

In vitro Antibacterial Efficacy of Non-Antibiotic Growth Promoters in Poultry Industry

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Antibiotic growth promoters (AGPs) have been used for many years as supplements in various livestock diets, including those for poultry. However, the use of AGPs in feed was also associated with an increasing number of antibiotic-resistant bacteria in livestock. In this study, the *in vitro* antibacterial efficacies of eight commercially available non-AGPs suitable for use in poultry were investigated. Assessments included a combination of antibacterial activity assays and estimations of the minimal inhibitory and bactericidal concentrations along with scanning electron microscopy analysis. The results showed that the probiotic, CloStat[®] exerted a bacteriostatic effect against all tested bacteria, namely *Salmonella* Typhimurium, *Escherichia coli*, *Staphylococcus aureus*, and *Clostridium perfringens*, whereas Gallipro Tect[®] and Bacillus Blend[®] demonstrated bacteriostatic activity towards most of the pathogens tested. Other commercial non-AGPs, Sangrovit[®], Fysal[®], and Mix oil blend[®] showed a stronger or equal antibacterial activity compared to the positive control (AGP Maxus[®] G100) againsts all bacteria tested, except *C. perfringens*. Nor-Spice AB[®] and Varium[™] did not show any significant effect against the tested bacteria. Several of the tested AGP substitutes exhibited good antibacterial efficiency against pathogenic bacteria and thus may be good candidates for second-stage *in vivo* investigations into reducing pathogen colonization in broilers.

Key words: antibiotic growth promoters, antibacterial activity, phytobiotics, poultry, probiotics

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Introduction

Over the last three decades, the poultry industry has witnessed a tremendous growth in production rates and is now one of the fastest developing segments of agriculture (Dhama *et al.*, 2014). However, an increased risk of food spoilage-associated exposure to foodborne pathogens poses a crucial challenge. This is a significant cause of morbidity and mortality among humans and animals worldwide (Radaelli *et al.*, 2016). According to World Health Organization (WHO), at least 61% of all human pathogens are derived from animal sources (WHO, 2006). In order to ensure food safety, antibiotic growth promoters (AGPs) are regularly supplemented in livestock diets, including those for poultry (Butaye *et al.*, 2003). Nevertheless, ever since the

initial use of AGPs in livestock production, there has been an increasing number of cases where antibiotic-resistant bacteria were isolated from livestock (Butaye *et al.*, 2003; Johnson *et al.*, 2006). Moreover, many AGPs administered to animals are in the same class with those used to treat human infections. This fact has raised serious public health concerns in several countries, leading to either a complete ban or strict restrictions on the use of AGPs (Smith *et al.*, 2003; Castanon, 2007).

With the elimination of AGP usage in several regions of the world, efforts are directed toward investigating possible equally efficacious alternatives, in order to reduce the incidence of bacterial infections and/or prevent the emergence of antimicrobial resistance. Non-antibiotic feed substitutes (or additives), such as probiotics, prebiotics, organic acids, enzymes, and phytobiotics have been evaluated for this purpose. Several studies have reported the antimicrobial properties of potential AGP alternatives. For instance, probiotics were shown to be effective in controlling *Clostridium perfringens* and *Salmonella* spp. colonization in chicken by their ability to produce substances with antimicrobial activity, such as organic acids (Teo and Tan, 2006; Abudabos *et al.*, 2013). Several *in vitro* studies have also demonstrated the capacity of probiotics to antagonize pathogens such as

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Salmonella Typhimurium and *Escherichia coli* (Servin, 2004; Ridwan *et al.*, 2008; Tejero-Sarinena *et al.*, 2012).

Phytobiotics (herbal extracts and essential oils) were also shown to have antimicrobial activity against different species of foodborne pathogens (Shan *et al.*, 2007; Karangiya *et al.*, 2016). Oussalah *et al.* (2007) observed that several commercial oils strongly inhibited the activity of certain pathogenic bacteria, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli* O157:H7, and *S. Typhimurium*. Kotan *et al.* (2007) showed that various oxygenated monoterpenes, found at high concentrations in essential oils, had antibacterial effects against 63 bacterial strains, including *S. Enteritidis*, *S. Typhimurium*, and *P. aeruginosa*. Despite these results, the *in vitro* antimicrobial activities of some commercially available products remain underreported and are still only incompletely understood due to the variations in testing methods, bacterial strains analyzed, and culture media conditions.

The aim of this study was to determine the *in vitro* antibacterial activity, minimum inhibitory concentration (MIC), and the minimum bactericidal concentration (MBC) of some commercial non-AGPs, including probiotics (CloStat[®], Gallipro Tect[®], and Bacillus Blend[®]) and phytobiotics (Sangrovit[®], Nor-Spice AB[®], Varium[™], Fysal[®], and Mix oil blend[®]) against a panel of foodborne pathogens associated with poultry products.

Materials and Methods

Bacterial Strains and Culture Conditions

A panel of four foodborne bacteria associated with poultry products was tested, including *Salmonella enterica* subsp. Typhimurium (ATCC 14028), *S. aureus* (ATCC 25923 AT), *E. coli* (ATCC 35218 EC), and *C. perfringens* (ATCC 13124). These strains were maintained at -50°C in a 13% (w/w) glycerol broth, activated by plating twice on trypticase soy agar supplemented with 5% sheep blood (Oxoid, CM 129), and incubated under conditions suitable for each bacterium (*S. Typhimurium*, *S. aureus*, and *E. coli* were incubated at 37°C for 24 h; *C. perfringens* was incubated at 37°C for 48 h under anaerobic conditions).

Preparation and Activation of Test Probiotics and Pathogenic Organisms

Commercial probiotic strains were obtained in a freeze-dried powder form. Probiotics included CloStat[®], a unique strain of *Bacillus subtilis* PB6 (Kemin Industries Inc., Des Moines, IA, USA); Gallipro[®] Tect, an organism isolated from soil; *Bacillus licheniformis* DSM 17236 (Biochem Co. Lohne, Germany); and Bacillus Blend[®], a stabilized powder spore formulation available from Parchem (www.parchem.com) containing various strains of both *B. subtilis* and *B. licheniformis* with concentrations ranging from 1 billion to 100 billion Colony Forming Units (CFU)/g. Upon retrieval, all probiotics were cultured twice on Luria-Bertani (LB) agar (Fisher Scientific Ltd., Loughborough, Leicestershire, UK), and incubated under aerobic conditions at 37°C . All pathogenic strains were freshly activated on appropriate general media [LB agar for *S. Typhimurium*, *S. aureus*, and *E. coli*;

neomycin blood agar (Oxoid Columbia Agar Base; CM331) for *C. perfringens*], and incubated aerobically at 37°C , except for *C. perfringens*, which was incubated at 37°C under anaerobic conditions. After incubation, one colony from each plate (for both probiotics and pathogens) was transferred to individual tubes containing 10 mL of LB broth for all probiotic strains and pathogens except for *C. perfringens*, which was transferred to 10 mL of Brain Heart Infusion (BHI) broth. The cultured broths were incubated under the same conditions as described above. *E. coli*, *S. Typhimurium*, and *S. aureus* were incubated at 37°C on a rotary shaker.

Initial Antimicrobial Activity Screening by Agar Spot Test/overlay Assay

A colony overlay assay was used to demonstrate antibacterial activity according to the procedure described by Barbosa *et al.* (2005). Overnight cultures (10^7 – 10^9 CFU/mL) of each probiotic strain were inoculated onto LB agar plates as $5\ \mu\text{L}$ spots (3 spots per plate) and incubated at 37°C for 24 h. After the development of probiotic colonies, the plates were exposed to chloroform vapor for 30 min to kill bacteria under aseptic conditions. All Petri dish lids were replaced with new sterile ones. The plates were left to aerate for 20 min under aseptic conditions prior to overlaying with 10 mL of 0.7% (w/v) LB and BHI agar warmed to 45°C , which was inoculated earlier with $10\ \mu\text{L}$ (10^7 – 10^9 CFU/mL) of an overnight culture of the tested pathogen. As a control, LB agar plates without a probiotic spot were also overlaid with 0.7% (w/v) LB agar containing $10\ \mu\text{L}$ of the tested pathogenic strain. All plates were then incubated aerobically at 37°C , except the *C. perfringens* plates, which were incubated anaerobically in an anaerobic jar with an AnaeroGen gas pack and an indicator strip (Oxoid, Basingstoke, Hampshire, UK) at 37°C for 48 h. Inhibition zones surrounding the spots were assessed at one time point (24 h for all pathogens except *C. perfringens*, which was tested at 48 h). The diameters of the inhibition zones were measured in millimeters. Finally, swabs taken from the inhibition zones around inoculation spots were sub-cultured into a suitable general media for an initial determination of the probiotic effect (whether bactericidal or bacteriostatic). Each experiment was carried out twice in triplicate.

Preparation of Cell-free Culture Supernatants (CFCS) from Probiotic Cultures

All probiotic strains were suspended in sterile normal physiological buffered saline to achieve an optical density of 0.220 at 600 nm (OD_{600}). One mL of this suspension was then transferred into 9 mL of LB broth and incubated at 37°C in a shaking incubator. After 24, 48, 72, and 96 h of incubation, probiotic cultures were centrifuged at 4000 g for 10 min at 4°C . The supernatants were then harvested and transferred to new sterile tubes after filter-sterilization using $0.2\ \mu\text{m}$ membrane syringe filters (Research Products International, Elk Grove Village, IL, USA). The pH values of the CFCS were measured and checked for the absence of viable cells by plating on trypticase soy agar (Oxoid) supplemented with 5% sheep blood. CFCS were then stored at -50°C until further use.

Disc Diffusion Assay

A disc diffusion assay was used to demonstrate inhibition of pathogen growth. An overnight culture of the test organism was suspended in sterile, normal physiological saline, adjusted to 0.5 McFarland turbidity standard and inoculated onto Mueller Hinton agar (MHA). Under aseptic conditions, sterilized discs (Whatman, 6 mm diameter) were impregnated with 100 μ L of CFCS at different time periods (24 h, 48 h, 72 h, and 96 h) and placed on the agar surface (4 discs per plate). Negative controls were prepared using sterile LB broth. Plates were incubated under appropriate conditions for each bacterium for 24 h, and antimicrobial activity was visually observed as growth-free inhibition zones around the discs containing the CFCS solution. All tests were performed in triplicate. The diameter of the inhibition zone (DIZ) is expressed in millimeters.

Preparation of Non-AGP Test Products

Five commercially available non-AGPs suitable for use in poultry farming were included (Sangrovit[®], Nor-Spice AB[®], Varium[™], Fysal[®], and MixOil[®]). The Nor-Spice AB[®] Powder (Nor-Feed Sud, Beaucauzé, France) is a phytochemical premixture of feed additive developed from lemon extract (Mendel *et al.*, 2017). Sangrovit[®] (Phytobiotics, Eltville, Germany) is an herbal extract prepared from the plume poppy (*Macleaya cordata*). Varium[™] (Amlan International, Inc., Chicago, USA) is a non-antibiotic promoter and Fysal[®] (Selko[®] Feed Additives) is formulated from a blend of organic acids and their ammonium salts with a high buffer capacity (Abudabos and Al-Mufarrej, 2014). The oil blend MixOil[®] (Animal Wellness Products, NE, USA) is formulated from a mixture of seven plant extracts: eucalyptus (*Eucalyptus saligna*), garlic (*Allium sativum*), lemon (*Citrus limonium*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), sweet orange (*Citrus aurantium*), and thyme (*Thymus vulgaris*) (Rivaroli *et al.*, 2016). Maxus[®] G100 (Avilamycin Maxus, Elanco Animal Health, Madrid, Spain) was used as the reference antibiotic (Mathlouthi *et al.*, 2012). All products were obtained in powder form and dissolved in 100% aqueous dimethyl sulfoxide (DMSO) at a final concentration of 200 mg/mL. All extracts were sterilized using a 0.22 μ m disposable sterile syringe filter membrane and stored in dark, sterile vials at 4°C.

Antibacterial Activity Assays

Both agar-well and disc diffusion methods were used (Ferraro, 2001). An overnight culture of the test organism was suspended in sterile normal saline, its density was adjusted to 0.5 McFarland turbidity standard and then inoculated on MHA plates. For *C. perfringens*, an overnight culture in BHI broth was diluted from 10^{-1} up to 10^{-3} , and 2 mL of the diluted culture was placed on BHI agar to obtain semi-confluent growth, while the excess liquid was removed after 15–20 min. Wells were cut out from the agar under aseptic conditions with a sterile glass Pasteur pipette (6 mm in diameter), and 70–100 μ L of each test solution was loaded separately into the wells. For the disc diffusion method, sterilized discs (Whatman, 6 mm diameter) were impregnated with an equal amount of extract and placed on the agar

surface. Negative controls were prepared using a 100% DMSO solution. Maxus[®] G100 (Avilamycin Maxus, Elanco Animal Health, Madrid, Spain) was used as a positive control. All plates were incubated aerobically at 37°C for 24 h except for *C. perfringens*, which was incubated anaerobically at 37°C for 48 h. Antibacterial activity was assessed by measuring the DIZ and expressed in millimeters. All tests were performed in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

The MICs of Sangrovit[®], MixOil[®], Fysal[®], and Maxus[®] G100 were estimated for each tested pathogen. This test was performed in sterile 96-well microplates having U-shaped wells following the procedure described by the Clinical and Laboratory Standards Institute (CLSI, 2006). All test products were dissolved in DMSO to obtain 200 mg/mL concentration, followed by a serial two-fold dilution in Mueller Hinton (MH) broth. Around 100 μ L of the diluted samples was then transferred to the wells and mixed properly. All products were tested at concentrations ranging from 100 to 0.195 mg/mL. Maxus[®] G100 was used as a positive control. Negative controls comprised either sterile MH broth or sterile test product at the same concentrations used in the dilutions. Each well was inoculated with 20 μ L of the 0.5 McFarland standard bacterial suspension. The plates were covered and incubated at 37°C for 24 h. Detection of bacterial growth was confirmed by visual observation of turbidity in the U-bottom wells. For *C. perfringens*, MIC values were detected on MHA supplemented with 5% sheep blood.

Determination of Minimum Bactericidal Concentration (MBC)

The MBCs of Sangrovit[®], MixOil[®], Fysal[®], and Maxus[®] G100 were defined by inoculating from non-turbid wells that showed the absence of microbial growth on blood agar medium. The plates were incubated under conditions suitable for each test organism for 24 h at 37°C. The presence and absence of microbial growth indicated bacteriostatic and bactericidal activity, respectively.

Scanning Electron Microscopy

To observe the morphological changes in bacterial cells, selected samples were examined using a scanning electron microscope (SEM). Small agar pieces were cut out from the inhibition zone, fixed in 50% (v/v) glutaraldehyde overnight at 4°C, and washed four times with sodium phosphate buffer. The pieces were then fixed in 1% (w/v) osmium tetroxide (OsO₄) for 1 h at 4°C and washed four times with buffer. Samples were dehydrated in a graded series of alcohol. After dehydration with an automated critical point dryer, the specimens were mounted onto metallic sample holders and examined using the Quanta 250 SEM (Thermo Fisher Scientific, Oregon, USA).

Statistical Analysis

Data were analyzed using the Statistical Analysis System (SAS, 2009) software. General linear models (GLM) procedure was utilized to test the treatment effects. Means showing significant differences in the analysis of variance were tested using the PDIFF option. The overall level of statistical significance was set at $P < 0.05$.

Results

Antibacterial Activity of Tested Probiotics and other Non-AGPs

There was a broad variation in the antibacterial activity and a significant difference between the DIZ measurements of the tested products. Inhibition zones larger than 7 mm in diameter were considered positive. Sangrovit[®] showed the highest antibacterial activity against gram-positive bacteria, which was statistically equal to that of AGP Maxus[®] G100 (DIZ ~ 27 mm), followed by Fysal[®] and MixOil[®] (Table 1) ($P < 0.05$).

However, gram-negative bacteria showed maximum sensitivity toward Fysal[®] (DIZ ~ 18 mm), followed by MixOil[®], which showed a statistically similar effect to AGP Maxus[®] G100 with marginal differences. For both gram-positive and gram-negative bacteria, Nor-feed AB[®] showed a marginal increase (with no statistical significance) in DIZ compared to the negative control, while VarumTM showed no effect at all (Table 1).

In general, the disc diffusion technique showed a larger DIZ except when using the anaerobic bacterium *C. perfringens*, where the well diffusion method had a greater DIZ (Table 2). The initial scanning of selected probiotics by the spot agar overlay method indicated that all the tested probiotics (CloStat[®], Gallipro Tect[®], and Bacillus Blend[®]) showed some bacteriostatic effect. CloStat[®] consistently showed the highest efficacy across all tested bacteria, while both Gallipro Tect[®] and Bacillus Blend[®] showed statistically significant, but relatively low effects ($P < 0.05$) (Table 3).

Most of the tested bacteria were sensitive to several non-AGPs. Of the eight products tested, seven showed antibacterial activity against at least one or more of the strains. *S. Typhimurium*, *E. coli*, *S. aureus*, and *C. perfringens* showed varying sensitivities to various non-AGPs. *S. aureus* growth was strongly inhibited by Sangrovit[®] (DIZ=33 mm) compared to *S. Typhimurium* and *E. coli* (DIZ=14 mm and 19 mm, respectively) when the disc diffusion method was used, but there was no inhibition with the well diffusion method (DIZ=6 mm). Fysal[®] showed maximum activity against *S.*

Table 1. Comparing zones of inhibition by the disk and well diffusion methods against gram-positive and gram-negative bacteria

Factor	Tested product	Diameter of inhibition zone (mm) ¹						
		Sangrovit [®]	MixOil [®]	NC (DMSO) ²	PC (Maxus [®] G100AGP) ²	Fysal [®]	Nor-feed AB [®]	Varum TM
Bacteria	Gram-negative	12.21 ± 5.98 ^{B c}	15.00 ± 4.42 ^{B b}	6.00 ± 0.00 ^{A d}	13.50 ± 4.25 ^{B bc}	18.08 ± 2.55 ^{A a}	7.29 ± 3.27 ^{A d}	6.00 ± 0.00 ^{A d}
	Gram-positive	27.68 ± 5.85 ^{A a}	21.89 ± 6.70 ^{A b}	6.00 ± 0.00 ^{A c}	28.88 ± 5.47 ^{A a}	19.42 ± 3.59 ^{A b}	6.58 ± 1.58 ^{A c}	6.00 ± 0.00 ^{A c}
Method ³	D	22.94 ± 8.31 ^{A a}	17.53 ± 8.11 ^{A c}	6.00 ± 0.00 ^{A d}	22.77 ± 8.65 ^{A ab}	18.67 ± 2.28 ^{A bc}	7.20 ± 3.17 ^{A d}	6.00 ± 0.00 ^{A d}
	W	15.96 ± 10.5 ^{B a}	19.29 ± 3.94 ^{A a}	6.00 ± 0.00 ^{A b}	18.58 ± 9.65 ^{B a}	18.75 ± 4.04 ^{A a}	6.63 ± 1.64 ^{A b}	6.00 ± 0.00 ^{A b}

¹ Values are the mean diameter of the inhibitory zone (mm) ± standard deviation (SD) of three replicates, the diameter of the paper disk and well (6 mm) is included. A diameter of an inhibition zone (DIZ) of 6 mm is considered as no antimicrobial activity. NC: negative control, PC: positive control. ³D: disc diffusion method; W: well diffusion method. ^{AB} Means within a factor column followed by different letters are statistically significant at $P < 0.05$. ^{abc} Means within a row followed by different letters are statistically significant at $P < 0.05$.

Table 2. Effect of the tested products against selected bacteria using disc and well diffusion methods

Bacteria	Method	NC (DMSO)	PC (Maxus [®] G100 AGP)	Product				
				Sangrovit [®]	MixOil [®]	Fysal [®]	Nor feed AB [®]	Varum TM
<i>S. Typhimurium</i>	D	6.00 ± 0.00 ^c	15.00 ± 1.15 ^b	14.75 ± 2.50 ^b	18.00 ± 4.08 ^{ab}	19.13 ± 1.11 ^a	8.25 ± 4.50 ^c	6.00 ± 0.00 ^c
	W	6.00 ± 0.00 ^c	6.00 ± 0.00 ^c	6.00 ± 0.00 ^c	15.66 ± 0.57 ^a	15.00 ± 0.00 ^b	6.00 ± 0.00 ^c	6.00 ± 0.00 ^c
<i>E. coli</i>	D	6.00 ± 0.00 ^c	17.00 ± 0.00 ^a	19.00 ± 1.41 ^a	10.75 ± 5.50 ^b	20.75 ± 1.19 ^a	8.25 ± 4.50 ^{bc}	6.00 ± 0.00 ^c
	W	6.00 ± 0.00 ^c	14.33 ± 1.15 ^b	6.00 ± 0.00 ^c	16.00 ± 0.00 ^a	16.00 ± 1.00 ^a	6.00 ± 0.00 ^c	6.00 ± 0.00 ^c
<i>S. aureus</i>	D	6.00 ± 0.00 ^c	29.5 ± 8.95 ^a	33.40 ± 5.67 ^a	28.00 ± 1.82 ^a	18.13 ± 2.84 ^b	6.00 ± 0.00 ^c	6.00 ± 0.00 ^c
	W	6.00 ± 0.00 ^d	23.66 ± 0.58 ^b	27.67 ± 1.52 ^a	24.67 ± 1.52 ^b	19.67 ± 2.08 ^c	6.67 ± 1.15 ^d	6.00 ± 0.00 ^d
<i>C. perfringens</i>	D	6.00 ± 0.00 ^c	31.83 ± 0.28 ^a	21.66 ± 0.57 ^b	12.00 ± 5.29 ^d	16.00 ± 0.00 ^c	6.00 ± 0.00 ^c	6.00 ± 0.00 ^c
	W	6.00 ± 0.00 ^d	30.33 ± 1.04 ^a	24.16 ± 1.04 ^b	20.83 ± 0.28 ^c	24.33 ± 2.02 ^b	7.83 ± 3.17 ^d	6.00 ± 0.00 ^d

Values are mean diameter of the inhibitory zone (mm) ± SD of triplicates, the diameters of paper disk and well (6 mm) are included. * A DIZ of 6 mm is considered as no antimicrobial activity. ^{abc} Means within a row followed by different letters are statistically significant at $P < 0.05$. D, disc diffusion method; W, well diffusion method; NC, negative control; PC, positive control.

Table 3. Effect of tested probiotic against bacteria using spot agar overlay method and CFSC test

Bacteria	Spot Agar Overlay Method		
	CloStat [®]	Gallipro Tect [®]	Bacillus Blend [®]
<i>S. Typhimurium</i>	24.00±0.00 ^{A a}	23.83±0.76 ^{A a}	0.00±0.00 ^{B b}
<i>E. coli</i>	26.00±1.42 ^{A a}	24.00±1.41 ^{A ab}	19.00±1.41 ^{A b}
<i>S. aureus</i>	23.75±1.06 ^{A a}	21.75±1.06 ^{A a}	15.50±0.71 ^{A b}
<i>C. perfringens</i>	24.33±1.16 ^{A a}	0.00±0.00 ^{B b}	9.83±6.64 ^{B b}

¹The different degrees of growth inhibition are expressed in mm as the mean of three replicates±SD. ^{AB}Means within probiotic columns followed by different letters are statistically significant at $P<0.05$. ^{abc}Means within a row followed by different letters are statistically significant at $P<0.05$. *CFSC: cell-free culture supernatants.

Table 4. MIC and MBC values of selected feed additives against foodborne bacteria

Tested product	Bacteria [MIC ¹ /MBC ² (mg/mL)]							
	<i>S. Typhimurium</i>		<i>E. coli</i>		<i>S. aureus</i>		<i>C. perfringens</i>	
	MIC ¹	MBC ²	MIC	MBC	MIC	MBC	MIC	MBC
Maxus[®] G100	12.5	100	0.78	25.0	1.50	3.12	>3.12	—
Sangrovit[®]	25.0	100	12.5	25.0	12.5	25.0	25.0	—
MixOil[®]	6.25	12.5	6.25	6.25	6.25	12.5	12.5	—
Fysal[®]	3.12	6.25	6.25	12.5	3.12	6.25	25	—

¹MIC, Minimum inhibitory concentration. ²MBC, Minimum bactericidal concentration.

Typhimurium (DIZ=19 mm), and the inhibition was statistically higher than that by AGP Maxus[®] G100 ($P<0.05$). Sangrovit[®] showed moderate inhibition of *E. coli*, which was comparable to that by AGP Maxus[®] G100. The organic acid Fysal[®], the phytobiotic- Sangrovit[®], and AGP Maxus[®] G100 all showed statistically similar results, followed by the reduced effects of the essential oil blend MixOil[®]. In the case of the anaerobic bacterium *C. perfringens*, none of the tested substitutes had an inhibitory activity greater than or equivalent to AGP Maxus[®] G100 (DIZ=31 mm). Overall, gram-positive bacteria demonstrated a higher sensitivity to the tested products than gram-negative bacteria. There was no growth inhibition observed using the solvent control (DMSO) (Table 2).

All the tested probiotics showed an inhibition zone against most of the pathogens. *S. Typhimurium* was susceptible to both probiotics, CloStat[®] and Gallipro Tect[®], but not to Bacillus Blend[®]. Comparable results were reported for *S. aureus*, with moderate effects observed for Bacillus Blend[®]. *E. coli* showed a zone of inhibition and responded to all three probiotics, CloStat[®], Gallipro Tect[®], and Bacillus Blend[®]. *C. perfringens* displayed a statistically significant susceptibility toward CloStat[®] followed by marginal inhibition with Bacillus Blend[®], but no effect was observed with Gallipro Tect[®]. Inhibition zone sizes ranged from 10 to 26 mm (Table 3). Using CFCS assay, marginal changes in pH levels were observed, and no growth inhibition was observed across all tested bacteria except for CloStat[®] vs. *C. perfringens* at the 96 h time point (DIZ=15.5 mm).

MICs and MBCs

Table 4 shows the MIC and MBC values of four selected products based on previous observations against reference bacterial strains. MIC and MBC values ranged from 1.50 to 25.0 mg/mL and from 3.12 to 100 mg/mL, respectively. The lowest MIC was observed with AGP Maxus[®] G100 (0.78 mg/mL). However, AGP Maxus[®] G100 yielded the highest MBC values among all tested products (100 mg/mL). In most cases, MIC and MBC results varied between test organisms except for MixOil[®] vs. *E. coli* where the MIC was equivalent to the MBC (6.25 mg/mL). MixOil[®] exhibited similar levels of bacteriostatic activity across all pathogens. Fysal[®] showed a significant antimicrobial effect against gram-negative *S. Typhimurium* and gram-positive *S. aureus* (MIC=3.12 mg/mL). Sangrovit[®] was the least potent product, as evidenced by higher MIC and MBC values ranging from 12.5 to 100 mg/mL.

SEM Observations

Bacterial cells treated with 200 mg/mL of different selected products (Sangrovit[®], Fysal[®], MixOil[®] blend, and AGP Maxus[®] G100) underwent substantial morphological changes compared to untreated cells (Fig. 1-5.). Bacterial cells appeared to have shrunk with no cellular content and dark, damaged spots were observed on cell walls and the remains were flaccid. Even though the examined samples were not quantitatively prepared, the number of cells in control samples observed under SEM at low magnification was clearly higher compared to treated samples.

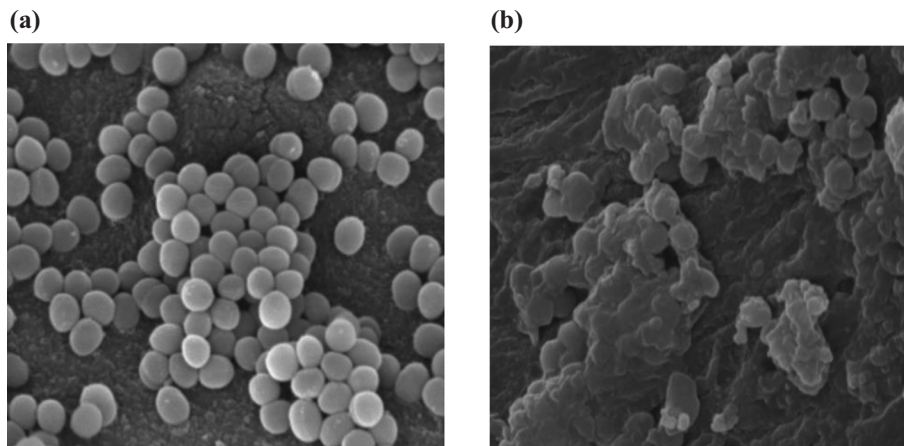


Fig. 1. Scanning electron microscopy (SEM) images showing morphological changes and cellular damage in *S. aureus* cells upon treatment with 200 mg/mL of the phytobiotic, Sangrovit[®]. (a) Naïve bacterial cells. (b) Treated bacterial cells. Enlargement: 24K.

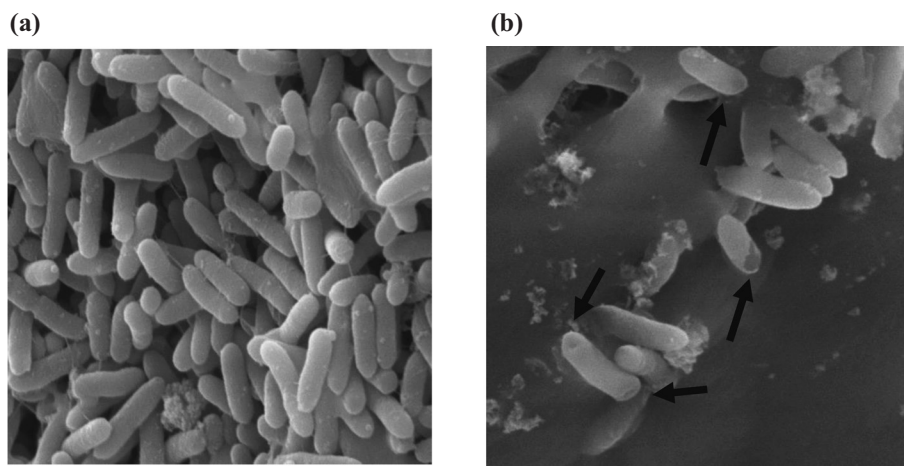


Fig. 2. SEM images of *S. Typhimurium* cells treated with 200 mg/mL of Fysal[®]. (a) Naïve bacterial cells. (b) Treated bacterial cells showing morphological changes and cellular damage (arrows). Enlargement: 24K.

Discussion

This study was conducted to evaluate the antibacterial activity of several commercially available non-AGP growth promoters for poultry production. Our findings indicated that compared to AGP Maxus[®] G100, Sangrovit[®] and Mix Oil[®] showed stronger/similar and weaker effects against gram-positive bacteria, respectively. Phytobiotics were previously proposed to show growth-promoting effects and found to be good substitutes for AGPs in aquaculture (Direkbusarakom, 2011; El-Sayed *et al.*, 2014). Herbal extracts and essential oils of various plants such as *Eucalyptus caryophyllata*, *Origanum vulgare*, and *Thymus vulgaris* have been shown to inhibit the growth of *E. coli*, *S. aureus*, *L.*

monocytogenes, and *S. Typhimurium* (Kalemba and Kunicka, 2003, Lopez *et al.*, 2005; Mith *et al.*, 2014). Sanguinarine, a quaternary benzo[c] phenanthridine alkaloid and one of the main active components of Sangrovit[®] (Dvorak and Simanek, 2007), has been reported to have anti-inflammatory (Dvořák *et al.*, 2006; Niu *et al.*, 2012) and antimicrobial properties (Newton *et al.*, 2002; Kosina *et al.*, 2010). Sangrovit[®] supplementation was previously shown to have a positive impact on the growth performance of animals, such as poultry (Vieira *et al.*, 2008). Although the mechanisms of action of phytobiotics remain unclear, some bioactive components, such as thymol, carvacrol, eugenol, and cinnamic aldehyde may be associated with antimicrobial properties (Shan *et al.*, 2007). Furthermore, the hydrophobicity is a key factor ena-

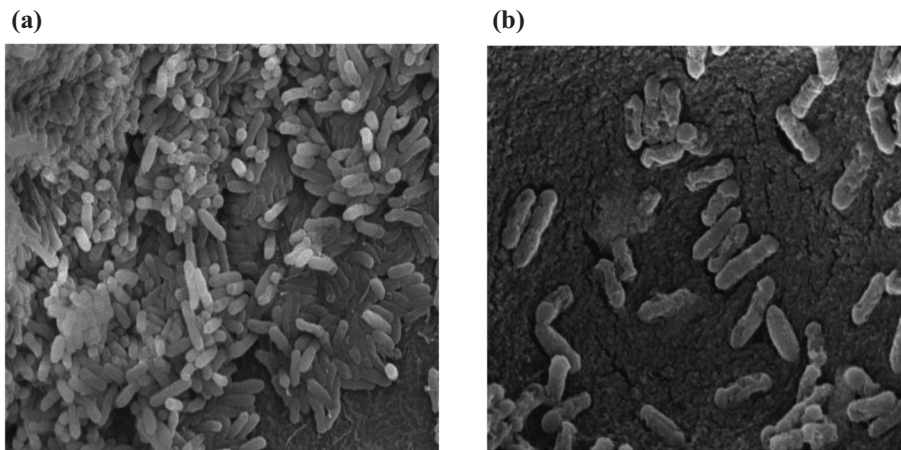


Fig. 3. SEM images of *E. coli* cells treated with 200 mg/mL of MixOil[®] blend. (a) Untreated bacterial cells, enlargement: 12K (b) Treated bacterial cells showing cell abnormality and cellular damage, enlargement: 24K.

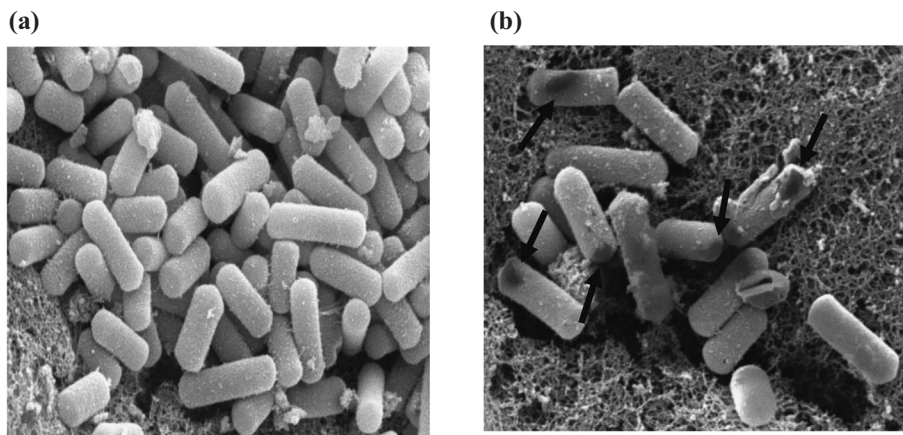


Fig. 4. SEM images showing morphological changes of *C. perfringens* cells upon treatment with 200 mg/mL of commercial AGP Maxus[®] G100. (a) Untreated bacterial cells. (b) Treated bacterial cell (arrows). Enlargement: 24K.

bling essential oils to penetrate the phospholipids present in membranes of bacterial cells and mitochondria. This leads to destruction of the cell structure and promotes extensive leakage of critical cell components and ions, eventually causing cell death (Prabuseenivasan *et al.*, 2006).

In contrast, gram-negative bacteria showed maximum sensitivity to Fysal[®]. Organic acids have recently gained attention as potential alternatives to AGPs, especially since the European Union approved their use in poultry production (Adil *et al.*, 2010). Several studies have demonstrated great antimicrobial potential of organic acids against a broad range of intestinal pathogens. In addition, they have been linked with lowering the pH levels of the gastrointestinal tract (GIT), improving nutrient metabolism, and enhancing poultry performance (Boling *et al.*, 2000; Huyghebaert *et al.*,

2011). Organic acids are hypothesized to interfere with electron transport in the cytoplasmic membrane, leading to a decline in ATP production and altered pH and electrical gradients over the cell membrane (Ricke, 2003). Hence, the variation in microbial resistance to organic acids may be due to the inherent capacity of specific pathogens to maintain their internal pH.

The MIC values for most of the tested products in this study were lower than their MBC values, implying that these AGP substitutes were bacteriostatic while being bactericidal at higher concentrations (Khan *et al.*, 2009). However, AGP Maxus[®] G100, our reference antibiotic, had a broad therapeutic index with a MBC several folds higher than the MIC.

Probiotics such as CloStat[®], Gallipro Tect[®], and Bacillus Blend[®] showed inhibitory effects against *S. Typhimurium*, *E.*

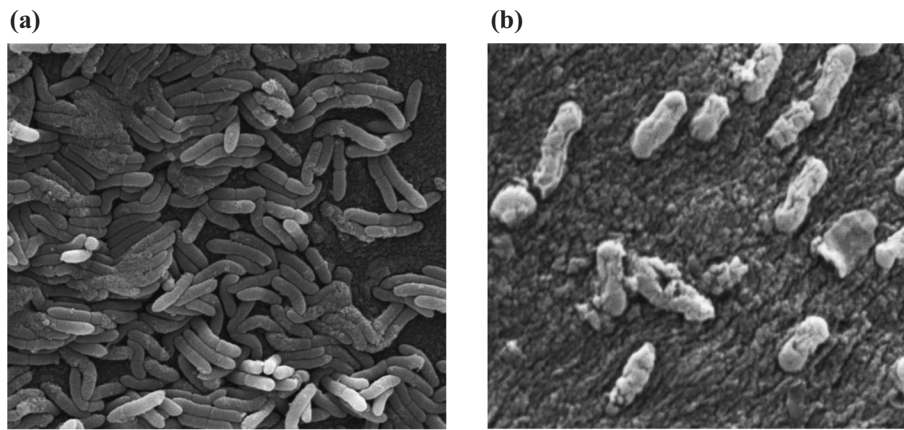


Fig. 5. SEM images of *S. Typhimurium* cells treated with 200 mg/mL of MixOil® blend. (a) Untreated bacterial cells, enlargement: 12K (b) Treated bacterial cells showing cell abnormality and cellular damage, enlargement: 24K.

coli, *S. aureus*, and *C. perfringens*. We ruled out the possibility that this inhibitory effect was due to competition for nutrients between probiotic and pathogenic bacteria as we had sterilized probiotic agar plates via chloroform vaporization before overlaying with the pathogen. Moreover, the CFCS supernatants were filter sterilized. A previous study on lactic acid-producing bacteria reported a strong correlation between the DIZ and low pH; the antagonistic effects were reduced when the tested supernatants were adjusted to pH 7 (Tejero-Sarinena *et al.*, 2012). In our study, the pH levels of broth media were marginally influenced by the tested probiotics. However, we were still able to observe the inhibitory effect of CFCS from the probiotic CloStat®, indicating that other mechanisms may be involved. For instance, Tejero-Sarinena *et al.* (2012) suggested that the antibacterial properties of the tested probiotics could be due to their ability to produce organic acids from glucose fermentation.

Our results indicated that gram-positive bacteria were more sensitive to the tested AGP substitutes than gram-negative bacteria. These observations are consistent with those of previous studies using various AGPs alternatives (Lopez *et al.*, 2005; Shan *et al.*, 2007). The variation in the responses of gram-positive and gram-negative bacteria may be attributed to the significant differences in their cell wall structures. Gram-negative bacteria may have a higher tolerance to AGPs due to their hydrophilic outer membrane, which prevents penetration by numerous hydrophobic molecules (Russell, 1998; Cox and Markham, 2007). Despite this, gram-negative *S. Typhimurium* and *E. coli* remained significantly sensitive to the tested AGP substitutes, as evidenced by the morphological alterations observed under SEM (Fig. 1-5.). Treatment with the tested products apparently caused bacterial cells to collapse either by losing their contents or by causing irreparable cell wall damage. Burt and Reinders (2003) and Lambert *et al.* (2001) obtained similar results upon testing the antibacterial effects of

oregano, carvacrol, and thyme essential oil.

The bacteria *S. Typhimurium*, *E. coli*, and *S. aureus* had larger DIZ when using the disc diffusion technique, while the anaerobic bacteria *C. perfringens* showed a larger DIZ when the well diffusion technique was used. This may be attributed to humidity levels in the anaerobic jars limiting the evaporation of the tested product. Further investigation is required to confirm this hypothesis.

Taken together, our observations indicate that AGP substitutes had antibacterial activities against pathogenic bacteria associated with consumable poultry products and may be good candidates for second-stage *in vivo* investigations into the development of approaches for reducing pathogen colonization in broilers. However, we only tested their efficacy against a limited number of bacterial genera. SEM images hinted at a potential mode of action of the tested non-AGPs, but this aspect needs further evaluation.

Overall, various products, such as probiotics, phytobiotics, organic acids, and other available AGP substitutes exhibit promising antibacterial activities against some pathogenic bacteria associated with poultry. However, it must be borne in mind that *in vitro* results are not enough to make a definitive judgment regarding whether a product will give the desired outcomes in animal models. Additional *in vivo* research is, therefore, needed to evaluate the efficacy of these products in improving poultry productivity and decreasing pathogen colonization in broilers.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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