compound systems to combinations.

REFERENCES

Aymerich, T., P. A. Picouet, et al. "Decontamination technologies for meat products." Meat Science **78**(1-2): 114-129.

Binks, B. P., J. H. Clint, et al. (1999). "Kinetics of Swelling of Oil-in-Water Emulsions Stabilized by Different Surfactants." Langmuir **15**(13): 4495-4501.

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

Gaysinksy, S., S. Suriyarak, et al. (In preparetion). "Emulsions as Antimicrobial Delivery Systems: Emulsion Stability, and Antimicrobial Activity".

Gutierrez, J., C. Barry-Ryan, et al. (2009). "Antimicrobial activity of plant essential oils using food model media: Efficacy, synergistic potential and interactions with food components." Food Microbiology **26**(2): 142-150.

Lawrence, M. J. and G. D. Rees (2000). "Microemulsion-based media as novel drug delivery systems." Advanced Drug Delivery Review(89-121).

Lemay, M.-J., J. Choquette, et al. (2002). "Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model." International Journal of Food Microbiology **78**(3): 217-226.

Meinders, M. B. J. and T. van Vliet (2004). "The role of interfacial rheological properties on Ostwald ripening in emulsions." Advances in Colloid and Interface Science **108-109**: 119-126.

Ouattara, B., R. E. Simard, et al. (1997). "Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms." International Journal of Food Microbiology **37**(2-3): 155-162.

Oussalah, M., S. Caillet, et al. (2006). "Antimicrobial effects of selected plant essential oils on the growth of a Pseudomonas putida strain isolated from meat." Meat Science **73**(2): 236-244.

Park, K. M. and C. K. Kim (1999). "Preparation and evaluation of flurbiprofen-loaded microemulsions for parenteral delivery." International Journal of Pharmaceutics(181): 173-179.

Taylor, P. (2003). "Ostwald ripening in emulsions: estimation of solution thermodynamics of the disperse phase." Advances in Colloid and Interface Science **106**(1-3): 261-285.

Weiss, J., S. Gaysinsky, et al. (2009). Nanostructured Encapsulation Systems: Food Antimicrobials. Global Issues in Food Science and Technology. b. Edited, B.-C. Gustavo, M. Alanet al. San Diego, Academic Press**:** 425-479.

Zhang, H., B. Kong, et al. (2009). "Antimicrobial activities of spice extracts against pathogenic and spoilage bacteria in modified atmosphere packaged fresh pork and vacuum packaged ham slices stored at 4 °C." Meat Science **81**(4): 686-692.