International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/1471-0307.12203

ORIGINAL RESEARCH

Evaluation of the potential synergistic antimicrobial effects observed using combinations of nanoparticled and non-nanoparticled agents on cheese-derived micro-organisms

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The objective of this study was to determine whether a combination of agents could produce a synergistic antimicrobial effect, by either targeting a greater spectrum of micro-organisms or reducing the concentration of antimicrobial required to cause inhibition. Five agents (nanoparticled solubilisates – sorbic acid, benzoic acid and rosemary extract, and non-nanoparticled chitosans – of 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 Antibacterial activity, Particle size, Cheese, Preservatives, Shelf life, Packaging.

INTRODUCTION

The driving force for the use of antimicrobial packaging for dairy foods, such as 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 purchasing these products on the 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 distribution distances, and poor distribution and storage conditions. These factors can cause changes to the physical and chemical characteristics of the cheese, including colour, texture, taste, oxidation, odour development, sweating, shape deformities, decrease in nutritional value and an increase in spoilage micro-organisms; 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 (De Kruijf *et al.* 2002). Different preservatives have been employed in antimicrobial packaging over the years, with polysaccharides, essential oils derived from herbs and plants, organic acids and their salts, and bacteriocins most commonly associated with cheese preservation (Kasrazadeh and Genigeorgis 1995; Scannell *et al.* 2000; Gammariello *et al.* 2008; Cerqueira *et al.* 2010; Hauser and Wunderlich 2011). In addition, a number of studies have examined the effect of various combinations of antimicrobials on cheese to determine whether synergistic antimicrobial relationships between agents could be achieved (Sinigaglia *et al.* 2008; Fajardo *et al.* 2010; Hanušová *et al.* 2010). The aim of utilising active agent combinations is to expand the antimicrobial spectrum reached, minimise toxicity, reduce concentration levels and obtain an overall synergistic antimicrobial activity (Song *et al.* 2003). 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 (Editorial 2012).

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The antimicrobial agents investigated in this study were selected based on results determined from previous work reported by O' Callaghan and Kerry (2014). The criteria for this selection comprised a balance of promising antimicrobial activity and/or enhanced solubility. Sorbic acid and benzoic acid nanoparticled solubilisates were chosen due to their increased solubility over normal-sized sorbic and benzoic acid. A study by Cruz-Romero *et al.* (2013) demonstrated the considerable antimicrobial activity of nanoparticled sorbic and benzoic acid solubilisates relative to their non-nano-equivalents. Nanoparticled rosemary extract solubilisate showed a notable balance of enhanced solubility and an antimicrobial affinity towards cheese-derived cultures and Gram-positive bacteria (O' Callaghan and Kerry, 2014). To our knowledge, no other studies have explored the antimicrobial effect of nanoparticled rosemary or rosemary extract. Non-nanoparticled chitosan is well established as having many applications as an antimicrobial agent (Rabea *et al.* 2003; Aider 2010). Previous work by No *et al.* (2002) and Zheng and Zhu (2003) have examined the influence of molecular weight on the degree of antimicrobial inhibition, but the impact of this characteristic on chitosan when used in combination with other antimicrobials has not been investigated as thoroughly.

Therefore, this study was undertaken to investigate the antimicrobial activity of nanoparticled benzoic acid, sorbic acid and rosemary extract 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.

MATERIALS AND METHODS

Materials and microbiological media

Aquanova AG (Darmstadt, Germany) supplied the four nanoparticled solubilisates (~30 nm) – 4% sorbic acid, 12% benzoic acid, 6% carnosolic acid (rosemary extract) and 4% sorbic acid/benzoic acid (1:1). 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, UK. Minimum inhibition concentration (MIC) was measured using 96-well tissue culture microplates (Sarstedt, Inc., Newton, NC, USA).

Cultures and their growth conditions

The bacterial strains used for MIC testing including the Gram-negative species *Escherichia coli* and *Pseudomonas fluorescens* and the Gram-positive species *Staphylococcus aureus* and *Bacillus cereus* were derived from cheese samples and cultivated on TSA slants. Prior to MIC testing, the microbial cultures were regenerated twice from the TSA slants into a growth media, MHB, and incubated for 18 h, at 30 °C for Gram-positive species and at 37 °C for Gram-negative species.

General cheese cultures were derived from both Emmental and cottage cheese. Emmental culture preparation involved homogenising 10 g of Emmental with 90 mL of sterile MHB in a Colworth Stomacher 400 (Seward Ltd., Worthing, UK). The homogenisate (1 mL) was transferred into 10 mL MHB and incubated for 18 h at 37 °C. Cottage cheese culture was prepared by swabbing the cottage cheese surface and transferring the swab into MHB (10 mL). The sample was then incubated for 18 h at 37 °C.

Antimicrobial preparation

The antimicrobials selected included three nanoparticles – sorbic acid (SASB), benzoic acid (BASB) and rosemary extract (ROSE), and two non-nanoparticled chitosans – low molecular weight chitosan (LMWC) (50 000–190 000 Da) and medium molecular weight chitosan (MMWC) (190 000–310 000 Da). The nanoparticled solubilisates were standardised at a concentration level of 0.5% (w/v). Both solubilisates and sterile distilled water were preheated to 40 °C prior to mixing. Non-nanoparticled chitosan was prepared at 0.25% (w/v) in a 1% (v/v) acetic acid in a sterile distilled water solution at room temperature. Stock solutions were prepared for each of the levels of antimicrobials used. These five agents were input into the statistical program Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, VA USA), which computed 32 different experimental mixtures. According to the mixtures computed via Statgraphics®, solutions from 1 to 33 were prepared (Table 1). Each solution was subjected to magnetic stirring to ensure homogeneity. In addition, another nanoparticled solubilisate – a mixture of sorbic acid and benzoic acid (SABASB) – was examined and labelled as solution 6.

Antimicrobial susceptibility assessment

Minimum inhibition concentration testing was used to determine the antimicrobial action of the prepared mixtures against various cultures through the microdilution method. This microdilution was executed via 96-well tissue culture microplates. Within the microplates, 100 μL of sterile MHB was pipetted into rows A to F, 1–12, with an additional aliquot of 200 μL of MHB into the well H 12. Quantities of the antimicrobial mixture (150 μL) were pipetted into to row G, with row H 1–11 containing 200 μL of the test culture. Dilution was performed by transferring 50 μL of the antimicrobial from row G and mixing it into row F. Subsequently, 50 μL of the resultant mixture from row F was extracted and mixed into row E. This same action was repeated until row B, from which 50 μL was discarded, thus creating a threefold serial dilution. Row A contained no antimicrobial and was used as a positive growth control.

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Table 1 Antimicrobial mixtures, concentration breakdown and the total concentration applied (% w/v)

	Antimicrobial mixtures	*Concentration breakdown*	*Total (% w/v)*
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.

Following dilution, each well from row A to G was inoculated with test culture (15 μL) from row H. Column 12 represented a no growth control as it contained no culture. The microplates were incubated for 18 h, at 30°C for *P. fluorescens* and *B. cereus*, and at 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. Minimum inhibition concentration was defined as the lowest concentration of antimicrobial agent showing a complete growth inhibition of the microbial culture tested and expressed as a % (w/v).

Statistical analysis

The experiment was performed twice in triplicate. The total number of data points for each antimicrobial solution being six. The experimental data were analysed on SPSS Statistics 20 (IBM, Armonk, NY, USA). The mean and standard deviation for each antimicrobial mixture were calculated. ANOVA and Tukey's post hoc tests were used to determine the statistical significance within treatments, and paired *t*-tests were used to determine the statistical significance between treatment means. The level of significance was set at $P \leq 0.05$–0.01 (significant), $P \leq 0.01$–0.001 (highly significant) and $P \leq 0.001$ (extremely significant). Means with the letters 'ns' are nonsignificant, $P > 0.05$.

RESULTS AND DISCUSSION

The antimicrobial effects of the 33 combinations of antimicrobial agents assessed against microbial cultures using the

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microdilution assay are shown in Table 2 (unless otherwise stated). It can be seen from Table 2 that all treatments, with the exception of sorbic acid/benzoic acid solubilisate (SABASB), exerted overall antimicrobial effects. It was also determined that not all treatments demonstrated a complete antimicrobial effect on all cultures.

In this study, the five most active antimicrobials against the microbial culture derived from cottage cheese were 0.25% MMWC (0.046), 0.25% LMWC (0.053), 0.5% ROSE (0.066), 0.75% ROSE + LMWC (0.102) and 0.5% LMWC + MMWC (0.111), with no significant differences determined between them. The five best functioning antimicrobial treatments against the Emmental-derived culture were 0.25% LMWC (0.046), 0.5% LMWC + MMWC (0.074), 0.25% MMWC (0.083), 0.75% ROSE + LMWC (0.111) and 1% ROSE + LMWC + MMWC (0.148), again with no significant differences determined between them. Chitosan and rosemary were the most effective antimicrobial agents assessed, with both substances previously reported to reduce bacterial counts on cheese (Coma *et al.* 2002; Mohamed *et al.* 2009). 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 ($P < 0.05$) (Table 3).

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 MICs generated, it can be seen that the cottage cheese-derived culture also presented a lower MIC, which implies that cottage cheese-derived culture was more sensitive to the treatments applied. This is an interesting finding as it shows differences in inherent resistances to chemical treatments by cultures derived from different cheese products. Such differences have been reported previously when chemical treatments have been applied to cheese products, and the reasons proposed for variations in antimicrobial efficacy have been attributed to the physical structure and composition of the cheese product in question. 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 micro-organisms. Selim (2011) suggested that differences in cheese morphology and composition, in particular fat, protein and level of water content, could be responsible for a diminished level of antimicrobial activity. A high fat content can impair the capacity of an antimicrobial to reduce a microbial population (Ribeiro *et al.* 2013). Specifically for cheese, Smith-Palmer *et al.* (2001) 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 (Mejlholm and Dalgaard 2002; Patel *et al.* 2005). Emmental has a higher total fat content (29.7 g) than cottage cheese (4.3 g) (Food Standards Agency 2002). The increased lipid levels may explain the lower inhibition observed for Emmental. Similarly, Emmental has a much lower water content than cottage cheese. Low water content may impair the movement of the antimicrobial agents to the active site of the bacterial cell (Smith-Palmer *et al.* 2001). These factors may explain why chemical treatments and their activities may be diminished when applied to cheese products, and the results presented here show clearly that inherent differences within cheese-derived cultures will present their own challenges and resistances when chemical treatments are applied.

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 MICs 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. Tsai and Su (1999) and Coma *et al.* (2003) have both demonstrated the inhibitory effect of chitosan on *E. coli* and *Pseudomonas* species, respectively. BASB + LMWC + MMWC (0.222) and SASB + LMWC (0.222) treatments make up the five most effective treatments against *E. coli*, suggesting that organic acids provide a marginal antimicrobial effect on *E. coli*. Within the five most effective treatments against *E. coli*, LMWC and MMWC were found to be significantly different from BASB + LMWC + MMWC ($P \leq 0.001$) and SASB + LMWC ($P \leq 0.001$). The remaining top-performing antimicrobial agents against *P. fluorescens* included ROSE + LMWC + MMWC (0.111) and SASB + LMWC + MMWC (0.222), with SASB + LMWC + MMWC being significantly different from LMWC, MMWC and LMWC + MMWC ($P < 0.001$), and ROSE + LMWC + MMWC ($P < 0.05$). A total of 10 and 11 treatments demonstrated antimicrobial activity at a concentration of 0.25% or below against *E. coli* and *P. fluorescens*, respectively. Nanoparticled rosemary extract demonstrates an acuteness for *P. fluorescens*, which it does not appear to possess for *E. coli*. An improvement in Gram-negative inhibition may be achievable, if rosemary was to be added at a higher concentration. Mendoza-Yepes *et al.* (1997) 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

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Table 2 Mean minimum inhibition concentration (MIC - %, w/v) and standard deviation of the antimicrobial soultions against the various cultures. Values correspond to mean data for six test samples each, ± corresponds to the standard deviation

	Antimicrobial Solution	*Cottage Cheese*	*Emmental*	*Escherichia coli*	*Pseudomonas fluorescens*	*Staphylococcus aureus*	*Bacillus cereus*	*Total*
1	LMWC 0.25%	**0.05 ± 0.04**	**0.05 ± 0.03**	**0.06 ± 0.03**	**0.03 ± 0.01**	**0.08 ± 0.00**	**0.04 ± 0.02**	**0.05 ± 0.03**
2	MMWC 0.25%	**0.05 ± 0.03**	**0.08 ± 0.00**	**0.07 ± 0.03**	**0.05 ± 0.03**	**0.08 ± 0.00**	**0.05 ± 0.03**	**0.06 ± 0.03**
3	SASB 0.5%	0.45 ± 0.14	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	0.49 ± 0.06
4	BASB 0.5%	0.45 ± 0.14	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	0.45 ± 0.14	0.48 ± 0.08
5	ROSE 0.5%	**0.07 ± 0.05**	0.37 ± 0.20	*0.50 ± 0.00*	*0.50 ± 0.00*	0.32 ± 0.21	**0.04 ± 0.02**	0.30 ± 0.22
6	SABASB 0.5%	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*	*0.50 ± 0.00*
7	LMWC/MMWC 0.5%	**0.11 ± 0.06**	**0.07 ± 0.05**	**0.13 ± 0.06**	**0.06 ± 0.00**	**0.17 ± 0.00**	**0.09 ± 0.06**	**0.11 ± 0.06**
8	SASB/LMWC 0.75%	0.21 ± 0.10	0.31 ± 0.23	**0.22 ± 0.07**	0.25 ± 0.00	0.50 ± 0.27	0.25 ± 0.00	0.29 ± 0.17
9	SASB/MMWC 0.75%	0.22 ± 0.07	0.25 ± 0.00	0.25 ± 0.00	0.22 ± 0.07	0.50 ± 0.27	0.25 ± 0.00	0.28 ± 0.15
10	BASB/LMWC 0.75%	0.22 ± 0.07	0.22 ± 0.07	0.25 ± 0.00	0.22 ± 0.07	0.50 ± 0.27	0.17 ± 0.09	0.26 ± 0.16
11	BASB/MMWC 0.75%	0.19 ± 0.10	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	**0.22 ± 0.07**	0.25 ± 0.00	0.24 ± 0.05
12	ROSE/LMWC 0.75%	**0.10 ± 0.08**	**0.11 ± 0.07**	0.25 ± 0.00	0.22 ± 0.07	0.47 ± 0.31	0.14 ± 0.09	**0.22 ± 0.18**
13	ROSE/MMWC 0.75%	0.16 ± 0.10	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	0.47 ± 0.31	**0.07 ± 0.03**	0.24 ± 0.18
14	SASB/BASB 1%	0.89 ± 0.27	*1.00 ± 0.00*	*1.00 ± 0.00*	*1.00 ± 0.00*	*1.00 ± 0.00*	*1.00 ± 0.00*	0.98 ± 0.11
15	SASB/ROSE 1%	0.62 ± 0.43	*1.00 ± 0.00*	*1.00 ± 0.00*	*1.00 ± 0.00*	0.67 ± 0.37	0.21 ± 0.14	0.75 ± 0.37
16	BASB/ROSE 1%	0.51 ± 0.40	*1.00 ± 0.00*	*1.00 ± 0.00*	*1.00 ± 0.00*	0.52 ± 0.38	0.19 ± 0.12	0.70 ± 0.39
17	SASB/LMWC/MMWC 1%	0.26 ± 0.12	0.30 ± 0.09	0.26 ± 0.12	**0.22 ± 0.12**	0.33 ± 0.00	0.33 ± 0.00	0.28 ± 0.09
18	BASB/LMWC/MMWC 1%	0.19 ± 0.12	0.26 ± 0.12	**0.22 ± 0.12**	0.26 ± 0.12	0.33 ± 0.00	0.19 ± 0.12	0.24 ± 0.11
19	ROSE/LMWC/MMWC 1%	0.19 ± 0.12	**0.15 ± 0.09**	0.28 ± 0.12	**0.11 ± 0.00**	**0.30 ± 0.09**	0.19 ± 0.12	**0.20 ± 0.11**
20	SASB/BASB/ROSE/LMWC/MMWC 1%	0.26 ± 0.12	0.33 ± 0.00	0.33 ± 0.00	0.33 ± 0.00	0.67 ± 0.37	0.33 ± 0.00	0.38 ± 0.20
21	SASB/BASB/LMWC 1.25%	0.37 ± 0.11	0.42 ± 0.00	0.42 ± 0.00	0.42 ± 0.00	*1.25 ± 0.00*	0.42 ± 0.00	0.55 ± 0.32
22	SASB/BASB/MMWC 1.25%	0.51 ± 0.38	0.56 ± 0.34	0.42 ± 0.00	0.42 ± 0.00	1.11 ± 0.34	0.42 ± 0.00	0.57 ± 0.34
23	SASB/ROSE/LMWC 1.25%	0.40 ± 0.44	0.42 ± 0.00	0.42 ± 0.00	0.42 ± 0.00	0.83 ± 0.46	0.22 ± 0.16	0.45 ± 0.31
24	SASB/ROSE/MMWC 1.25%	0.32 ± 0.14	0.70 ± 0.43	0.42 ± 0.00	0.42 ± 0.00	0.83 ± 0.46	0.28 ± 0.15	0.49 ± 0.32
25	BASB/ROSE/LMWC 1.25%	0.36 ± 0.15	0.46 ± 0.41	0.42 ± 0.00	0.42 ± 0.00	0.65 ± 0.48	0.23 ± 0.14	0.42 ± 0.28
26	BASB/ROSE/MMWC 1.25%	0.32 ± 0.14	0.42 ± 0.00	0.42 ± 0.00	0.42 ± 0.00	0.83 ± 0.46	0.19 ± 0.11	0.43 ± 0.27
27	SASB/BASB/ROSE 1.5%	0.76 ± 0.60	*1.50 ± 0.00*	*1.50 ± 0.00*	*1.50 ± 0.00*	1.33 ± 0.41	0.45 ± 0.14	1.17 ± 0.51
28	SASB/BASB/LMWC/MMWC 1.5%	0.45 ± 0.14	0.50 ± 0.00	0.45 ± 0.14	0.50 ± 0.00	0.67 ± 0.41	0.50 ± 0.00	0.51 ± 0.19
29	SASB/ROSE/LMWC/MMWC 1.5%	0.37 ± 0.20	0.33 ± 0.18	0.39 ± 0.17	0.45 ± 0.14	0.83 ± 0.52	0.17 ± 0.00	0.42 ± 0.31
30	BASB/ROSE/LMWC/MMWC 1.5%	0.31 ± 0.21	0.56 ± 0.49	0.50 ± 0.00	0.45 ± 0.14	1.00 ± 0.55	0.33 ± 0.18	0.52 ± 0.38
31	SASB/BASB/ROSE/LMWC 1.75%	1.04 ± 0.79	0.58 ± 0.00	0.58 ± 0.00	0.58 ± 0.00	1.56 ± 0.48	0.58 ± 0.00	0.82 ± 0.51
32	SASB/BASB/ROSE/MMWC 1.75%	0.65 ± 0.57	0.58 ± 0.00	0.58 ± 0.00	0.58 ± 0.00	1.36 ± 0.60	0.52 ± 0.16	0.71 ± 0.44
33	SASB/BASB/ROSE/LMWC/MMWC 2%	0.89 ± 0.88	0.67 ± 0.00	0.67 ± 0.00	0.67 ± 0.00	1.11 ± 0.69	0.67 ± 0.00	0.78 ± 0.46
	Total MIC for each culture	0.38 ± 0.38	0.46 ± 0.34	0.45 ± 0.30	0.45 ± 0.31	0.67 ± 0.49	0.39 ± 0.22	

Data in the columns highlighted in bold are the top five treatments for that culture. Italicized figures indicate if a treatment failed to work.

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Table 3 Paired samples test – difference between pairs

	Pairs	*Significance*
Cottage Cheese	Emmental	*
Escherichia coli	*Pseudomonas fluorescens*	ns
Staphylococcus aureus	*Bacillus cereus*	***

The level of significance is denoted by these asterisks; *$P < 0.05$–0.01 (significant), **$P < 0.01$–0.001 (highly significant) and ***$P < 0.001$ (extremely significant). Means with the letters 'ns' are nonsignificant, $P > 0.05$.

associated with chitosan is attributed to chitosan's ability bind to the outer membrane of the bacterial cell and subsequently disrupt barrier function (Helander *et al.* 2001). Even though chitosan provided the greatest antimicrobial effect for both micro-organisms, *P. fluorescens* had noticeably lower MIC values. This could be due to the *E. coli* possessing an early warning defence mechanism against antimicrobial attack (Rowbury 2001). In any case, when MIC data for *E. coli* and *P. fluorescens* were compared and no significant differences were found (Table 3).

Unlike the treatment similarities observed for Gram-negative bacteria, there was a stark contrast in results between *S. aureus* and *B. cereus*. *Staphylococcus 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 for both *B. cereus* – 0.5% ROSE (0.037), 0.25% LMWC (0.037), 0.25% MMWC (0.046), 0.75% ROSE + MMWC (0.065) and 0.5% LMWC + MMWC (0.093) and *S. aureus* – 0.25% LMWC (0.083), 0.25% MMWC (0.083), 0.5% LMWC + MMWC (0.167), 0.75% BASB + MMWC (0.222) and 1% ROSE + LMWC + MMWC (0.296). However, as can be readily observed, the treatment levels required to deliver antimicrobial effects were very different ($P < 0.001$, Table 3). 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 nonactive treatments. Conversely, 28 treatments had an antibacterial effect on *S. aureus*; however, only four 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. Staphylococci can illicit efficient mechanisms to neutralise antimicrobials (Lowy 2003). For example, *S. aureus* has been known to use intercellular communication to induce virulence factors (Sifri 2008). However, in this study, *S. aureus* tolerance to the antimicrobials was most likely due to the natural variance within the microbe assessed rather than an actual stable resistance.

Bacillus 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 the nanoparticled rosemary extract performed just as strongly as chitosan in treatments. Ivanovic *et al.* (2012) also determined that *B. cereus* and other *Bacillus* species were very susceptible to rosemary compared to other bacteria tested. Rosemary extract also impacted on *S. aureus*, but at a higher MIC level (0.315). Del Campo *et al.* (2000) 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%).

Overall, the five best performing antimicrobial treatments were determined to be 0.25% LMWC, 0.25% MMWC, 0.5% LMWC + MMWC, 0.75% ROSE + LMWC and 1% ROSE + LMWC + MMWC. They had MICs of 0.060, 0.076, 0.120, 0.207 and 0.238, respectively. This correlates with previous work reported by O' Callaghan and Kerry (2014), which showed that LMWC, MMWC and nanoparticled rosemary extract all showed the greatest antimicrobial activities of the 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, which is in contrast to the findings reported by Shin *et al.* (2001) who found that an increase in bacterial reduction as the molecular weight of chitosan increased. Overall, LMWC and MMWC functioned more effectively as antimicrobial substances when used on their own than when used in combination treatments. The nanoparticled rosemary extract itself exerted a moderate antimicrobial activity, working particularly well for both cheese-derived cultures and 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). Da Rocha *et al.* (2014) 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 agents assessed, a commensal influence was more evident. No combination treatment attained the same antimicrobial effectiveness as that produced by a single antimicrobial treatment. Gutierrez *et al.* (2008) also reported that various chemical combinations

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assessed in their study showed no synergism, but resulted in many additive, and some indifferent patterns. Park *et al.* (2004) suggested that chitosan has great compatibility with other antimicrobials due to its chemical structure. Studies have previously demonstrated that chitosan, when used in combination with other substances, has the capacity to enhance greater antimicrobial activity than either agent applied individually (Duan *et al.* 2007; Del Rosario Moreira *et al.* 2011). 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 (Pranoto *et al.* 2005). The reduction in antimicrobial activity observed when chitosan and an organic acid are used in combination may be due to the decreased ability of chitosan to interact with the bacterial membrane (Vásconez *et al.* 2009).

Gutierrez *et al.* (2008) 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, this 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. The use of a dispersing agent could enhance the contact of solubilisates with the microbial cells, especially in foods with a high fat content, such as Emmental cheese (Smith-Palmer *et al.* 2001). Additionally, the incorporation of natural chelators or enzymes could be used to disrupt the membrane of Gram-negative bacteria.

CONCLUSION

Chitosan, of low and medium molecular weight, and nanoparticled rosemary extract provided the most interesting and effective inhibition across all cultures 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 nanoparticled rosemary extract treatments into packaging and applying the treated packaging to cheese products.

ACKNOWLEDGEMENT

This work was financially supported by the Irish Research Council via the EMBARK Postgraduate Research Scholarship Scheme.

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