Dual role of select plant based nutraceuticals – as an antimicrobial agent to mitigate food borne pathogens and as food preservatives

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Electronic Supplementary Information

Experimental Methods

Chemicals and plant material

Root bark of *Arnebia nobilis* was obtained from local market in Thanjavur, India. Aerial parts of *Cotoneaster buxifolia* was obtained from ornamental farms of Nilgiris, Tamilnadu. Leaves of *Adhatoda vasica* were harvested from medicinal plant farms in Thanjavur, India. Leaves of *Desmostachya bipinnata* were obtained along the river beds of River Cauvery in and around Thanjavur, India. The plant materials were authenticated by Dr. Jayendran, Department of Botany, Government Arts College, Ootacamund, India. Voucher specimens (JDB1422 of *D. bipinnata*, JDB1425 of *A. nobilis*, JDB 1426 of *C. buxifolia,* JDB1512 of *A. vasica*) were deposited in Government Arts College, Ootacamund, India. Laboratory grade solvents, Silica gel and TLC pre-coated plates were purchased from Merck Ltd., India. Glass wares were purchased from Borosil Ltd. India. Microbial standard cultures were purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. Food isolates were provided by Royal Biotech Research centre, Chennai, India. Nisin was purchased from Sigma-Aldrich.

Isolation of Nutraceuticals

β-Sitosterol-D-glucopyranoside from *D. bipinnata*, Acetyl shikonin and β,β dimethylacryl shikonin from *A. nobilis* were isolated through our previously developed processes[1]. Vasicine from *A. vasica* was isolated through acid-base extraction technique[2]. Ursolic acid from *C. buxifolia* was isolated using conventional column chromatography method. Briefly, Hydro-methanolic extracts (70%) (25g) was taken for column chromatography with silica gel (60-120 mesh) (150 g) packed in a glass column of 4 x 60 cm with bed height of 30 cm. Elution was started with Hexane, followed by increasing ethyl acetate (EA)- hexane combinations (5,10,25,50 and 80% EA in hexane) and finally with EA followed by MeOH. The column elution was monitored by TLC and fractions were pooled based on similar TLC profiles. In total, 11 fractions were collected and were concentrated under reduced pressure in a Rotary evaporator. Fraction 3 that eluted at 10% ethyl acetate-hexane afforded to produce precipitate which was characterised to be Ursolic acid.

Characterisation

All reagents were purchased from Sigma-Aldrich. TLC was monitored with silica gel-precoated aluminum sheets (Type 60 F254, Merck, Darmstadt, Germany) and the spots were visualized in the ultraviolet light chamber, Iodine chamber, 5% MeOH-H_2SO_4 mixture. Elemental analyses were carried out on an automatic Flash EA 1112 Series, CHN Analyzer (Thermo). All melting points are measured using Buchi-545. ^{1}H NMR and ^{13}C NMR spectra were determined on a Bruker-400 NMR spectrometer and chemical shifts were expressed as part per million against TMS as internal reference. For FTIR analysis, 1 mg of compounds were ground along with 100mg of Potassium Bromide (KBr- pre-ground and desiccated at 500°C for 12hr) and the disc was prepared by Hydraulic pressure method. FTIR (Perkin Elmer) analysis was performed at wave number range of 400-4000 cm^{-1}.

Antimicrobial studies

Pathogens and antimicrobial agents

Staphylococcus aureus (MTCC 96), *Bacillus subtilis* (MTCC 441), *Enterococcus faecalis* (MTCC 439), *Listeria monocytogenes* (MTCC 657), *Salmonella enterica* (MTCC 9844), *Escherichia coli* (MTCC 723), *Vibrio cholera* (MTCC 3904), *Klebsiella pneumonia* (MTCC 432), *Shigella dysenteriae* (ATCC 23513), *Pseudomonas aeruginosa* (MTCC 1688) were used for the antimicrobial studies. Commercial antibiotics were purchased from Sigma-aldrich, India which included Amoxicillin (AMX), Ampicillin (AMP), Methicillin (MET), Ciprofloxacin (CIP), Gentamicin (GEN), Chloramphenicol (CHL), Azithromycin (AZM), Erythromycin (ERY), Tetracycline (TET), Polymyxin B (PMB). All pathogens were maintained on nutrient agar slants at 4°C.

Inoculum preparation

All procedures for determination of Antimicrobial activity were done and inoculum size was standardized according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1993). Mueller Hinton Broth (MHB; HiMedia, Mumbai, India) was used to prepare inoculum and grown in incubator orbital shaker at 37°C for 4-8 h until the cultures attained turbidity of 0.5 McFarland Unit. Inoculum size was adjusted and standardized to 5×10^5 CFU ml^{-1} throughout the experiments.

Antibacterial studies

Minimum inhibitory concentrations of isolated compounds against food pathogens were carried out in triplicate using Resazurin Microtitre Assay (REMA) (Martin et al., 2003) with some modifications. Stock solutions of samples at 512 µg/ml were prepared by dissolving the samples in 10% Dimethyl sulphoxide (DMSO). The test samples were diluted in MH broth. The concentration range was 4-512 µg/ml. In 96-well microtiter plates, 100 µl of each of the compound dilutions was added to a mixture of 90 µl of MHB and 10 µl of bacterial inoculum. The negative control consisted of 100µl of 10% DMSO, 90 µl of MHB and 10 µl of cell suspension; the positive control had the addition of ciprofloxacin (2-256 µg/ml). Upon the incubation of the test plates at 30°C for 24 h, cell viability

was determined by the addition of 15 µl of a 0.01% (wt/vol) Resazurin solution to each of the wells, following an extra incubation period of 2 h at 30°C. Viable microorganisms reduced the blue dye to a pink color, which was detected by fluorescence scanning using a microfluorimeter (FLX-800 fluorimeter, BioTek, Winooski, VT) set to an excitation/emission profile of 530 nm/590 nm.

The MIC plates after incubation were taken for determination of minimum bactericidal concentrations (MBCs) which is the lowest concentration of agent that kills 99.9% of the test bacteria. This was done by plating out aliquots onto each appropriate agar plate. Ciprofloxacin was used as control.

Combination studies

To study the interaction of isolated bioactive compounds with other antimicrobial agents, combinations of the compound with commercial antibiotics were assessed by the checkerboard test. Pure compound combined with antibiotics at concentrations ranging from 1/32 × MIC to 4 × MIC were prepared in MHB with standard inoculum size of 5×10^5 CFU ml^{-1}. The fractional inhibitory concentration index (FICI) was found as the sum of the FICs of each of the drugs. FIC is defined as the MIC of each drug used in combination divided by the MIC of the drug when used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5; additive if the FIC index was greater than 0.5 and less than or equal 1.0; indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0. All experiments were done in triplicates and data represented in arithmetic average.

Since Nisin (NSN) is an approved food preservative used commercially, the combination studies were done for the compounds with Nisin and the observations were recorded.

Time kill curves

To assess the rate of growth inhibition of isolated bioactive compounds against pathogens, bactericidal action was evaluated using time-kill curves. Tubes containing Nisin-compound combinations at MIC were inoculated with a suspension of pathogens yielding the final bacterial concentration in broth at 4.5-5.5 $\times 10^5$ CFUs. The tubes were thereafter incubated at 37°C and viable counts were performed at 0, 0.5, 1, 2, 3, 4, 5, 6, 12, 16, 20 and 24 h after the addition of isolated compound. Aliquots of culture broth were taken, serially diluted and spread over agar plates and incubated for up to 48 h at 37°C. Repeated washing and centrifugation was done to minimize antibiotic carry over. Colony counts were done in triplicate and data represented in arithmetic mean.

Food preservation studies

Preparation of fruit juices

Real fruit juices of apple, orange and red grapes were selected for the studies. Two sets of each fruit juice were considered for the study (i) Fresh juices extracted from fresh fruits (Un-pasteurised) (ii) Commercial real fruit juices Tropicana® Pure premium and Dabur Real® (Pasteurized). For Un-pasteurised juices, Fresh fruits were obtained from the market, washed thoroughly and the juices were extracted using domestic benchtop juice extractor and Grinder. The juices were filtered for insolubles, seeds and the clear filtrates were considered for studies. For pasteurized juices, the above brands were selected since they contain no added flavours and preservatives. These juices were directly used for studies.

Antimicrobial studies

Initial experiments were done on fruit juices by plating these fruit juices onto Nutrient agar plates after regular serial dilution technique. The microbial plate counting was done to find the initial amount of microbes present in the juices. Colonies were enumerated on plates containing Nutrient agar using spread plate technique. The detection limit for the plating technique was found to be 1-2 CFU/ml. Therefore, this technique was further used to evaluate 5 log reduction studies. 5 log reduction studies were done on 3 pasteurised fruit juices (Apple, orange and red grapes) of 2 brands (Tropicana and Dabur). Two gram positive strains, *S. aureus* and *L. monocytogenes*; and two gram negative strains *S. enterica* and *E. coli* were inoculated into fruit juices at 4-5 × 10^5 CFU/ml. Concentrations of Compounds at 4 × MBC – 1/32 × MBC were added to different sets of juices. Combinations with Nisin were also experimented where combination ratios were taken from previous synergistic profile studies. The juices were kept at room temperature for 24 h. Then, aliquots were taken and plated onto nutrient agar and the concentrations which gave 5 log reduction (<5 CFU/ml) over 24 h were tabulated.

To juices with no initial microbial contamination as detected by enumeration technique, an initial inoculum of 4-5 ×10^5 CFU/ml of select pathogens, *L. monocytogenes,* and *S. enterica* was added in fruit juices. Concentrations of Compounds which gave 5 log reductions over 24 h were added to different sets of juices and growth curve analysis was done for 14 days. Compounds and the compound-nisin synergistic observations which best inhibited the microbes in juices were determined.

Further studies were done to check the extension of shelf life of juices. In this study, isolated compounds and nisin-compound ratios at concentrations that gave 5 log reductions were added to 50 ml of juices. Then microbial counts

were performed by taking aliquots from juices at regular time intervals (every 12 h). For Un-pasturized juices, studies were conducted for 7 days and for pasteurized juices it was 15 days. The juices were maintained at room temperature and a similar set of juices were maintained under refrigeration at 4°C. The actual food preservation profiles were tabulated through this study.

Table S1 Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of tested compounds

Pathogens	Nisin	Allicin	BDMS	AS	UA	Vasicine
Staphylococcus aureus (MTCC 96)	8/16	16/32	64/128	48/64	128/512	32/64
Staphylococcus aureus	8/16	16/32	64/128	48/64	128/512	32/64
Staphylococcus aureus	4/8	8/12	48/64	32/64	128/512	16/32
Listeria monocytogenes (MTCC 657)	8/8	16/32	128/256	32/64	>512/>1000	48/64
Listeria monocytogenes	8/16	16/32	128/256	32/64	>512/>1000	48/64
Bacillus subtilis (MTCC 441)	4/12	2/8	256/512	32/64	128/256	32/64
Bacillus subtilis	8/16	4/16	256/512	32/64	128/256	32/64
Bacillus cereus (MTCC 1272)	8/24	4/16	256/>512	48/128	128/>1000	32/64
Bacillus cereus	16/32	4/8	256/>512	48/128	128/>1000	32/64
Enterococcus faecalis (MTCC 439)	32/64	4/8	512/>1000	32/64	64/128	32/64
Enterococcus faecalis	16/32	4/8	512/>1000	32/64	64/128	16/32
Salmonalla enterica (MTCC 9844)	NE	8/16	512/>1000	64/128	128/512	16/32
Salmonalla enterica	NE	16/32	512/>1000	48/128	128/512	16/32
Escherichia coli (MTCC 723)	NE	2/8	32/64	16/32	48/64	16/32
Escherichia coli	NE	4/8	64/128	12/32	64/64	32/64
Escherichia coli	NE	4/12	64/128	12/32	64/128	32/64
Vibrio cholera (MTCC 3904)	NE	16/32	128/256	32/64	64/128	64/128
Vibrio cholera	NE	16/32	128/256	32/64	64/128	64/128
Klebsiella pneumonia (MTCC 432)	NE	8/32	256/512	32/64	64/128	32/64
Klebsiella pneumonia	NE	16/32	256/512	32/64	64/128	32/64
Shigella dysenteriae (ATCC 23513)	NE	8/32	128/512	24/64	>512/>1000	16/64
Shigella dysenteriae	NE	8/16	128/512	24/64	512/>1000	16/64
Pseudomonas aeruginosa (MTCC 1688)	NE	16/32	256/512	32/64	>512/>1000	32/64
Pseudomonas aeruginosa	NE	8/32	128/512	16/32	512/>1000	16/32

NE- Not effectivethe Strains apart from MTCC are stable laboratory derived isolates from food stuffs.

Table S2 Synergistic antimicrobial profiles of isolated nutraceuticals in combination with commercial antibiotics

Pathogens		AMX[#]	AMP	MET	CIP	GEN	CHL	AZM	ERY	TET	PMB
Staphylococcus aureus (MTCC 96)	1	A	A	A	S	A	A	N	A	N	N
	2	A	A	A	S	A	A	A	A	A	A
	3	A	A	A	A	A	A	A	A	A	A
	4	A	A	S	S	N	N	A	S[1]	A	N
Listeria monocytogenes (MTCC 657)	1	A	A	S	S	A	A	N	N	N	N
	2	A	S	S	S	A	A	A	A	N	A
	3	A	A	A	A	A	A	A	A	A	A
	4	A	A	S[1]	S	A	N	A	S[1]	A	A
Bacillus subtilis (MTCC 441)	1	A	A	A	A	A	A	A	N	N	N
	2	A	A	A	A	A	A	A	A	A	A
	3	A	A	A	A	A	A	A	N	A	A
	4	A	A	S	S	A	N	N	S[1]	A	N
Bacillus cereus (MTCC 1272)	1	A	A	A	A	A	A	A	N	N	N
	2	A	A	A	A	A	A	A	A	A	A
	3	A	A	A	A	A	N	N	A	N	A
	4	A	A	S	S	N	N	N	S	A	N
Enterococcus faecalis (MTCC 439)	1	A	A	S	S	A	A	A	A	A	A
	2	A	S	S	S	A	A	A	A	A	A
	3	A	A	A	A	A	A	A	N	A	A
	4	N	A	S	S	N	N	N	S	A	A
Salmonalla enterica (MTCC 9844)	1	A	A	S	S	A	A	A	A	A	N
	2	A	S	S	S	A	A	A	A	A	A
	3	A	A	A	A	A	N	A	A	A	A
	4	N	A	S[1]	S	A	N	N	S	A	N
Escherichia coli (MTCC 723)	1	S[1]	S	S	S	A	A	A	A	A	A
	2	S[1]	S	S	S	A	A	A	A	A	N
	3	A	A	A	A	A	A	A	N	A	A
	4	N	A	S	S	N	N	N	S[1]	A	A
Vibrio cholera (MTCC 3904)	1	A	A	A	A	A	N	N	N	N	N
	2	A	N	S	S	A	A	N	A	N	N
	3	A	A	A	A	A	A	A	N	N	A
	4	N	A	S	S	N	N	A	S	A	A
Klebsiella pneumonia (MTCC 432)	1	A	A	S	A	A	A	A	N	N	N
	2	A	S	S[1]	A	A	A	A	N	A	A
	3	A	A	A	A	A	A	A	N	N	A
	4	A	A	S	S	N	N	A	S[1]	A	A
Shigella dysenteriae (ATCC 23513)	1	N	A	A	A	N	N	N	A	A	N
	2	N	S	S	S	A	A	A	A	A	N
	3	A	S	S[1]	S	A	A	S	A	A	S
	4	A	A	S	S	N	N	A	S	A	A
Pseudomonas aeruginosa (MTCC 1688)	1	N	A	A	A	N	A	N	A	N	N
	2	A	A	S	A	N	A	A	A	N	N
	3	A	A	A	A	A	N	A	A	A	S
	4	A	A	S[1]	S	N	N	A	S[1]	A	A

[#]**Commercial Antibiotics** –AMX- Amoxicillin, AMP- Ampicillin, MET- Methicillin, CIP- Ciprofloxacin, GEN-Gentamycin, CHL- Chloramphenicol, AZM- Azithromycin, ERY- Erythromycin, TET- Tetracycline, PMB-Polymyxin-B. 1- β,β dimethylacryl

shikonin, 2- Acetyl shikonin, 3- Ursolic acid, 4-vasicine. S-Synergistic, A- Additive, N- No interaction, S^1- Concentrations of Compound:Antibiotic = 1:4. S- Concentrations of Compound : Antibiotic = 1:1.

Table S3 (a) Concentrations (ppm l^{-1}) at which 5 log reductions of *S. aureus* was observed

Juice	Fruit	BDMS	AS	UA	VN	N	N+BDMS	N+AS	N+UA	N+VN
Tropicana	Apple	128	64	512	64	16	32	24	24	24
	Orange	96	64	512	64	16	24	18	24	18
	Grapes	128	64	512	64	16	32	24	24	24
Dabur Real	Apple	128	64	512	64	16	32	24	24	24
	Orange	96	64	512	64	16	32	18	24	18
	Grapes	128	64	512	64	16	32	24	24	24

BDMS- β,β dimethylacryl shikonin, AS- Acetyl shikonin, UA- Ursolic acid, VN- Vasicine, N- Nisin.

Table S3 (b) Concentrations (ppm l^{-1}) at which 5 log reductions of *L. monocytogenes* was observed

Juice	Fruit	BDMS	AS	UA	VN	N	N+BDMS	N+AS	N+UA	N+VN
Tropicana	Apple	1000	64	128	32	32	32	32	48	32
	Orange	768	56	128	32	32	28	32	48	32
	Grapes	1000	64	128	32	32	32	32	48	32
Dabur Real	Apple	1000	64	128	32	32	32	32	48	32
	Orange	768	56	128	32	32	28	32	48	32
	Grapes	1000	64	128	32	32	32	32	48	32

BDMS- β,β dimethylacryl shikonin, AS- Acetyl shikonin, UA- Ursolic acid, VN- Vasicine, N- Nisin.

Table S3 (c) Concentrations (ppm l^{-1}) at which 5 log reductions of *S. enterica* was observed

Juice	Fruit	BDMS	AS	UA	VN	N	N+BDMS	N+AS	N+UA	N+VN
Tropicana	Apple	1000	128	512	48	NE	128	96	128	64
	Orange	896	96	512	32	NE	96	84	128	56
	Grapes	1000	128	512	48	NE	128	96	128	64
Dabur Real	Apple	1000	128	512	48	NE	128	96	128	64
	Orange	896	96	512	32	NE	96	84	128	56
	Grapes	1000	128	512	48	NE	128	96	128	64

BDMS- β,β dimethylacryl shikonin, AS- Acetyl shikonin, UA- Ursolic acid, VN- Vasicine, N- Nisin.

Table S3 (d) Concentrations (ppm l^{-1}) at which 5 log reductions of *E. coli* was observed

Juice	Fruit	BDMS	AS	UA	VN	N	N+BDMS	N+AS	N+UA	N+VN
Tropicana	Apple	64	32	64	32	NE	32	24	32	24
	Orange	56	24	64	24	NE	24	24	24	24
	Grapes	64	32	64	32	NE	32	24	32	24
Dabur Real	Apple	64	32	64	32	NE	32	24	32	24
	Orange	56	24	64	24	NE	24	24	24	24
	Grapes	64	32	64	32	NE	32	24	32	24

BDMS- β,β dimethylacryl shikonin, AS- Acetyl shikonin, UA- Ursolic acid, VN- Vasicine, N- Nisin.

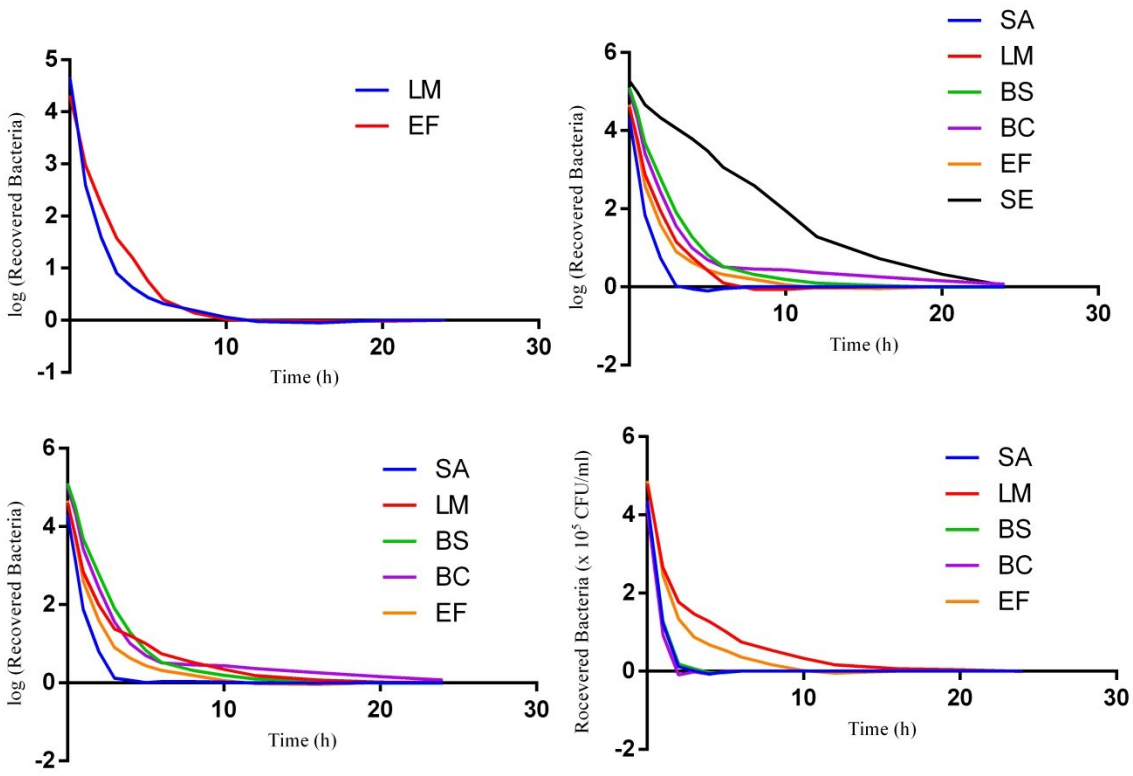

Fig S1: Time kill curves of combinations of nisin with BDMS, AS, UA and Vasicine respectively against food pathogens.

SA- *S. aureus*, LM – *L. monocytogenes*, EF- *E. faecalis*, BS- *B. subtilis*, BC- *B. cereus*, SE- *S. enterica*.

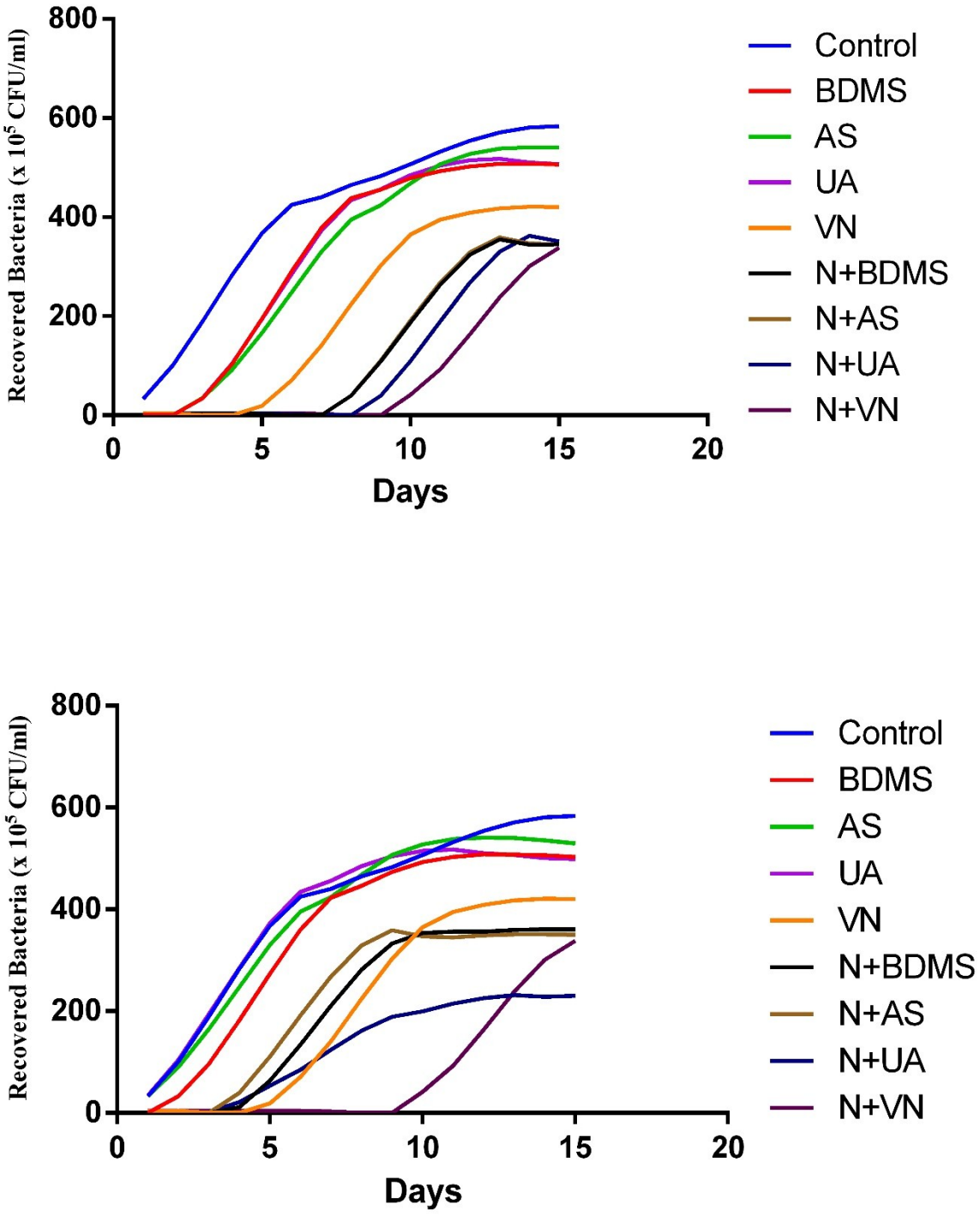

Fig S2: Growth curves of *L. monocytogenes* and *S. enterica* in grape juice containing compounds and nisin (individual and in combination)