Evaluation of the Potential Synergistic Antimicrobial Effects Observed using Various Combinations of Agents (Nanoparticled and Non-Nanoparticled) on a Selected Panel of Cheese-Derived Microorganisms.

K. A-M. O' Callaghan* and J. P. Kerry**

Food Packaging Group, School of Food and Nutritional Sciences, University College Cork, Ireland,
*k.a.ocallaghan@umail.ucc.ie, **joe.kerry@ucc.ie

ABSTRACT

This objective of this study was to determine if a combination of chemical agents could produce a synergistic antimicrobial effect, by either targeting a greater spectrum of microorganisms, or by reducing the amount of antimicrobial required to cause inhibition. Five agents (nanoparticled solubilisates - sorbic acid, benzoic acid and rosemary, and non-nanoparticled chitosans, two different molecular weights) were selected based on promising antimicrobial activity and/or enhanced solubility. Combinations of these agents were examined against cultures derived from cheese. The study found the top performing antimicrobials contained chitosan and/or rosemary, individually or in combination. These findings encourage their use as active agents in cheese packaging.

Keywords: antimicrobial, nanoparticles, cheese, rosemary, chitosan

1 INTRODUCTION

The driving force for the antimicrobial packaging of dairy foods, like cheese, is due to the increase in demand for such products globally, with global consumers requiring the same standard of quality and safety as those receiving the products in the home manufacturing domestic market. Exportation of cheese, like any other perishable product, is accompanied by many challenges. The problems imposed include increased exposure to fluctuating temperatures and humidities, increased handling, excessive distances, and poor distribution and storage conditions. These factors can cause changes to the physical and chemical characteristics of the cheese, including; colour, texture and taste, oxidation, odour development, sweating, shape deformities, decrease in nutritional value, and an increase in spoilage microorganisms; all of which can lead to a decrease in shelf-life and a compromised quality, providing a final product of an unacceptable standard.

The use of active packaging changes the condition of the packaged food. Active packaging extends the shelf-life, improves food safety or alters the sensory properties; whilst maintaining the quality of the packaged food [1]. Different preservatives have been employed in antimicrobial packaging over the years, with organic acids and bacteriocins most commonly associated with cheese preservation [2, 3]. A number of studies have examined the effect of various combinations of antimicrobials on cheese[4, 5], with the aim of utilising active agent combinations is to expand the antimicrobial spectrum reached, minimise toxicity, reduce concentration levels, and to obtain an overall synergistic antimicrobial activity [6]. However, many of these combinations to date have contained synthetic chemical agents, whereas the demand in active packaging for food applications is for natural antimicrobials. Additionally, there is an increased drive for the incorporation of nanotechnology into smart packaging design, as the area encompassing nano-based research is rapidly growing [7].

The antimicrobial agents investigated in this study were selected based on results determined from previous work [8]. Criteria for this selection combined a balance of promising antimicrobial activity and/or enhanced solubility. Therefore, this study was undertaken in order to investigate the antimicrobial activity of nanoparticled benzoic acid, sorbic acid and rosemary solubilisates, and non-nanoparticled low molecular weight chitosan and medium molecular weight chitosan, when applied individually and in combination against cheese-derived cultures, including both Gram-negative and Gram-positive varieties.

2 MATERIALS AND METHODS

2.1 Materials and microbiological media

Aquanova AG (Darmstadt, Germany) supplied the nanoparticled solubilisates (~30nm) - 4% Sorbic acid, 12% Benzoic acid, 6% Carnosolic acid (rosemary), 4% Sorbic acid/4% Benzoic acid. Both chitosans, low molecular weight (50-190 kDa) and medium molecular weight (190-310 kDa) were sourced from Sigma-Aldrich, St. Louis, MO, USA. Acetic acid (Fisher Scientific UK Ltd., Leicestershire, UK) was used to improve the solubility of chitosan in water. Emmental and Cottage cheese were both sourced locally. Tryptone Soya Agar (TSA) and Mueller-Hinton Broth (MHB) were obtained from Oxoid Ltd., Basingstoke, Hampshire, England. Minimum Inhibition Concentration (MIC) was measured using 96-well tissue culture microplates (Sarstedt, Inc., Newton, NC, USA).

2.2 Antimicrobial Preparation

The antimicrobials selected included; three nanoparticles – sorbic acid (SASB), benzoic acid (BASB) and rosemary (ROSE), and two non-nanoparticled chitosans – low molecular weight chitosan (LMWC) (50,000 to 190,000 Da) and medium molecular weight chitosan (MMWC) (190,000 to 310,000 Da). These five agents were input into the statistical program Statgraphics, which computed 32 different experimental mixtures. According to the mixtures computed via Statgraphics, solutions from 1 to 32 were prepared (Table 1). Additionally, a nanoparticled solubilisate - a blend of sorbic acid and benzoic acid (SABASB) was also examined, and labelled as solution 6.

Table 1

	Antimicrobial Mixtures	Concentration Breakdown	%
1	LMWC	0.25	0.25
2	MMWC	0.25	0.25
3	SASB	0.5	0.5
4	BASB	0.5	0.5
5	ROSE	0.5	0.5
6	SABASB	0.5	0.5
7	LMWC + MMWC	0.25 + 0.25	0.5
8	SASB + LMWC	0.5 + 0.25	0.75
9	SASB + MMWC	0.5 + 0.25	0.75
10	BASB + LMWC	0.5 + 0.25	0.75
11	BASB + MMWC	0.5 + 0.25	0.75
12	ROSE + LMWC	0.5 + 0.25	0.75
13	ROSE + MMWC	0.5 + 0.25	0.75
14	SASB + BASB	0.5 + 0.5	1
15	SASB + ROSE	0.5 + 0.5	1
16	BASB + ROSE	0.5 + 0.5	1
17	SASB + LMWC + MMWC	0.5 + 0.25 + 0.25	1
18	BASB + LMWC + MMWC	0.5 + 0.25 + 0.25	1
19	ROSE + LMWC + MMWC	0.5 + 0.25 + 0.25	1
20	SASB + BASB + ROSE + LMWC + MMWC	0.25 + 0.25 + 0.25 + 0.125 + 0.125	1
21	SASB + BASB + LMWC	0.5 + 0.5 + 0.25	1.25
22	SASB + BASB + MMWC	0.5 + 0.5 + 0.25	1.25
23	SASB + ROSE + LMWC	0.5 + 0.5 + 0.25	1.25
24	SASB + ROSE + MMWC	0.5 + 0.5 + 0.25	1.25
25	BASB + ROSE + LMWC	0.5 + 0.5 + 0.25	1.25
26	BASB + ROSE + MMWC	0.5 + 0.5 + 0.25	1.25
27	SASB + BASB + ROSE	0.5 + 0.5 + 0.5	1.5
28	SASB + BASB + LMWC + MMWC	0.5 + 0.5 + 0.25 + 0.25	1.5
29	SASB + ROSE + LMWC + MMWC	0.5 + 0.5 + 0.25 + 0.25	1.5
30	BASB + ROSE + LMWC + MMWC	0.5 + 0.5 + 0.25 + 0.25	1.5
31	SASB + BASB + ROSE + LMWC	0.5 + 0.5 + 0.5 + 0.25	1.75
32	SASB + BASB + ROSE + MMWC	0.5 + 0.5 + 0.5 + 0.25	1.75
33	SASB + BASB + ROSE + LMWC + MMWC	0.5 + 0.5 + 0.5 + 0.25 + 0.25	2

Antimicrobial abbreviations were assigned as follows: SASB - Sorbic Acid Solubilisate, BASB - Benzoic Acid Solubilisate, LMWC - Low Molecular Weight Chitosan, MMWC - Medium Molecular Weight Chitosan, SABASB - Sorbic Acid/Benzoic Acid Solubilisates.

Table 1: Antimicrobial mixtures, concentration breakdown and the total % concentration applied.

2.3 Cultures and their Growth Conditions

The bacterial strains used for MIC testing included; Gram-negative species *Escherichia coli* and *Pseudomonas fluorescens*, and Gram-positive species, *Staphylococcus aureus* and *Bacillus cereus*, were derived from cheese samples and cultivated on TSA slants. Prior MIC testing, the microbial cultures were regenerated twice from the TSA slants into a growth media, MHB, and incubated for 18 hours, at 30°C for Gram-positive species, and at 37°C for Gram-negative species. Cheese cultures were derived from both Emmental and cottage cheese. Emmental culture preparation involved homogenising 10g of Emmental with 90mls of sterile MHB in a Colworth Stomacher 400 (Seward Ltd., England). The homogenisate (1ml) was transferred into 10ml MHB and incubated for 18 hours at 37°C. Cottage cheese culture was prepared by swabbing the cottage cheese surface and transferring the swab into MHB (10ml). The sample was then incubated for 18 hours at 37°C.

2.4 Antimicrobial Susceptibility Assessment

MIC testing was used to determine the antimicrobial action of the prepared mixtures against various cultures through the micro-dilution method. Within the 96-well tissue culture microplates, 100µl of sterile MHB was pipetted into rows A to F, 1-12, with an additional aliquot of 200µl of MHB into the well H 12. Quantities of the antimicrobial mixture (150µl) were pipette into to row G, with row H 1-11 containing 200µl of the test culture. Dilution was performed by transferring 50µl of the antimicrobial from row G and mixing it into row F. Subsequently, 50µl of the resultant mixture from row F was extracted and mixed into row E. This same action was repeated until row B, from which 50µl was discarded, thus creating a three-fold serial dilution. Row A contained no antimicrobial and was used as a positive growth control. Following dilution, each well from row A to G was inoculated with test culture (15µl) from row H. Column 12 represented a no growth control as it contained a no culture. The microplates were incubated for 18 hours, 30°C for *P. fluorescens* and *B. cereus*, and 37°C for *E. coli, S. aureus*, and both Emmental- and cottage cheese-derived cultures. Turbidity was identified as an indication of growth, which was evaluated visually after incubation. MIC was defined as the lowest concentration of antimicrobial agent showing a complete growth inhibition of the microbial culture tested.

3 RESULTS AND DISCUSSION

All treatments, with the exception of SABASB exerted antimicrobial effects. The five best performing antimicrobial agents for each culture tested is listed in Table 2.

Table 2

Antimicrobial Solution	Cottage Cheese	Emmental	*E. coli*	*P. fluorescens*	*S. aureus*	*B. cereus*	Total
LMWC 0.25%	0.053	0.046	0.062	0.025	0.083	0.037	0.051
MMWC 0.25%	0.046	0.083	0.065	0.046	0.083	0.046	0.062
ROSE 0.5%	0.066					0.037	
LMWC/MMWC 0.5%	0.111	0.074	0.131	0.056	0.167	0.093	0.105
SASB/LMWC 0.75%			0.222				
BASB/MMWC 0.75%					0.222		
ROSE/LMWC 0.75%	0.102	0.111					0.216
ROSE/MMWC 0.75%						0.065	
SASB/LMWC/MMWC 1%				0.222			
BASB/LMWC/MMWC 1%			0.222				
ROSE/LMWC/MMWC 1%		0.148		0.111	0.296		0.202

Table 2: Mean MIC of the top five best functioning antimicrobial solutions for each culture.

It can be seen from Table 2, chitosan and rosemary, singly and in combination, provided the most active results against

both Emmental and cottage cheese cultures. Although no significance was determined between antimicrobial treatments applied against cottage cheese- or Emmental-derived cultures individually, when the antimicrobial activities observed between both cheese culture types were compared, a significant difference in the effectiveness of treatments was found ($P < 0.05$). Emmental microflora showed a greater resistance than cottage cheese to the treatments used. In total, seven treatments produced no antimicrobial effect against the Emmental-derived culture, whereas, only one treatment (SABASB) failed to produce an antimicrobial effect against the cottage cheese-derived culture. From the MIC's generated, it can be seen that the cottage cheese-derived culture also presented a lower overall MIC, which implies that cottage cheese-derived culture was more sensitive to the treatments applied. It has been proposed that components present within the cheese may provide a level of protection which might prevent interaction between the antimicrobial substance and the target microorganisms. Specifically for cheese, [9] found a reduced antimicrobial activity in higher fat cheeses. The fat present can form a protective barrier around the bacteria and additionally the antimicrobial agent could dissolve into the lipid fraction which decreases the concentration of antimicrobial available, thereby reducing its capacity to act against bacteria in the aqueous phase [10]. Emmental has a higher total fat content (29.7g) than cottage cheese (4.3g) [11]. The increased lipid levels may explain the lower inhibition observed with Emmental.

The results produced from the antimicrobial testing of Gram-negative bacteria show remarkable similarities between the inhibition of *E.coli* and *P.fluorescens*. The overall MIC's for *E.coli* and *P.fluorescens* are relatively comparable at 0.456 and 0.445 respectively, as are the three most effective working treatments – LMWC, MMWC and LMWC + MMWC. [12, 13] have both demonstrated the inhibitory effect of chitosan on *E.coli* and *Pseudomonas* species, respectively. As seen in Table 2, the remainder of treatments having the greatest effect on *E.coli* include organic acids. In comparsion, rosemary features within *P.fluorescens* top five functioning treatments. Rosemary demonstrates an acuteness for P. *fluorescens*, which it does not appear to possess for E.coli. An improvement in Gram-negative inhibition may be achieveable, if rosemary were to be added at a higher concentration. [14] found that increased levels of essential oils were required to inhibit Gram negative compared to the levels needed to inhibit the Gram positive range of bacteria present. However, the strongest antimicrobial effects exerted on Gram-negative bacteria in this study were seen for chitosan-based treatments. The antimicrobial mechanism associated with chitosan is attributed to chitosan's ability bind to the outer membrane of the bacterial cell and subsequently disrupt barrier function [15]. Even though chitosan provided the greatest antimicrobial effect for both microorganisms, *P.fluorescens* had noticeably lower MIC values. This could be due to *E.coli* possessing an early warning defence mechanism against antimicrobial attack [16]. In any case, when MIC data for *E.coli* and *P.fluorescens* were compared, no significant differences were found.

Unlike the treatment similarities observed for Gram-negative bacteria, there was a stark contrast in results between *S.aureus* and *B.cereus*. *S.aureus* endured the highest overall MIC (0.667) amongst all samples tested, whereas *B.cereus* experienced the lowest MIC (0.308). The five most effective antimicrobial treatments for both Gram-positive bacteria assessed were similar (Table 2). However, as can be readily observed, the treatment levels required to deliver antimicrobial effects were very different ($P<0.001$). For *B.cereus*, a total of 30 active antimicrobial combinations were evident from screening; 18 of which had a MIC of less than 0.250, with only SASB, SASB + BASB and SABASB proving to be non-active treatments. Conversely, 28 treatments had an antibacterial effect on *S.aureus*, however, only 4 of these treatments were effective at a concentration of less than 0.25%. Generally Gram-positive bacteria are considered less resistant to antimicrobial substances than Gram-negative bacteria as they do not possess an outer membrane. However, certain Gram-positive microbes have been known to develop a protective response to compensate for the absence of this outer cell membrane. For example, *Staphylococcus aureus* has been known to use intercellular communication to induce virulence factors [17]. However, in this study, *S.aureus* tolerance to the antimicrobials is most likely caused due to natural variance within the microbe assessed rather than an actual stable resistance. Interestingly, *B.cereus* was the only microbe tested which showed sensitivity to an active antimicrobial treatment which did not possess chitosan as part of the treatment; SASB + ROSE (MIC - 0.210). Another unique point with respect to the control of *B.cereus* was that Rosemary performed just as strongly as chitosan in treatments. [18] also determined that *B.cereus* and other *Bacillus* species were very susceptible to rosemary compared to other bacteria tested. Rosemary also impacted on *S.aureus*, but at a higher MIC level (0.315). [19] examined the antimicrobial effect of a commercial rosemary extract, and similar to our findings, found that much lower concentrations of rosemary were needed to inhibit *B.cereus* (0.06%) compared to *S.aureus* (0.5%).

The five best overall performing antimicrobial treatments are also outlined in Table 2. LMWC, MMWC and rosemary all showed the greatest antimicrobial activities of the chemical agents assessed. Chitosan is evidently the most effective broad-spectrum antimicrobial in this study due to its low MIC levels, and as evidenced by its presence in all of the five most effective active treatments, used either on its own or in combination. Chitosan of a lower molecular weight performed slightly better than medium molecular weight chitosan. Rosemary itself exerted a moderate antimicrobial activity, working particularly well for both cheese-derived cultures and pure Gram-positive cultures. The organic acid solubilisates demonstrated only a marginal

effect. Of the two organic acids tested, BASB (MIC = 0.486) performed better than SASB (MIC = 0.493). [20] showed similar results for both organic acids against Gram-positive and Gram-negative bacteria following incorporation into packaging films. Although, it was hoped that stronger synergistic effects would be achieved between the chemical agents assessed, a commensal influence was more evident. No combination treatment attained the same antimicrobial effectiveness as that produced by a single antimicrobial treatment. [21] also reported that various chemical combinations assessed in their study showed no synergism, but resulted in many additive patterns. In general, the antimicrobial effects of the chitosan combinations, particularly those with rosemary, proved stronger than the chitosan-organic acid combinations. This has also been seen when chitosan was used in combination with garlic oil and potassium sorbate. The activity of chitosan was substantially improved using the essential oil, but a reduced action was reported when chitosan was combined with the organic acid salt [22]. [21] suggested that agents with a similar composition and structure may not provide synergistic effects. Although rosemary and organic acids do not have similar chemical compositions, nanoparticled solubilisates have related physical structures. Equally LMWC and MMWC have similar structures and when used together in different combinations, they provided antimicrobial action but none of these combinations were as antimicrobially effective as either form of chitosan applied individually. Conversely, his could also explain why combinations of chitosan and solubilisates had an additive effect; owing to the different physical and chemical structures associated with these substances. In addition to chemistry and structure affecting efficacy, potency can also be affected by environmental conditions. Adjusting pH may be key to achieving synergism with solubilisates in the future. Additionally, the incorporation of natural chelators or enzymes could be used to disrupt the membrane of Gram-negative bacteria.

4 CONCLUSION

Chitosan, of low and medium molecular weight, and rosemary provided the most effective inhibition across all samples examined. Overall, chitosan was the best performing antimicrobial of all screened agents, providing strong results when used singly or in combination; with low molecular weight chitosan functioning slightly better than medium molecular weight chitosan. Rosemary appeared to be more antimicrobially selective in its inhibition behaviour, providing a favourable effect against cheese-derived cultures and Gram-positive bacteria. No treatment combination proved to be synergistic. Lowering pH or incorporating membrane perturbing substances could be employed to improve solubilisate activity. Future work will concentrate on the incorporation of chitosan and/or rosemary treatments into packaging and applying the treated packaging to cheese products.

REFERENCES

[1] N.N. De Kruijf, M. van Beest, R. Rijk, T. Sipiläinen-Malm, L.P. Paseiro and B. De Meulenaer, Food Additives and Contaminants, 19, 144-162, 2002.

[2] C. Hauser and J Wunderlich, Procedia Food Science, 1, 197-202, 2011.

[3] A.G.M. Scannell, C. Hill, R.P. Ross, S. Marx, W. Hartmeier and E.K. Arendt, International Journal of Food Microbiology, 60, 241-249, 2000.

[4] K. Hanušová, M. Šťastná, L. Votavová, K. Klaudisová, J. Dobiáš, M. Voldřich and M. Marek, Journal of Food Engineering, 99, 491-496, 2010.

[5] M. Sinigaglia, A. Bevilacqua, M. Rosaria Corbo, S. Pati and M.A. Del Nobile, International Dairy Journal, 18, 624-630, 2008.

[6] W. Song, H.J. Woo, J.S. Kim and K.M. Lee, International Journal of Antimicrobial Agents, 21, 8-12, 2003.

[7] Editorial, Technovation, 32, 157-160, 2012.

[8] K.A-M. O' Callaghan and J.P. Kerry, International Journal of Dairy Technology, Manuscript submitted for publication, 2013.

[9] A. Smith-Palmer, J. Stewart and L. Fyfe, Food Microbiology, 18, 463-470, 2001.

[10] O. Mejlholm and P. Dalgaard, Letters in Applied Microbiology, 34, 27-31, 2002.

[11] Food Standards Agency, "McCance and Widdowson's The Composition of Foods, 6th edition," Royal Society of Chemistry, 2002.

[12] G.J. Tsai and W.H. Su, Journal of Food Protein, 62, 239-243, 1999.

[13] V. Coma, A. Deschamps and A. Martial-Gros, Journal of Food Science, 68, 2788-2792, 2003.

[14] M.J. Mendoza-Yepes, L.E. Sanchez-Hidalgo, G. Maertens and F. Marin-Iniesta, Journal of Food Safety, 17, 47-55, 1997.

[15] I.M. Helander, E-L. Nurmiaho-Lassila, R. Ahvenainen, J. Rhoades and S. Roller, International Journal of Food Microbiology, 71, 235-244, 2001.

[16] R. Rowbury, Advanced Microbiology Physiology, 44, 215-257, 2001.

[17] C.D. Sifri, Clinical Infectious Diseases, 47, 1070-1076, 2008.

[18] J. Ivanovic, D. Misic, I. Zizovic and M. Ristic, Food Control, 25, 110-116, 2012.

[19] J. Del Campo, M-J. Amiot and C. Nguyen-The, Journal of Food Protection, 10, 1315-1449, 2000.

[20] M. Da Rocha, M.R. Loiko, E.C. Tondo and C Prentice, Food Hydrocolloids, 37, 213-220, 2014.

[21] J. Gutierrez, C. Barry-Ryan and P. Bourke, International Journal of Food Microbiology, 124, 91-97, 2008.

[22] Y. Pranoto, S.K. Rakshit and V.M. Salokhe, LWT, 38, 859-865, 2005.