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Efficacy of nisin A and nisin V semi-purified preparations alone and in combination with plant essential oils to control *Listeria monocytogenes*

Des Field[1*], Karen Daly[1], Paula M. O'Connor[2], Paul D. Cotter[2,3], Colin Hill[1,3] and R. Paul Ross[2,3]**

[1]Department of Microbiology, University College Cork, Cork, Ireland.

[2]Teagasc, Moorepark Food Research Centre, Fermoy, Co. Cork, Ireland.

[3]Alimentary Pharmabiotic Centre, University College Cork, Ireland.

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For correspondence.

*des.field@ucc.ie: Tel: +353-(0)21-4903146; Fax. +353-(0)21-4903101

** c.hill@ucc.ie; Tel. +353-(0)21-4901373; Fax. +353-(0)21-4903101

Summary

The foodborne pathogenic bacterium Listeria is known for relatively low morbidity and high mortality rates reaching up to 25-30%. *Listeria* is a hardy organism and its control in foods represents a significant challenge. Many naturally occurring compounds, including the bacteriocin nisin and a number of plant essential oils, have been widely studied and are reported to be effective as antimicrobial agents against spoilage and pathogenic microorganisms. The aim of this study was to investigate the ability of semi-purified preparations (spp) containing either nisin A or an enhanced bioengineered derivative nisin V, alone and in combination with low concentrations of the essential oils thymol, carvacrol and trans-cinnamaldehyde, to control *L. monocytogenes* in both laboratory media and model food systems. Combinations of nisin V-containing spp (25 µg/ml) with thymol (0.02%), carvacrol (0.02%) or cinnamaldehyde (0.02%) produced a significantly longer lag phase than any of the essential oil/nisin A combinations. In addition, the log reduction in cell counts achieved by the nisin V + carvacrol or nisin V + cinnamaldehyde combinations was twice that of the equivalent nisin A + essential oil treatment. Significantly, this enhanced activity was validated in model food systems against *L. monocytogenes* strains of food origin. We conclude that the fermentate form of nisin V in combination with carvacrol and cinnamaldehyde offers significant advantages as a novel, natural and effective means to enhance food safety by inhibiting foodborne pathogens such as *L. monocytogenes*.

Introduction

The growing consumer demand for food products that are minimally processed and free of chemical preservatives presents a difficult challenge for food processors. Consequently, there has been a focus on the application of naturally produced antimicrobial compounds as a more acceptable means to control the growth of undesirable microorganisms in food (1, 2). Bacteriocins (ribosomally-produced, small, heat-stable peptides that are active against other bacteria) derived from generally regarded as safe organisms provide one potential solution. However, only two bacteriocins have been commercialized to any extent. These are nisin, produced by *Lactococcus lactis* and pediocin PA-1, produced by *Pediococcus acidilactici* (3, 4). Of these, nisin is used in a wide variety of dairy and non-dairy products including cream and cheese products, soups, liquid egg, mayonnaises, salad dressings, tomato products and beer (5). Nisin A exhibits antibacterial activity against a wide range of Gram positive bacteria, including food-borne pathogens such as staphylococci, bacilli, clostridia and *Listeria* (6, 7). Indeed, the success of nisin A from discovery (8) through to regulatory approval and finally to commercial application has spurred researchers to exploit its gene-encoded nature and to attempt to 'bioengineer' variants with altered biological, chemical and physical properties. Over the last decade several studies have described the discovery of new nisin derivatives with enhanced activity against a range of food-related pathogenic micro-organisms (9, 10) Of these, nisin M21V, subsequently designated nisin V, was noteworthy by virtue of its enhanced antimicrobial activity against a wide range of targets, including medically significant pathogens and food-borne pathogens such as *B. cereus* and *L. monocytogenes* (10, 11). Significantly, this enhanced activity against *L. monocytogenes* was also apparent in a food setting (11). The increasing trend towards minimally processed and ready to eat (RTE) refrigerated foods means that more robust strategies are required to control the growth and survival of *Listeria monocytogenes*. Indeed, recent strategies for controlling spoilage and pathogenic microorganisms lean towards hurdle technology, whereby different preservation methods are combined to inhibit microbial growth and

improve food safety. To this end, numerous studies have been carried out highlighting the potential of nisin in conjunction with other hurdle technologies including organic acids, salt, EDTA, heat, high hydrostatic pressure, modified atmosphere packaging and pulsed electric fields (for reviews see 12, 13). Similarly, aromatic plant oils have been widely studied due to their antimicrobial activities and have found various applications including the preservation of raw and processed foods (14), pharmaceuticals (15) and as natural therapies. Notably, several studies have demonstrated the synergistic activities of nisin and essential oil combinations including thymol (16), carvacrol (17, 18), and cinnamaldehyde (19-21), amongst others.

In this study we created a stable nisin V producer of an industrial nisin production strain to enable the generation of fermentates of nisin V for comparative studies with its nisin A equivalent. We examine their solo activity, as well as in combination with the essential oils thymol, carvacrol and trans-cinnamaldehyde, in terms of their ability to control *L. monocytogenes* strains of food-borne origin in laboratory media and in model food systems. Notably, we demonstrate that the previously observed enhanced specific activity of purified nisin V over nisin A against *L. monocytogenes* is retained by the fermentate versions in both microbiological media and in food matrices. In addition, the essential oil and nisin V combinations display significantly increased efficacy compared to either compound alone or to the Nisin A + essential oil combinations.

Materials and Methods

Bacterial Strains and Growth Conditions

L. lactis strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30˚C. *E. coli* was grown in Luria-Bertani broth with vigorous shaking or agar at 37˚C. *Listeria* strains were grown in Brain Heart Infusion (BHI) or BHI agar at 37˚C. Antibiotics were used where indicated at the following concentrations: Chloramphenicol at 10 and 20 µg ml^{-1}, respectively for *L. lactis* and *E. coli.* Erythromycin was used at 150 µg/ml and 5 µg/ml for *E. coli* and *L. lactis*, respectively. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 40 µg/ml. Stock concentrations of Thymol (Sigma) at 50 mg/ml was made up in 50% ethanol and stored at -20ºC. Caravcrol (Sigma) was diluted from stock (0.976 g/ml) in 100% ethanol to the desired concentration. Trans-cinnamaldehyde (Sigma) was diluted from stock (1.05 g/ml) in 100% ethanol to the desired concentration. In all experiments the concentration of ethanol did not exceed 2% (vol/vol).

Conversion of an industrial Nisin producing strain to a Nisin V producer

Mutagenesis of the *nis*A gene was carried out as described previously (11). Briefly, to introduce the desired mutations within the hinge-region of the *nis*A gene, the plasmid pDF06 (a 774 bp product encompassing approx. 300 bp either side of the *nis*A gene cloned into the vector pORI280) was amplified with the QuickChange system (Stratagene) using the primers nisinVFor 5'GAGCTCTGATGGGTTGTAACGTTAAAACAGCAACTTGTCATT3' and nisinVRev 5'CAATGACAAGTTGCTGTTTTAACGTTACAACCCATCAGAGCT3' (codon changes underlined). The resulting PCR products were transformed into *E. coli* EC101 (RepA+). To detect altered pORI280-*nis*A transformants, candidates were screened by PCR using a specific 'check' primer (nisinV check 5'GCTCTGATGGGTTGTAACG) designed to amplify mutated plasmid template only and a reverse primer oDF106 5'TAGAATTCAACAGACCAGCATTA3'. Plasmids from positive candidates were sequenced (Sourcebioscience UK) using the primers pORI280FOR 5'CTCGTTCATTATAACCCTC3' and pORI280REV 5'CGCTTCCTTTCCCCCCAT3' to verify the deliberate mutation and to

confirm no other changes had been introduced. pDF08 (pORI280-*nis*M21V) was then introduced into *L. lactis* DGCC 10042 pVE6007 by electroporation (22) and transformants were selected by growth on GM17-Ery-X-gal plates at 30°C. Integration of pDF08 by single crossover recombination and curing of the temperature sensitive plasmid pVe6007 was achieved by growth at 37°C in GM17-Ery broth and plating on GM17-Ery-X-gal agar at the same temperature. Selected colonies were checked for their inability to grow on GM17-Cm agar at 30°C and then subcultured in GM17 at 37°C. Each subculture was spread on GM17-X-gal plates to identify candidates where pORI280 had excised and was lost ($LacZ^-$) due to a second crossover event. Mutant and wild-type revertants were distinguished by deferred antagonism assays. Bac^+ candidates were analysed by Mass Spectrometry to verify production of the mutant Nisin peptide.

Mass Spectrometry

For Colony Mass Spectrometry (CMS) bacteria were collected with sterile plastic loops and mixed with 50 µl of 70% isopropanol adjusted to pH 2 with HCl. The suspension was vortexed, the cells spun down in a benchtop centrifuge at 14,000 r.p.m. for 2 min, and the supernatant was removed for analysis. Mass Spectrometry in all cases was performed with an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10 mg ml^{-1} in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target and left for 1-2 min before being removed. The residual solution was then air dried and the sample solution (resuspended lyophilised powder or CMS supernatant) was positioned onto the precoated sample spot. Matrix solution (0.5 µl) was added to the sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode.

Generation of Nisin A- and Nisin V-containing fermentate.

Laboratory scale fermentations were carried out by DuPont (Beaminster, UK) using the nisin producing strain *L. lactis* DGCC10042 and the newly created nisin V producing strain *L. lactis* DGCC 10042::*nisV*. Analysis of the resultant nisin A and nisin V containing spp revealed equivalent quantities of nisin peptides by HPLC (81.9 mg/gram and 82.6 mg/gram respectively). This is in agreement with previous analysis relating to production levels of bioengineered nisin derivatives (10).

Minimum Inhibitory Concentration assays

Minimum inhibitory concentration (MIC) determinations were carried out in triplicate in 96 well microtitre plates. 96 well microtitre plates were pre-treated with bovine serum albumin (BSA) prior to addition of the nisin fermentates. Briefly, to each well of the microtitre plate 200 µL of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (PBS/BSA) was added and incubated at 37°C for 30 min. The wells were washed with 200 µL PBS and allowed to dry. Target strains were grown overnight in the appropriate conditions and medium, subcultured into fresh broth and allowed to grow to an OD_{600} of ~0.5, diluted to a final concentration of 10^5 cfu ml^{-1} in a volume of 0.2 ml. The nisin spp were resuspended in BHI broth to a stock concentration of 10 mg/ml. Wild type nisin and nisin V peptides were adjusted to a 1.25 mg/ml starting concentration and 2-fold serial dilutions of each peptide were made in 96 well plates for a total of 10 dilutions. The target strain was then added and after incubation for 16 h at 37°C the MIC was read as the lowest peptide concentration causing inhibition of visible growth.
MICs were carried as above for the three essential oils against selected Listeria strains with some minor modifications as the 96 well plates did not require treatment with BSA. Target strains were grown overnight, subcultured and added at a final concentration of 10^5 cfu ml^{-1} in a volume of 0.2 ml. The various oils were diluted accordingly and added to 0.2 mls at a starting concentration of 2.5 mg/ml, 2-fold serial dilutions were subsequently carried out and

the target strain was then added. Following incubation for 16 h at 37°C the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

Growth/Kill experiments

For growth experiments, overnight cultures were transferred (10^7 cfu ml^{-1} in a volume of 1.0 ml.) into BHI supplemented with 25 μg/ml of nisin fermentates in combination with one of the essential oils being tested ie. thymol, carvacrol or cinnamaldehyde with concentrations ranging from 152 μg/ml - 304 μg/ml. 0.2 mls was subsequently transferred to 96 well microtitre plates (Sarstedt). Cell growth was measured spectrophotometrically over 24-h periods by using a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, Calif.). For kill assays, fresh overnight cultures were transferred (10^7 cfu ml^{-1} in a volume of 1.0 ml.) into BHI broth containing 50 μg/ml nisin A fermentate or nisin V fermentate alone and in combination with of thymol, carvacrol and cinnamaldehyde respectively. The samples were incubated for 180 mins at 37°C. Cell growth was measured by performing viable cell counts by diluting cultures in one-quarter-strength Ringer solution and enumeration on BHI agar plates.

Model Food Trials

A commercially produced chocolate milk drink and a fresh chilled commercially produced chicken noodle soup product were streaked on *Listeria* Selective Agar (LSA) (Oxoid) to check for the presence of *Listeria*. Aliquots of chocolate milk and chicken noodle soup were aseptically transferred to 1.5 ml Eppendorf tubes and inoculated with approx 1 x 10^7 cfu ml^{-1} *Listeria monocytogenes* EGDe or *Listeria monocytogenes* F2365 repectively. Chocolate milk samples were treated with 50 μg ml^{-1} of fermentates of nisin A and nisin V, alone and in combination with trans-cinnamaldehyde at a concentration of 210 μg/ml to achieve final volumes of 1 ml. Samples with trans-cinnamaldehyde alone at the same concentration served as controls. Chicken soup samples were treated with 50 μgml^{-1} of fermentates of nisin A and nisin V, alone and in combination with carvacrol at a concentration of 195.2

μg/ml to achieve final volumes of 1 ml. In both experiments, samples were incubated at 37ºC for 3 hours. The kill effect of nisin fermentates alone and in combination with either trans-cinnamaldehyde and carvacrol against *Listeria* was examined by serial dilution and plate count technique using *Listeria* Selective Agar (Oxoid Ltd). All tests were conducted in triplicate.

Statistical analysis

Statistical analysis was carried out using the software package Sigmastat 3.5. Groups were compared by Kruskal Wallis One Way Analysis of Variance, with post hoc comparison by the Student Neuman Keuls method (except where group sizes were unequal; Dunn's comparison method was used in this case). $P < 0.05$ was considered to be significant in all cases.

Results

Conversion of an industrial nisin A production strain to a nisin V producer.

To initiate studies that would more accurately reflect the situation in which nisin is used commercially (as a nisinpreparation such as Nisaplin®), it was necessary to generate a nisin V-containing spp for experimental analysis and comparison. To that end we created a genetically stable nisin V producer of the commercial Nisaplin® production strain *L. lactis* DGCC 10042 through double crossover homologous recombination. This 'self cloning' strategy means that following excision and gene replacement, no heterologous DNA is present in the final constructs. The *nisV* gene (generated by PCR based mutagenesis) was inserted at the appropriate location in the chromosome of the industry stain, *L. lactis* DGCC 10042, *via* double crossover recombination to generate *L. lactis* DGCC 10042::*nisV*. Results of deferred antagonism assays with *L. monocytogenes* LO28 indicated that gene replacement had successfully occurred since the bioactivity profile of the newly constructed strain was enhanced relative to that of *L. lactis* DGCC 10042 (Fig.1a). Mass spectrometry analysis confirmed the production of peptides with a mass corresponding to nisin V (3322

Da) (Fig. 1b). We also confirmed the absence of the pORI280 shuttle vector employed to facilitate the recombination process (data not shown).

MIC-based assessment of nisin A and V fermentates and essential oils against *L. monocytogenes* used in this study.

Nisin A exhibits antimicrobial activity against *L. monocytogenes* but it is not very potent (23, 24). Indeed, in our previous examinations of nisin A and nisin derivatives against Gram positive pathogens, we observed that *Listeria* strains exhibited the greatest natural resistance to nisin A (11). Thus a decision was made to investigate the sensitivity of a wider range of *Listeria* isolates, and in particular those associated with food-borne outbreaks (Table 1), to the nisin-containing fermentates for direct comparison. Additionally, the sensitivity of the strains to the essential oils thymol, carvacrol and cinnamaldehyde was assessed to establish suitable concentrations for combinatorial studies. Previous studies have demonstrated the enhanced efficacy of nisin V to nisin A using equimolar concentrations of purified peptides (10, 11). In this study, a semi-purified preparation containing nisin V obtained through fermentation was produced for the first time at laboratory-scale for direct comparison with the nisin A containing equivalent. These investigations revealed that the nisin V spp is indeed two-fold more active (MIC of 39 µg/ml) than its nisin A equivalent (78 µg/ml) against *L. monocytogenes* EGDe, *L. monocytogenes* LO28 and *L. innocua* FH1848 (Table 2). Similarly, nisin V was also two-fold more active (MIC of 62.5 µg/ml) against *L. monocytogenes* F2365 and *L. monocytogenes* 33013 than nisin A (125 µg/ml).

The minimum inhibitory concentration for each of the essential oils under investigation was also determined for all *L. monocytogenes* isolates (Table.2). Thymol was found to completely inhibit growth at concentrations of 156 μg/ml (EGDe, 33013 and 33413) and 312 μg/ml (F2365 and LO28). The MIC of carvacrol was determined to be 156 μg/ml for *L. monocytogenes* 33413 and 625 μg/ml for both *L. monocytogenes* F2365 and *L. monocytogenes* 33013. Both *L. monocytogenes* EGDe and LO28 exhibited MIC values of 312 μg/ml. In experiments conducted with trans-cinnamaldehyde, MICs ranged from 156 μg/ml for *L. monocytogenes* LO28 to 312 μg/ml for all remaining *L. monocytogenes* isolates (EGDe, F2365, 33013 and 33413). These values are in close agreement with previously established values (25).

Growth and kill curve-based comparisons

Having demonstrated the increased specific activity of the nisin V spp against all the *Listeria* strains utilised in this study through MIC determinations, we sought to examine (i) the impact on bacterial growth of the spp alone and in combination with the selected essential oils through growth curve analysis, and (ii) the ability of the combinations to kill the bacteria using time-kill curve analysis. Both *L. monocytogenes* EGDe and *L. monocytogenes* LO28 were selected as test strains for growth curve experiments. For growth curves, a sub-lethal concentration of 25 μg/ml was used (as previously employed by Field et al., 2010 (11)) for each of the nisin spp in combination with a range of concentrations (100-330 μg/ml) for each essential oil. In the presence of identical concentrations of spp (25 μg/ml) *L. monocytogenes* EGDe and *L. monocytogenes* LO28 had a longer lag time for nisin V compared to nisin A in all experiments. When combined with 100 μg/ml thymol (Fig. 2), carvacrol (304 μg/ml and 152 μg/ml for EGDe (Fig. 3A) LO28 (Fig. 3B)) or trans-cinnamaldehyde (327.6 μg/ml and 327.6 μg/ml for EGDe (Fig. 4A) and LO28 (Fig. 4B)), a significantly longer delay in growth was observed for the nisin A + essential oil combination than with either compound used alone. The combination of nisin A with either carvacrol or cinnamaldehyde produces similar

growth inhibition to nisin V alone (Fig. 4 and Fig. 5). Notably, the most profound delay in growth was observed for the combination of nisin V with either thymol (Fig. 2), carvacrol (Fig. 3) or trans-cinnamaldehyde (Fig. 4) against both LO28 and EGDe.

In order to compare the bactericidal activity of nisin A and nisin V fermentates over a defined period of time, *L. monocytogenes* EGDe (1 x 10^7 cfu/ml) was exposed to 50 μg/ml of each fermentate in combination with 195.2 μg/ml carvacrol (Fig. 5A), 100 μg/ml thymol (Fig. 5B) and 210 μg/ml trans-cinnamaldehyde (Fig. 5C) in BHI broth for a period of 3 hours at 37ºC. In all cases and at the concentrations used, cell numbers remained static or slightly increased when the nisin A spp, thymol, carvacrol and trans-cinnamaldehyde were used singly (Fig. 5). In contrast, the nisin V fermentate resulted in a 1 log reduction of EGDe over the 3 hour period. With respect to combinations of antimicrobials, the antimicrobial potency of nisin A was significantly enhanced when used in combination with all three essential oils bringing about a 1 log (+thymol, 1.8 x 10^6 cfu/ml) (Fig. 5B), 2 log (+trans-cinnamaldehyde, 2.57 x10^5 cfu/ml) (Fig. 5C) or 3 log (+carvacrol, 8.53 x 10^3 cfu/ml) (Fig. 5A) reduction in bacterial cell counts. However, the combination of nisin V with all three essential oils proved most effective in that a 2 log (+thymol, 1.28 x 10^5 cfu/ml) (Fig. 5B), 4 log (+cinnamaldehyde, 3.15 x 10^3 cfu/ml) (Fig. 5C) and greater than 5 log (+carvacrol, not detected) (Fig. 5A) reduction in *L. monocytogenes* EGDe cell counts was achieved.

Investigation of the anti-*Listeria* activity of nisin spp and essential oil combinations in a food matrix

Having established the enhanced potency of nisin V alone and in combination with essential oils against a wide range of *L. monocytogenes* isolates using a variety of laboratory-based assays, we wished to ascertain whether this enhanced effectiveness could be translated to a

food setting. We conducted two food trials, one involving a commercially produced chilled chocolate milk beverage and the other a commercially produced fresh chilled chicken noodle soup. Although *L. monocytogenes* is not frequently associated with soup, the organism has been isolated from rice soup with cream, lettuce and meat products (26). The chocolate milk was spiked with *L. monocytogenes* EGDe to evaluate the efficacy of the nisin spp alone and in combination with trans-cinnamaldehyde, while the soup was spiked with *L. monocytogenes* F2365 to evaluate nisin spp and carvacrol combinations. For both food-based assays, chocolate milk or soup was aseptically transferred to containers to which was directly added the powdered nisin spp A or V alone (50 μg/ml) or in combination with trans-cinnamaldehyde (210 μg/ml) or carvacrol (195.2 μg/ml). *L. monocytogenes* EGDe or *L. monocytogenes* F2365 was added at a concentration of 1 x 10^7 cfu/ml. Following incubation at 37˚C for 3 hours, bacterial growth was monitored by serial dilution and plate counts on Listeria Selective Agar (LSA). In chocolate milk with trans-cinnamaldehyde alone, *L. monocytogenes* EGDe numbers remained static relative to initial inoculum levels (1.33 x 10^7 cfu/ml) (Fig. 6A), while a very slight increase in cell numbers was observed for the sample containing the nisin A spp (3.73 x 10^7 cfu/ml). A 1 log reduction (3.33 x 10^6 cfu/ml) was achieved by the nisin A and cinnamaldehyde combination. The best results were obtained for the nisin V spp alone (a 2 log reduction to 3.33 x 10^5 cfu/ml) or nisin V in combination with cinnamaldehyde (a 4 log reduction to 5.0 x 10^3 cfu/ml) (Fig. 6A).

In chicken noodle soup both the nisin A and nisin V spp (50 μg/ml) alone resulted in a 1 log reduction (2.0 x 10^6 cfu/ml and 1.67 x 10^6 cfu/ml, respectively). The combination of either nisin A or nisin V (50 μg/ml) with carvacrol (195.2 μg/ml) demonstrated a potent effect, reducing cell numbers by 5 log (4.33 x 10^2 cfu/ml) and >5 log (not detected), respectively (Fig. 6B). While the model food results are in close agreement with the broth-based kill curve experiments, more importantly they demonstrate that the enhanced nature of nisin V when applied as a commercial-like fermentate is maintained even within the complex environment of food. Even more notable is the combined effect observed between nisin V and

combinations of both trans-cinnamaldehyde and carvacrol to control *L. monocytogenes* in food, reducing cell numbers significantly more than either compound alone and at least 2 logs greater than nisin A or nisin A + essential oil combinations.

Discussion

There is an increasing need to develop economical, natural and effective food preservative systems to meet the public demand for convenient, safe, healthy and nutritious food products. Such demand has opened up new opportunities for the use of natural antimicrobials derived from plant, animals or microbial sources. Examples of investigated compounds include lactoperoxidase, lysozyme, plant essential oils, organic acids, bacteriocins and chitosan (12, 25, 27). However, while the preservative action of these compounds alone in a food system is unlikely to ensure comprehensive protection, combinations of natural antimicrobials with other non-thermal processing technologies within the hurdle concept could prove invaluable to food manufacturers and consumers in terms of food safety, shelf life, quality and nutritional properties. The synergistic effect of nisin in combination with the plant essential oils carvacrol, and thymol to inhibit the growth of food spoilage and pathogenic organisms such as *L. monocytogenes and B. cereus* has been previously reported (16, 17, 19, 28). Here we demonstrate the ability to provide even greater protection against *L. monocytogenes* by combining for the first time an enhanced nisin derivative (nisin V) in the form of a fermentate with a selection of essential oils. Although the enhanced activity of the nisin V containing spp compared to nisin A against several listerial targets is in agreement with our previous study (11), the enhanced partnership observed when nisin V was combined with carvacrol or cinnamaldehyde is remarkable. In all instances, the nisin V and essential oil combination (thymol, carvacrol, cinnamaldehyde)

outperformed their nisin A equivalents as observed by the extended lag phase of several hours. In time-kill assays, a 2-log decrease in cell numbers over and above that achieved by the nisin A combination was observed (carvacrol and cinnamaldehyde) against the target *L. monocytogenes* EGDe. The fact that this interaction is maintained in a food setting is noteworthy and serves to highlight the potential of enhanced nisin derivatives when utilized in multi-hurdle systems to effectively control *L. monocytogenes* in food. *L. monocytogenes* is of particular concern to the food industry. Although listeriosis is a relatively rare disease, mortality rates associated with outbreaks are high (29). Apart from the risk to human health, food product recalls due to *Listeria* contamination present an enormous financial burden, estimated to cost between $1.2-2.5 billion per year in the United States (30). Notably, in 2003 the Food Safety and Inspection Services (FSIS) published a ruling requiring manufacturers of ready-to-eat foods to provide additional post processing control measures to kill *Listeria* or prevent its growth, placing increased pressure on food manufacturers with respect to food safety. Therefore, any new technologies or means to enhance the control of *L. monocytogenes* in foods are particularly desirable. The consistency of the nisin V and essential oil combination to control *L. monocytogenes* isolates is striking, especially since the level of essential oil used is bacteriostatic rather than bacteriocidal. Notably, despite the demonstrated efficacy of essential oils and their components *in vitro*, their use as food preservatives has been limited because of the high concentrations needed to achieve sufficient antimicrobial activity. Additionally, their intense aroma, even at low concentrations, can produce adverse organoleptic effects exceeding the threshold acceptable to consumers. Consequently, the observed co-operation of nisin V and the essential oils carvacrol and cinnamaldehyde at these low concentrations (equivalent to approx. 0.02%) may provide the key to implementing essential oils in food preservation without simultaneous organoleptic effects. Crucially, we have demonstrated that this relationship is still maintained in complex food matrices, an important finding given that the hydrophobic nature of essential oils can lead to reduced efficacy by interactions with food matrix components such as fat (31), starch, (32) and the level of microbial contamination (33). It is worth noting that our studies

employed a high initial inoculum (1 x 10^7 cfu ml^{-1}) which is much higher than would be expected in a food processing plant (~20 cfu/g). The enhanced co-operation of the nisin V spp and essential oil mixture under these 'abusive' conditions suggests that were it to be incorporated with good manufacturing processes and other hurdle technologies, this combination could provide very effective anti-*Listeria* protection in food preservation. Additionally, the use of natural food grade antimicrobials in this way could provide a more acceptable solution for consumers wishing to buy more 'natural' products. In order for the food industry to fully make the change from the use of artificial sources to purely natural sources research must address the limits of antimicrobial activity, the overall cost, the most effective concentration, optimization of the antimicrobial, use of hurdle technology and address regulatory concerns. This study has begun the process of answering some of those questions.

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Strain (equivalent names)	Food vehicle	Source	Reference
***L. monocytogenes* EGDe**	n/a	Isolated from Guinea pigs (Cambridge, England)	(34)
***L. monocytogenes* LO28**	n/a	Clinical isolate (Faeces of healthy pregnant woman)	
***L. monocytogenes* F2365 (J-119, Ts43)**	Jalisco soft cheese	Human clinical (California Outbreak 1985)	(35)
***L. monocytogenes* 33413 (Ts45)**	Paté	United Kingdom outbreak, 1988)	(36)
***L. monocytogenes* 33013 (Scott A)**	Pasteurised Milk	Human Clinical (Massachusetts outbreak, 1983)	(37)
***L. innocua* FH1848**	Food (fish paste/smoked haddock)	Food (fish paste/smoked haddock) (UCC Culture Collection)	UCC Culture Collection

Table. 1. Listeria strains utilised in this study

Table. 2. Minimum inhibitory concentration determinations of nisin A and nisin V spp and the essential oils thymol, carvacrol and trans-cinnamaldehyde against a selection of *Listeria* strains.

Indicator organism	nisin A µg/ml	nisin V µg/ml	Thymol µg/ml	Carvacrol µg/ml	Cinnamaldehyde µg/ml
L. monocytogenes EGDe	78	39	156	625	312
L. monocytogenes LO28	78	39	312	625	156
L. monocytogenes F2365	125	62.5	312	312	312
L. monocytogenes 33413	62.5	31.25	156	156	312
L. monocytogenes 33013	125	62.5	156	312	312
L. innocua FH1848	78	39	nd	nd	nd

Fig. 1 Deferred antagonism assays of the Nisin A producing strain *L. lactis* DGCC10042 and the stable nisin derivative producing strain *L. lactis* DGCC10042::*nis*V against the indicator *L. monocytogenes* LO28 and (below) Colony Mass Spectrometry analysis of the nisin A (3354 amu) and Nisin V (3322 amu) producing strains utilised in this study.

Fig. 2: Growth curve analysis of strains (A) *L. monocytogenes* EGDe and (B) *Listeria monocytogenes* LO28 in 25µg/ml spp of Nisin A (open square), Nisin V (open diamond), 100µg/ml thymol (open circle) and combinations of Nisin A and thymol (closed square) and Nisin V and thymol (closed diamond).

Fig. 3: Growth curve analysis of strains (A) *L. monocytogenes* EGDe and (B) *Listeria monocytogenes* LO28 (right) in 25µg/ml spp of Nisin A (open square), Nisin V (open diamond), 304µg/ml carvacrol (open circle) and combinations of Nisin A (25µg/ml) and 304µg/ml carvacrol (closed square) and Nisin V (25µg/ml) and 304µg/ml carvacrol (closed diamond).

Fig. 4: Growth curve analysis of strains (A) *L. monocytogenes* EGDe (left) and (B) *Listeria monocytogenes* LO28 (right) in 25µg/ml spp of Nisin A (open square), Nisin V (open diamond), 327.6µg/ml cinnamaldehyde (open circle) and combinations of Nisin A (25µg/ml) and 327.6µg/ml cinnamaldehyde (closed square) and Nisin V (25µg/ml) and 327.6µg/ml cinnamaldehyde (closed diamond).

Fig. 5: Kill curve analysis of strain *L. monocytogenes* EGDe (initial inoculum 1 X 10^7 cells) upon exposure to 50 µg/ml of each spp alone and in combination with **(A)** carvacrol (CA) 195.2 µg/ml **(B)** thymol (THY) 100 µg/ml and **(C)** trans-cinnamaldehyde (CN) 210 µg/ml in BHI broth for a period of 3 hours at 37ºC. Cell growth/kill was measured by performing viable cell counts by diluting cultures in one-quarter-strength Ringer solution and enumeration on BHI agar plates. ND = not detected. Asterisks indicate statistically significant differences between groups (*= $p < 0.05$).

Fig. 6: Survival of *L. monocytogenes* EGDe (initial inoculum 1 X 10^7 cells) in **(A)** a commercial Chocolate Milk product in the presence of cinnamaldehyde (CN) 210 µg/ml plus 50 µg/ml nisin spp A and V, and **(B)** *L. monocytogenes* F2365 (initial inoculum 1 X 10^7 cells)in a commercial chicken noodle soup product in the presence of carvacrol (CA) 195.2 µg/ml plus 50 µg/ml nisin spp A and V. Samples were incubated at 37º C for 3 hours prior to plate count analysis on Listeria Selective Agar (LSA). ND = not detected. Asterisks indicate statistically significant differences between groups (*= $p < 0.05$).

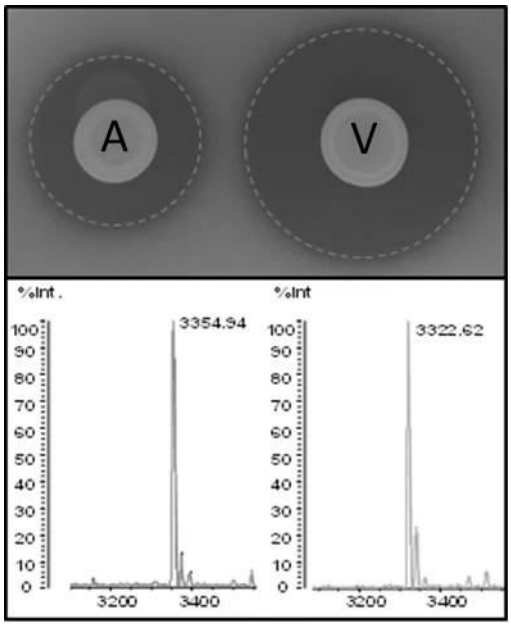

Fig. 1 Deferred antagonism assays of the Nisin A producing strain *L. lactis* DGCC10042 and the stable nisin derivative producing strain *L. lactis* DGCC10042::*nis*V against the indicator *L. monocytogenes* LO28 and (below) Colony Mass Spectrometry analysis of the nisin A (3354 amu) and Nisin V (3322 amu) producing strains utilised in this study.

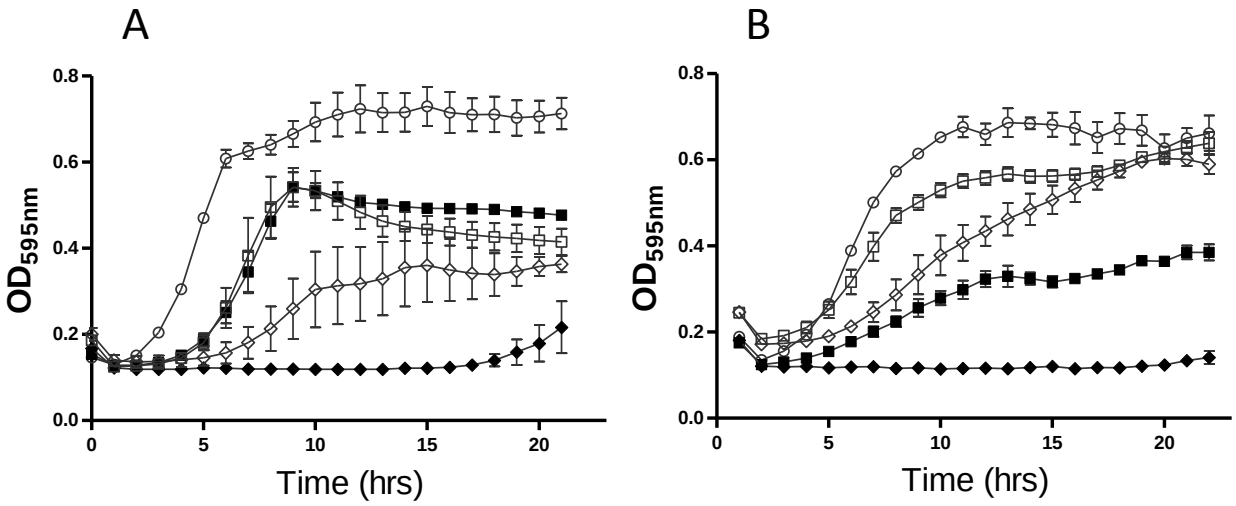

Fig. 2: Growth curve analysis of strains (A) *L. monocytogenes* EGDe and (B) *Listeria monocytogenes* LO28 in 25 μg/ml semi-purified preparation of Nisin A (open square), Nisin V (open diamond), 100 μg/ml thymol (open circle) and combinations of Nisin A and thymol (closed square) and Nisin V and thymol (closed diamond).

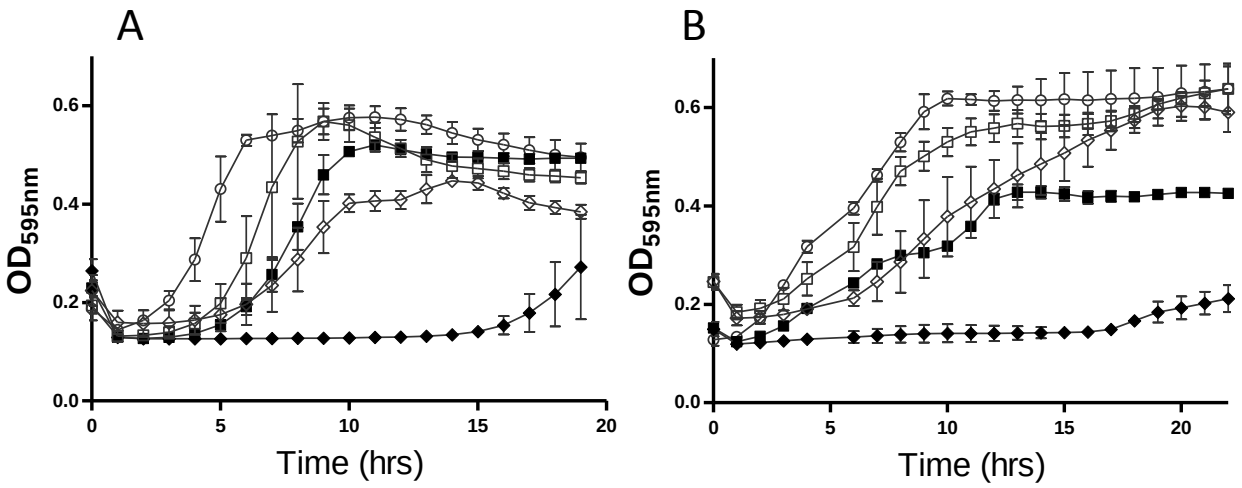

Fig. 3: Growth curve analysis of strains (A) *L. monocytogenes* EGDe and (B) *Listeria monocytogenes* LO28 (right) in 25 µg/ml semi-purified preparation of Nisin A (open square), Nisin V (open diamond), 304 µg/ml carvacrol (open circle) and combinations of Nisin A (25 µg/ml) and 304 µg/ml carvacrol (closed square) and Nisin V (25 µg/ml) and 304 µg/ml carvacrol (closed diamond).

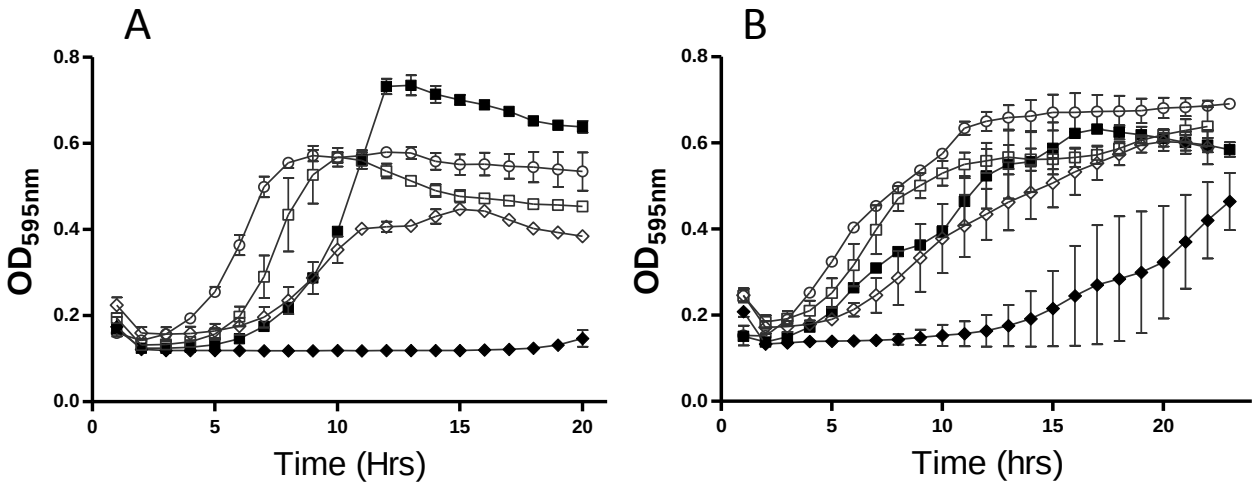

Fig. 4: Growth curve analysis of strains (A) *L. monocytogenes* EGDe (left) and (B) *Listeria monocytogenes* LO28 (right) in 25 µg/ml semi-purified preparation of Nisin A (open square), Nisin V (open diamond), 327.6 µg/ml cinnamaldehyde (open circle) and combinations of Nisin A (25 µg/ml) and 327.6 µg/ml cinnamaldehyde (closed square) and Nisin V (25 µg/ml) and 327.6 µg/ml cinnamaldehyde (closed diamond).

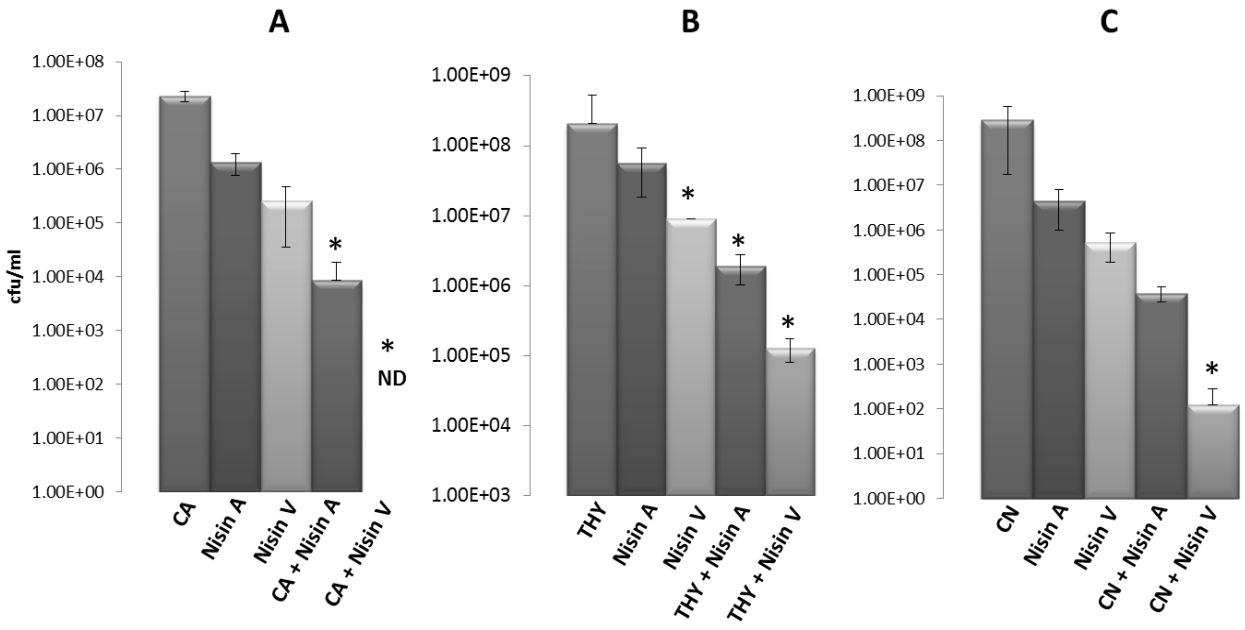

Fig. 5: Kill curve analysis of strain *L. monocytogenes* EGDe (initial inoculum 1 X 10^7 cells) upon exposure to 50 µg/ml of each semi-purified preparation alone and in combination with **(A)** carvacrol (CA) 195.2 µg/ml **(B)** thymol (THY) 100 µg/ml and **(C)** trans-cinnamaldehyde (CN) 210 µg/ml in BHI broth for a period of 3 hours at 37ºC. Cell growth/kill was measured by performing viable cell counts by diluting cultures in one-quarter-strength Ringer solution and enumeration on BHI agar plates. ND = not detected. Asterisks indicate statistically significant differences between groups (*= $p < 0.05$).

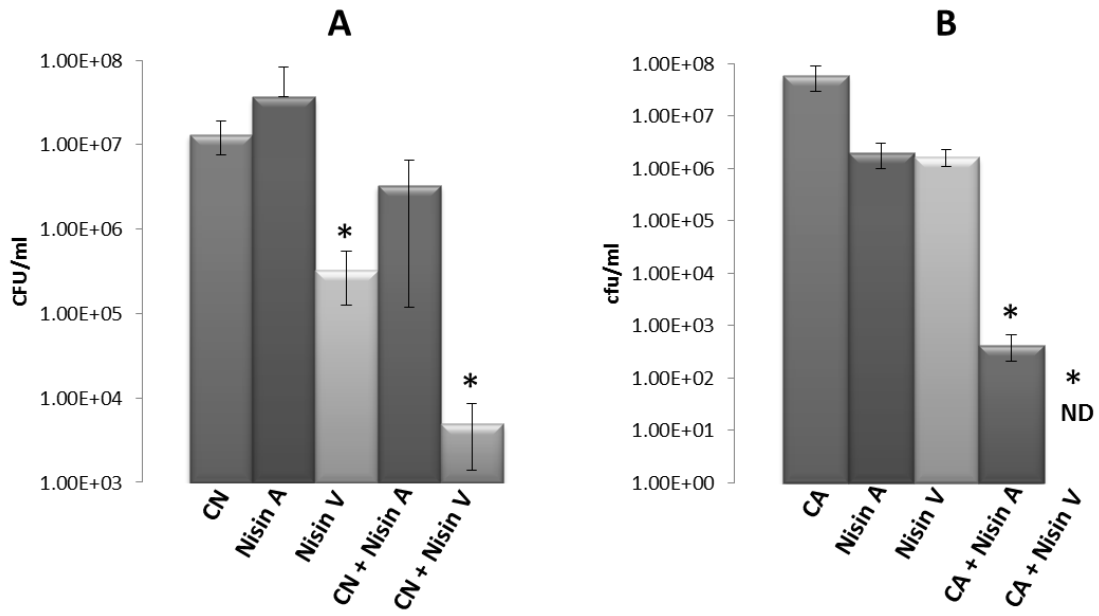

Fig. 6: Survival of *L. monocytogenes* EGDe (initial inoculum 1 X 10^7 cells) in **(A)** a commercial Chocolate Milk product in the presence of cinnamaldehyde (CN) 210 µg/ml plus 50 µg/ml nisin spp A and V, and **(B)** *L. monocytogenes* F2365 (initial inoculum 1 X 10^7 cells) in a commercial chicken noodle soup product in the presence of carvacrol (CA) 195.2 µg/ml plus 50 µg/ml nisin spp A and V. Samples were incubated at 37º C for 3 hours prior to plate count analysis on Listeria Selective Agar (LSA). ND = not detected. Asterisks indicate statistically significant differences between groups (*= $p < 0.05$).