Full Length Research Paper

Preliminary study related to specific nutrient synergy-modulation of antimicrobial resistance of bacteria isolated from dairy products

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Increasing resistance to currently used antimicrobials has resulted in the evaluation of other methods to rectify this growing health concern. Herein, we present an investigation to examine the modulation of antimicrobial resistance in bacteria grown in the presence of a specific nutrient synergy (SNS) known as Epican Forte (EF). We have conducted tests on thirty colonies of each of the following bacterial species: *Staphylococcus aureus*, *Salmonella*, *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Staphylococcus saprophyticus*. Bacterial cells were grown in brain heart infusion broth (BHI) in the presence and absence of EF at 37°C for 1, 2 and 3 h. The antimicrobial resistance patterns of the SNS-exposed and SNS-deprived isolates were examined and evaluated by Wilcoxon tests to assess the significance of the percentage increase in antimicrobial susceptibility. Our results indicate a significant increase in the inhibition zone diameters of most SNS-exposed bacterial colonies, demonstrating a rise in drug-susceptibility. Whilst this analysis has indeed provided significant insights, further investigations are required to identify the mechanisms involved in nutrient synergy reduction of bacterial resistance to drugs.

Key words: Antimicrobial resistance, Epican Forte, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*, *Yersinia enterocolitica*, specific nutrient synergy.

INTRODUCTION

Antimicrobial resistance is currently recognized as a prime public health problem. Resistance in bacteria to exposed drugs is a natural biological phenomenon (Pontes et al., 2009). Nosocomial and community acquired agents have successfully proven their ability to

develop resistance mechanisms against commonly used antimicrobials (Jones, 2010). The supplementation of animal food with antimicrobials at low preventative levels as growth promoter, interrupted courses of antimicrobials in human treatment, and poor implementation of infection

Table 1. Selective media used for bacterial isolation and the expected morphologies.

Selective media	Bacteria Isolated	Expected colonies morphology	Reference
MC	*Escherichia coli*	Pink to violet colour	Hayes et al., 2001
SMC	Pathogenic *E. coli*	Colourless to gray colour	Galland et al., 2001
CIN	*Yersinia enterocolitica*	Bulls eye morphology	Saida et al., 1998
LSA	*Listeria monocytogenes*	Black to brown colonies surrounded by black halos	Aygun and Pehlivanlar, 2006
MSA	*Staphylococcus aureus*	Golden yellow colonies surrounded by a yellow hallow	Harakeh et al., 2006b
MSA	*Staphylococcus saprophyticus*	White mucoid colonies	Harakeh et al., 2006b
BSA	*Salmonella*	Black colonies with pronounced metallic sheen	Harakeh et al., 2005
BGA	*Salmonella*	Pink colonies surrounded by brilliant red zone	Harakeh et al., 2009

MC, MacConkey agar; SMC, Sorbitol MacConkey agar; CIN, Cefsulodin-Irgasan-Novobiocin agar; LSA, *Listeria* selective agar; MSA, mannitol salt agar; BSA, bismuth sulfite agar; BGA, brilliant green agar.

control measures in hospitals lead to the development and spread of multi-drug resistant bacteria (Checkley et al., 2010; Taylor et al., 2002). There are only few studies which reported antimicrobial resistance level in countries where this problem exists. Previous studies conducted in Lebanon showed very high levels of antimicrobial resistance in bacteria isolated from water and other environmental samples (Harakeh et al., 2005; Harakeh et al., 2006). Thus, the search for methods to rectify this major health problem is urgently required.

In this work, we evaluated the effects of specific nutrient synergy (SNS) on the resistance patterns of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Staphylococcus saprophyticus*. SNS is a unique combination of antioxidants, amino acids, and other nutrients; among those are epigallocatechingallate (EGCG), ascorbic acid (AA), L-lysine and L-proline. It has been previously reported that EGCG (a major constituent of green tea) showed an increase in sensitivity of methicillin-resistant *S. aureus* (MRSA) to antimicrobials (Stapleton and Taylor, 2002; Stapleton et al., 2004).

Ascorbic acid is one of the components of SNS as well as an important antioxidant. It has been proved in a previous study to abrogate P-glycoprotein (P-gp) mediated multidrug resistance in *E. coli* (El-Masry and Abou-Donia, 2003). In another paper, it has been demonstrated that *Enterobacter cloacae* could increase its susceptibility to ampicillin if incubated anaerobically in the presence of increasing concentrations of ascorbic acid (Shoeb et al., 1995). In addition, the effect of ascorbic acid on plasmid-coded antimicrobial resistance in *S. aureus* was investigated by Amabile-Cuevas*et* et al. (1991). Their work showed that the presence of ascorbic acid induced 50 to 75% decrease in minimal inhibitory concentrations of different antimicrobials used

against different test strains (Amábile-Cuevas et al., 1991). Prompted by these previous studies, the individual effect of ascorbic acid on antibiotic growth inhibition of our bacterial species was also investigated.

MATERIALS AND METHODS

Bacterial isolation and identification

A total of 164 samples of dairy-based foods were collected from the north-eastern area of Lebanon where most dairy products are produced. The collection of samples has been described by Saleh et al. (2009).

A variety of selective media were used to isolate different bacterial types including *E. coli*, *Salmonella*, *Y. enterocolitica*, *L. monocytogenes* and *Staphylococcus* spp. (*S. aureus* and *S. saprophyticus*) (Table 1). The number of suspected colonies investigated per sample depended on the number of colony forming units per gram of food. In general, only two colonies were selected from each dairy-based food sample showing less than 10^2 cfu/g, four were selected from each sample showing 10^2 to 10^4 cfu/g, and eight from each sample showing more than 10^4 cfu/g. Each selected colony was purified by subculturing onto its specific agar medium and incubated at 37°C for 24 h. Plates were finally stored at 4°C until further investigation.

Chosen suspected colonies on the selective media were confirmed using a series of biochemical key tests. *E. coli*, *L. monocytogenes*, *S. aureus* and *S. Saprophyticus* isolates were molecularly confirmed via polymerase chain reaction (PCR) method as reported in our previous studies (Harakeh et al., 2009; Saleh et al., 2009; Zouhairi et al., 2010).

In the case of *Salmonella* and *Y. enterocolitica*, Gram staining was first conducted followed by Gram-negative rods for biochemical tests. Final confirmation was carried out using the API 20E biochemical system (BioMérieux, Marcy l'Étoile) (Saleh et al., 2012). Among the biochemically identified isolates, an equal number of colonies of each bacterial type (30 colonies) were selected to detect the effect of SNS on their antimicrobial resistance patterns. The remaining colonies were molecularly investigated in other studies.

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Table 2. Percentage of antimicrobial resistance isolates to different antimicrobials.

Antimicrobial	Percentage of antimicrobial resistant isolates				
	Staphylococcus	*Salmonella*	*E. coli*	*L. monocytogenes*	*Y. enterocolitica*
Ampicillin	-	66.7	71.8	63.3	-
Ciprofloxacin	-	27.8	31.9	-	43.7
Cefotaxime	-	61.1	30.9	-	-
Cefuroxime	-	55.6	63.8	-	-
Clindamycin	93.5	-	-	43.3	-
Chloramphenicol	-	-	69.1	60	62.5
Erythromycin	-	-	-	26.6	-
Gentamicin	67.4	50	51.0	6.6	68.7
Kanamycin	-	-	-	-	81.2
Methicillin	84.8	-	-	-	-
Nalidixic acid	-	77.8	58.5	-	31.2
Ofloxacin	93.5	66.7	44.7	-	-
Oxacillin	-	-	-	93.3	-
Penicilline	-	-	-	90.0	-
Streptomycin	-	-	-	-	87.5
Teicoplanin	76.1	-	-	-	-
Tetracycline	-	-	84.0	20.0	-
Trimethoprim/ sulfamethoxazole	-	33.3	42.6	16.7	37.5
Vancomycin	71.7	-	-	26.6	-

-, Isolates were not tested for their susceptibility of the listed antimicrobial.

Reagents

SNS of Epican Forte (EF) is a formulation containing the following: 900 µM of ascorbic acid, 1.1 mM of L-lysine, 1.1 mM of L-proline, 500 µM of L-arginine, 250 µM of N-acetyl L-cysteine, 150 µM of epigallocatechingallate, 85 µM of selenium, 7 µM of copper and 4 µM of manganese (Dr Rath Research Institute, Santa Clara, California, USA).

Bacterial cultures and their growth conditions

Isolates were grown overnight in 5 ml BHI. A total of 65 µl of the overnight culture were added to 5 ml of brain heart infusion (BHI) broth containing SNS at a concentration of 25 mg/ml. Different SNS concentrations were previously tested for their effect on bacterial growth and the optimum concentration that did not affect the growth was used in this work. The tubes were incubated in a 37°C shaker. Samples were taken at three different time-points of 1, 2, and 3 h. A second solution of BHI and ascorbic acid was prepared to test the ascorbic acid individual effect, to compare it to that of the components synergy in the first solution, the concentrations of ascorbic acid in both solutions should be the same. Therefore, concentration of ascorbic acid in the SNS solution was calculated using the known formula of EF. The result was used in the preparation of the second solution and 65 µl of the bacterial culture were incubated in 5 ml BHI containing ascorbic acid. Samples were also taken at the three different time-points.

Antimicrobial effect of EF and ascorbic acid

To study the antimicrobial effects of SNS and ascorbic acid, overnight bacterial cultures were used to inoculate Mueller Hinton plates. Disks impregnated with SNS and then with ascorbic acid of concentrations equal to those used in the above test were then added. Plates were incubated for a period of 18 to 24 h and the inhibition zones were measured.

Antimicrobials resistance test

Disk diffusion method was utilised to evaluate the antimicrobial resistance level of the selected isolates (30 isolates of each bacterial type) before and after treatment with EF and ascorbic acid. The antimicrobials used for each bacterial species are displayed in Table 2. At each time-point, a volume of 100 µl of the mixture was placed on Mueller Hinton plates (MH) and disks impregnated with various antimicrobials were added. As a control, the antimicrobial resistance of these bacteria was tested by the same method in the absence of SNS and ascorbic acid. Plates were incubated for a period of 18 to 24 h and the inhibition zone diameters in millimeters were then recorded. Using the National Committee for Clinical Laboratory Standards (NCCLS) guidelines, organisms were classified as resistant (not inhibited), intermediately resistant (not completely inhibited), or susceptible (inhibited) to the antimicrobials. The percentage change in the resistance pattern was calculated. Finally, the percentage of bacteria that showed an increase in the diameter of their inhibition zones was also calculated.

Statistical analysis

A statistical examination was carried out using SAS 9.1. to evaluate if the percentage increase in the antimicrobials susceptibility after SNS treatment was significant. Primarily, Wilcoxon tests were carried out. Friedman tests followed by Nemenyi test were

conducted to examine the significance of variation in the percentages of susceptible isolates among different antimicrobials within each bacterial species.

RESULTS

Bacterial isolation and identification

Based on their morphologies on selective media, colonies were selected for further identification using biochemical and molecular tests. Selected suspected colonies of each of the following bacterial species include, 173 *E. coli*, 54 *Y. enterocolitica*, 30 *L. monocytogenes*, 321 *S. aureus* and *S. saprophyticus*, and 35 *Salmonella.* A set of specific key tests were used for each bacterial type.

The results of these tests showed that among the suspected colonies, 102 were confirmed as *E. coli*, 36 as *Y. enterocolitica*, 30 as *L. monocytogenes*, 29 as *S. aureus*, 17 as *S. saprophyticus*, and 32 as *Salmonella*. To make the comparison among the different bacterial types possible, the same number of colonies was used in the investigation. The lowest number of identified colonies was 30 colonies in the case of *Listeria*. Hence, 30 colonies of each bacterial type were selected and tested for their antimicrobial resistance level before and after the application of SNS and ascorbic acid.

Antimicrobial resistance pattern of the tested isolates

All tested isolates showed a high level of antimicrobial resistance. Percentages of resistant isolates are illustrated in Table 2. No previous studies were conducted in Lebanon on environmental isolates. Therefore, the results of this study provide baseline information on how serious is the level of antimicrobial resistance among bacteria that may cause food-borne illnesses. It was striking to find that 97.8% of the tested staphylococcal isolates were resistant to at least one antimicrobial. Sixty-one percent showed resistance to all 6 antimicrobials used. Staphylococcal isolates displayed the highest resistance to oxacillin (93.5%) and clindamycin (93.5%), followed by methicillin (84.8%). The identification of such a high percentage of antimicrobial resistant environmental isolates is of major public health concern, especially when encountering a high percentage of methicillin-resistant *S. aureus (*MRSA).

As for *Salmonella*, all isolates were resistant to at least one of the antimicrobials used with the highest patterns of resistance against nalidixic acid (77.78%). Resistance to tetracycline was significantly the highest in *E. coli* isolates. Among the β-lactames, resistance to ampicillin (seen in 72% of isolates) and cefuroxime (64%) were considerably more common than resistance to cefotaxime (31%). As for quinolones, resistance to nalidixic acid (59%) and ofloxacin (45%) was higher in comparison with ciprofloxacin (32%).

In the case of *L. monocytogenes*, all tested isolates were found resistant to at least one of the antimicrobial agents. Significantly, more isolates were resistant to oxacillin (93.33%), penicillin (90%), and ampicillin (60%) compared with the rest of the evaluated antimicrobials. Finally, Yersinia isolates also displayed high level of antimicrobial resistance, with the highest level of resistance being streptomycin (87.5%) and kanamycin (81.2%).

Antimicrobial effect of SNS and ascorbic acid on different bacterial types

SNS and ascorbic acid of concentration identical to those used with the antimicrobials did not exhibit an effect on the growth of any of the studied bacteria (data not shown).

Effect of SNS on the antimicrobial resistance of isolates

The effect of SNS on the antimicrobial resistance profile of the tested bacterial strains was more evident at the 1 h time-point. At the two remaining time-points, the diameters of inhibition zones showed a slight increase or remained fixed. Therefore, analysis was conducted using the first time-point readings. The percentages of previously resistant isolates that increase susceptibility after 1 h of incubation in SNS were calculated. *Yersinia* isolates showed a significant increase in their susceptibility to all the tested antimicrobials ($p<0.05$) except for ciprofloxacin to which only three isolates were originally resistant. Similarly, all the tested antimicrobial agents demonstrated a marked improvement in the modulation of antimicrobial resistance for both *E. coli* and *Salmonella* isolates ($p<0.05$) (Wilcox test, data not shown). These results indicated that one or a combination of the nutrients present in SNS has a statistically crucial effect on the reduced antimicrobial resistance pattern of Gram-negative bacteria. In the case of Gram-positive bacteria, isolates showed a significant increase in their susceptibility to most of the tested antimicrobials with a p-value of lower than 0.05. The unaffected antimicrobial susceptibilities are those of penicillin and gentamicin in the case of *L. monocytogenes* and clindamycin whereas oxacillin in the case of *S. aureus* (data not shown).

Comparing the SNS effect among different bacterial types

The percentages of Gram-negative isolates that displayed an increase in their susceptibility level were compared among each other where comparison was possible. The effect of SNS on the resistance pattern was
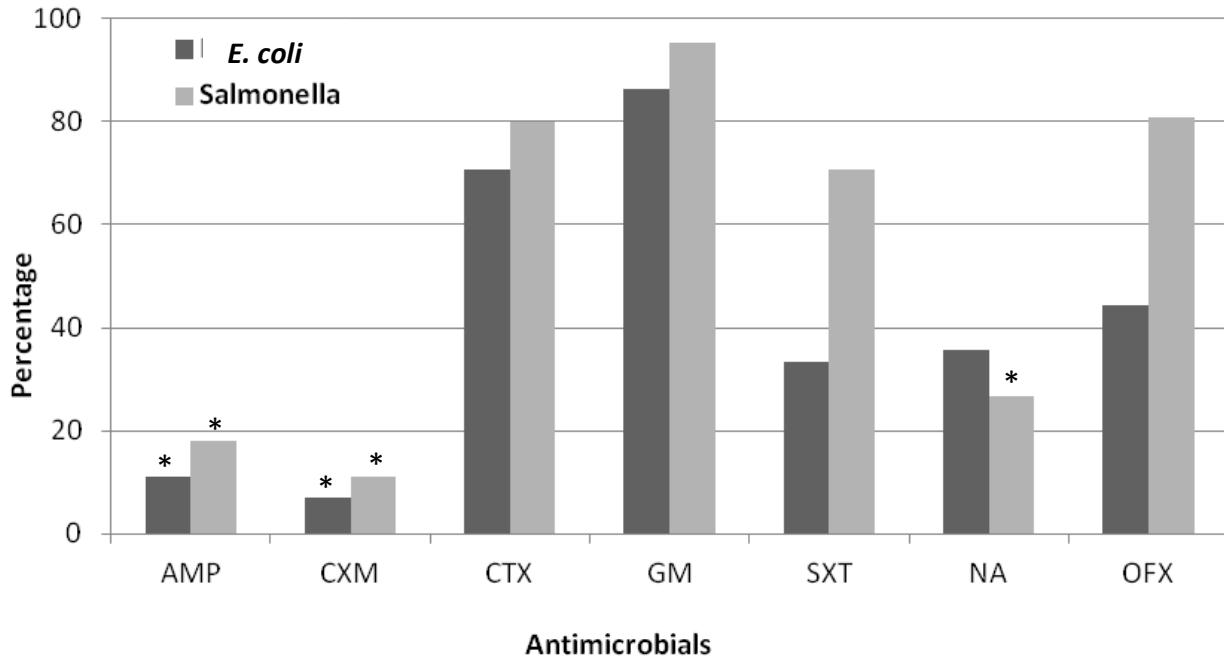
Figure 1. Percentages of *E. coli* and *Salmonella* isolates that showed an increase in their susceptibility to seven different antimicrobials. AMP, Ampicillin; CXM, cefuroxime; CTX, cefotaxime; GM, gentamicin; SXT, trimethoprim-sulfamethoxazole; NA, nalidixic acid; OFX, ofloxacin; *, statistically non-significant percentage of isolates with an increase in drug susceptibility.

evaluated using 10 different antimicrobials for the *E. coli* isolates and eight different antimicrobials for the *Salmonella* isolates. Seven of those drugs were used for both bacterial types. The percentage increases in antimicrobial susceptibility of the two species were compared and similar patterns were clearly observed (Figure 1). The Friedman test demonstrated that the highest percentage increase in antimicrobial susceptibility was gentamicin followed by cefotaxime for both *E. coli* and *Salmonella* ($p < 0.05$). The lowest percentage increase in antimicrobial susceptibility was cefotaxime and ampicillin which were not significantly affected by SNS ($p > 0.05$). The only difference between the two species was in the case of nalidixic acid for which only 26.6% of the resistant *Salmonella* isolates showed an increase in their susceptibility and this percentage was not significant as indicated by the Wilcoxon test. Another comparison was conducted between the *E. coli* and the *Yersinia* isolates where four common antimicrobials were tested for both species. Similar patterns were also observed with the highest level of percentage increase in antimicrobial susceptibility again with gentamicin for both species ($p < 0.05$) (Figure 2). In the case of *Yersinia*, ofloxacin showed the lowest percentage increase (27.3%) followed by trimethoprim-sulfamethoxazole (45.5%).

The effect of SNS on the antimicrobial resistance patterns of *L. monocytogenes* and *S. aureus* were compared taking into account the four antimicrobials

tested in common for both species. Although Figure 3 shows a similar pattern, the Wilcoxon test demonstrated that the percentage increase in *S. aureus* susceptibility to clindamycin was not significant (6.6%), noting that the 16.6% increase in susceptibility to clindamycin by *L. monocytogenes* isolates was considered as significant ($p < 0.05$). Furthermore, the highest level of percentage increase in susceptibility shown in Figure 3 by gentamicin is also misleading. The 100% increase of *L. monocytogenes* susceptibility to gentamicin was not significant ($p > 0.05$), since only three out of the 30 tested isolates were shown to be gentamicin resistant. Even though the three isolates were susceptible in the presence of SNS, the number was still considered as statistically not significant. It is worth mentioning that even the previously susceptible *L. monocytogenes* isolates showed an increase in the diameters of the inhibition zones in the presence of SNS.

Effect of ascorbic acid on the antimicrobial resistance patterns

The individual effect of ascorbic acid on the susceptibility of various bacterial species to different antimicrobials was also evaluated. Results showed that ascorbic acid modified significantly the resistance pattern of *Yersinia* isolates to streptomycin and ciprofloxacin. Both *E. coli* and *Salmonella* significantly increased their susceptibility
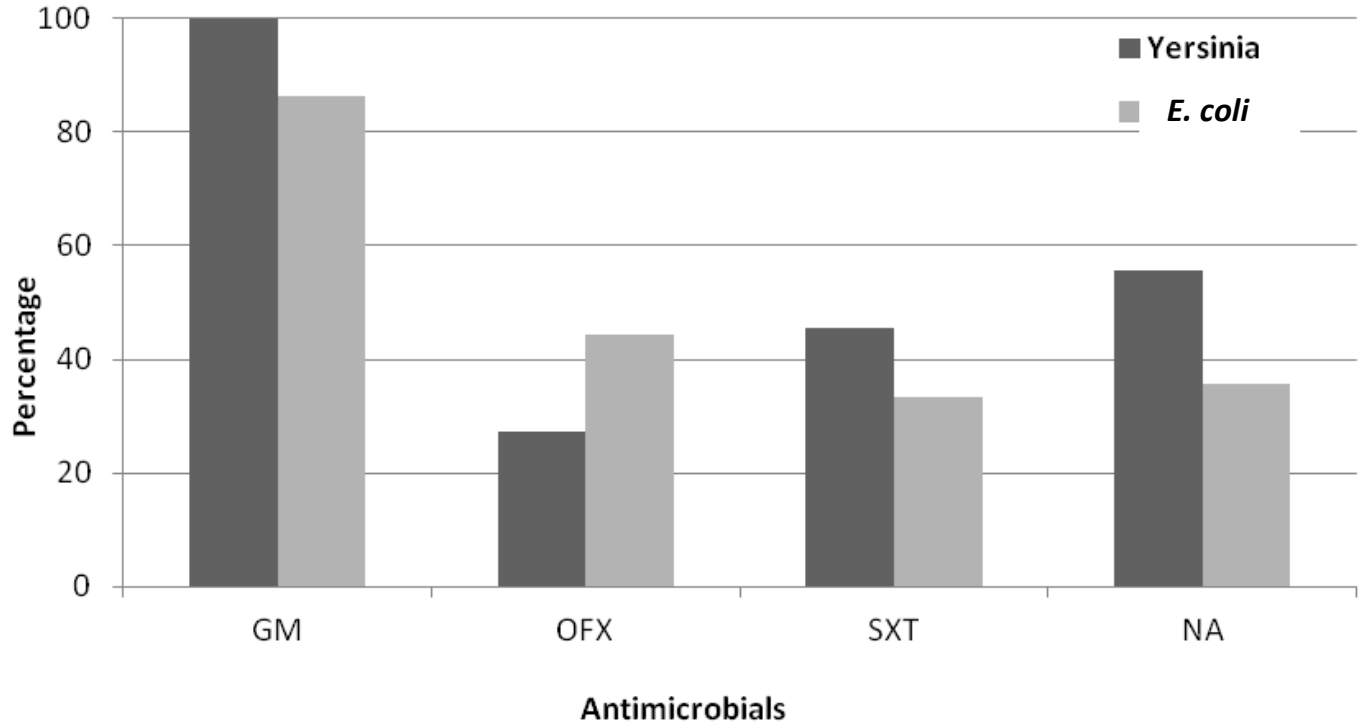
Figure 2. Percentages of *Yersinia* and *E. coli* isolates that showed an increase in their susceptibility to four different antimicrobials. GM, Gentamicin; OFX, ofloxacin; STX, trimethoprim-sulfamethoxazole; NA, nalidixic acid.
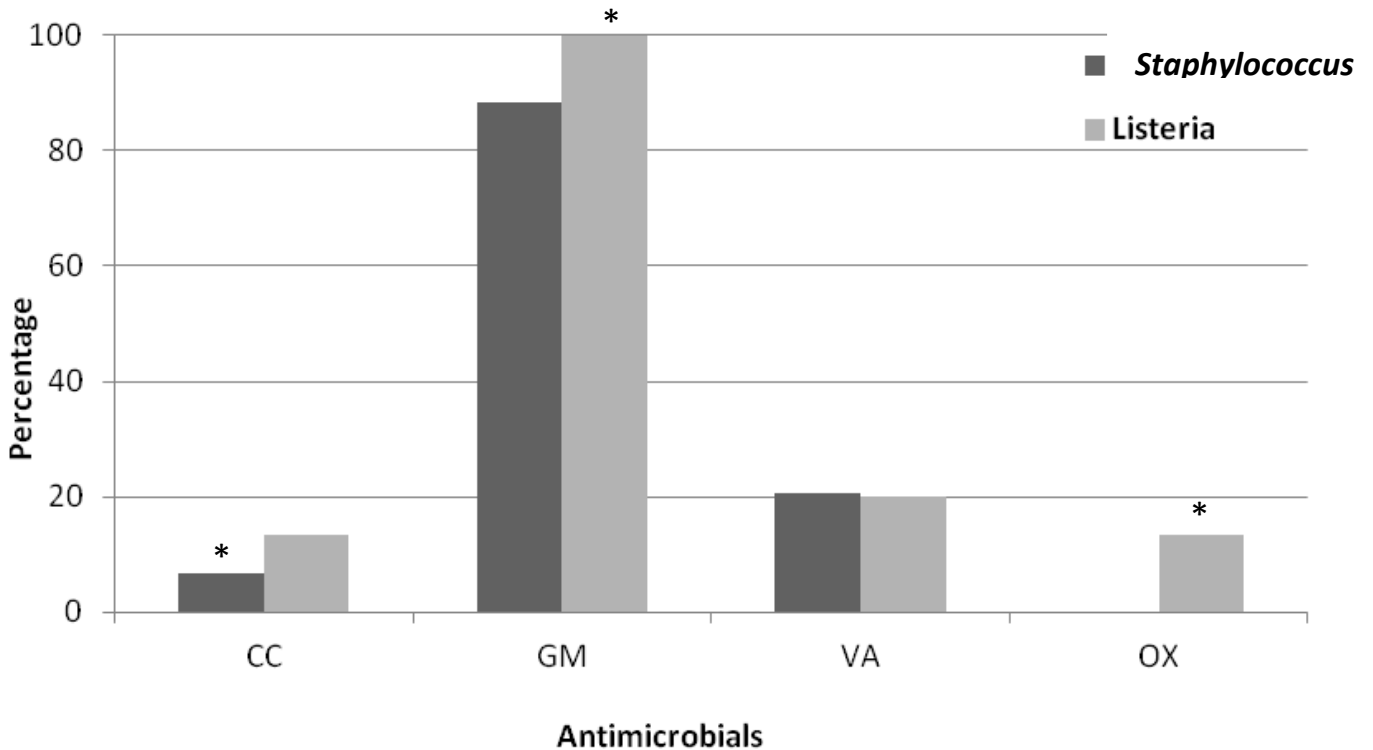

Figure 3. Percentages of *Staphylococcus* and *Listeria* isolates that showed an increase in their susceptibility to four different antimicrobials. CC,Clindamycin ; GM, gentamicin ; VA, vancomycin ; OX, oxacillin; *, statistically non-significant percentage of isolates with an increase in drug susceptibility.

Table 3. Antimicrobials affected by the presence of ascorbic acid.

Bacterial type	*E. coli*	*Yesinia*	*Listeria*	*Staphylococcus*	*Salmonella*
Antimicrobials affected by ascorbic acid	CTX	S CIP	C TE	None	NA CTX OfX

CTX, Cefotaxime; S, streptomycin; CIP, ciprofloxacin; TE, tetracycline; D, doxycycline; C, chloramphenicol; NA, nalidixic-acid; OFX, ofloxacin.

to cefotaxime when treated with this antioxidant. Antimicrobials affected by the ascorbic acid are listed in Table 3.

DISCUSSION

Driven by the known effect of antioxidants in suppressing mutations, we chose this combination of antioxidants and nutrients to be studied. In current times, antioxidants present in natural food products and others are tested for their efficacy in reversing antimicrobial resistance in bacteria. Wang et al. have reported that the antioxidants in ginger are able to reverse tetracycline resistance in *Acinetobacter baumanii* (Wang et al., 2010) while others reported the effect of caffeic acid in reducing gentamicin MIC by 4-fold in *Pseudomonas aeruginosa* (Sakharkar et al., 2009). These studies as well as others highlight the importance of antioxidant effects in this domain.

In this work, a combination of nutrients and antioxidants was employed which included the following compounds: L-lysine, ascorbic acid, ECGC, and EGCG, some of which have already been tested for their effect on the antimicrobial resistance pattern of multi-drug resistant bacteria. For instance, it has been demonstrated that certain concentrations of ascorbic acid and tomato lectin considerably ($p<0.05$) abrogated the multidrug resistance protein P-glycoprotein mediated resistance against mitomycin C (Galland et al., 2001). Furthermore, EGCG (a polyphenolic compound present in green tea) was found to reduce resistance of methicillin-resistant *S. aureus* (MRSA) isolates to all types of β-lactams, including benzylpenicillin, ampicillin, oxacillin, methicillin and cephalexin (Zhao et al., 2001). The results of this work illustrate that antioxidants do not only have individual effects on the level of antimicrobial resistance but also have a powerful effect when used in combination.

The results reported herein indicate that the effect of EF is not restricted to one family of antimicrobials and confirms different patterns based on bacterial types. Gram-negative bacteria showed similar patterns in their percentage increase in susceptibility when common drugs were used. However, Gram-positive bacteria reacted differently to the same antimicrobials in the presence of SNS. This could possibly reveal a difference in the mechanisms involved in drug susceptibility of

Gram-positive versus Gram-negative bacteria.

Some resistant isolates showed an increase in their susceptibility to various antimicrobials simultaneously, which could indicate that the ingredients of EF are affecting a mechanism that is involved in antimicrobial resistance to different drugs. It is worth noting that resistance to the antimicrobial gentamicin was the most affected. This aspect may be investigated further for a better understanding of the positive effect of SNS on the mechanism of resistance to this antimicrobial drug. When ascorbic acid was examined, few antimicrobial susceptibilities were affected which reflects the significance of the rest of the SNS ingredients. It is possible that each of the antimicrobials was affected by one of the SNS components and it is also likely as indicated previously that a synergic effect of a group of components on a multi-drug resistance mechanism is leading to this increase in drug-susceptibility. In all cases, further investigation is required to understand the real mechanisms behind these observations and to make the revival of some ineffective antimicrobials valuable again.

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