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IN VITRO SCREENING OF ANTIMICROBIALS ON BACTERIA CULTURED FROM MARKET-AGED BROILER'S DIGESTA AND *SALMONELLA ENTERICA* SEROVAR *HEIDELBERG* PURE CULTURE

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ABSTRACT: *Easily digested diets based on maltodextrin have been evaluated as a diet used in clearance of digestive tracts in poultry prior to shipping for slaughter. There is potential to include antimicrobial in this type of diet in effort to reduce the bacterial load in the gut and potentially improve food safety. In order to establish an effective inclusion level for bacteria inhibition in broiler digesta, a lysozyme product and allicin from garlic were tested in vitro. These ingredients were evaluated in a broth solution containing bacteria cultured from digesta from broilers fed maltodextrin or a Salmonella enterica serovar Heidelberg pure culture. Lysozyme was tested in two trials and allicin in the second trial. The antimicrobials were added at levels of 0, 50, 100 and 150 mg in 9 ml of buffered peptone water. Samples were incubated for 6 h at 35°C and plated on 3M petrifilm aerobic plates. Aerobic bacterial numbers were transformed into* log_{10} *values and analyzed using Proc Mixed of SAS. Lysozyme reduced bacteria numbers in both the Salmonella pure culture and the culture generated from chicken digesta. Allicin had no effect on aerobic bacteria numbers for all levels evaluated but lysozyme reduced numbers by 1.65, 4.15 and 3.91* log_{10} *cfu for 50, 100 and 150 mg respectively. It is concluded that lysozyme will be effective in controlling bacteria in the digestive tract of market-aged broilers when delivered through a maltodextrin diet than allicin.*

KEY WORDS: aerobic bacteria; allicin, lysozyme; maltodextrin*; Salmonella enterica* serovar *Heidelberg*.

INTRODUCTION

Alternative antimicrobials are evaluated in poultry research to improve intestinal health, growth performance, and ensure that the health and welfare of the bird is maintained by controlling bacteria in the GIT (Buchanan et al., 2008; Roberts et al., 2015). However, the continual use of

antibiotics has increased the prevalence of antibiotic-resistant bacteria (Matthew et al., 2007). This has led to disease control problems in both humans and animals (Thai et al., 2012). Government regulation in Europe and consumer pressure in North America has led to an increased interest in producing antibiotic-free poultry meat (Mehdi et al., 2018; Marshall and Levy, 2011). Often the first stage in evaluation of alternatives to antimicrobials is to determine the effective dosage. The Canadian Council on Animal Care requires that the 3R (reduce, refine and replace animal use) rules be strictly observed during animal experimentation (CCAC, 2009). A strategy to comply with the 3R rules of animal use is to initially conduct *in vitro* experiments (Kulpa-Eddy et al., 2011).
In vitro methods of testing antimicrobials have been used on numerous occasions to determine inhibitory effects of these substances on bacterial isolates (Citron et al., 2003, Zhang et al., 2006). In vitro methods of evaluation are typically less expensive compared to in vivo methods and often less time is required (Zips et al., 2005). The microbroth dilution assay in which the wells of a microtitre plate are used as the container of the test material and bacteria, is an example of an in vitro method used to evaluate the minimal inhibitory concentrations of antimicrobials (Anil and Samaranayake, 2002, Hall et al., 2011).

Lysozyme from egg white was tested in this way against three clinical isolates of *Clostridium perfringens* type A that were associated with severe necrotic enteritis in chickens (Zhang et al., 2006). They found the method was effective for determining dosage required for *Clostridium perfringens* inhibition. Research regarding the potential use of lysozyme as an alternative to antimicrobials have been performed on monogastric animals. Gong et al. (2016) reported that dietary lysozyme reduced *E, coli* in the ileum of poultry birds. Li et al. (2018) suggested that fumigating broiler hatching eggs with lysozyme product (Inovapure) reduced *E. coli* on the eggshells. Addition of lysozyme to a maltodextrin-based feed was effective in altering bacteria population in ileal contents of market-aged broilers (Asante et al., 2019). Lysozyme is a suitable alternative to growth-promoting subtherapeutic antibiotic use in swine feed. Feeding dietary lysozyme to swine improved gastrointestinal health, and alter the gastrointestinal bacteria ecology (Oliver and Wells, 2015).

Allicin (diallylthiosulfinate) is a potential antimicrobial substance, produced by garlic tissues, which was effective against pathogens (Reiter et al., 2019). A study was conducted to determine the potential of garlic extract as an antimicrobial agent. The garlic extract was effective against *Pseudomonas aeruginosa and Staphylococcus aureu* bacteria in vitro (Boboye and Alli, 2008). Garlic extract can be used as meat preservative as it reduced Salmonella and *E. coli* present on skinless chicken legs during storage (Sarma, 2004). Salmonella is an important source of foodborne illness (Nisar et al., 2017) and the highest incidence is found in chicken meat (Antunes et al., 2016; Nisar et al., 2017).

To facilitate determination of the optimal inclusion level of the two antimicrobials in the maltodextrin feed supplement, an in vitro evaluation was performed in the current research. This was performed to select the antimicrobial that will be effective in controlling bacteria in the gastrointestinal tract of market-aged broilers when delivered through the maltodextrin diet. Maltodextrin is a highly digestible feed supplied to broilers during feed withdrawal period. Maltodextrin feed helps to reduce digesta content in the gastrointestinal tract of poultry in

preparation for shipping (Asante et al.,2019). When the gastrointestinal tract is full, the meat can become contaminated with pathogenic bacteria through digesta leakage during processing (Asante et al., 2019). Bacteria cultured from the digesta of broilers fed maltodextrin and/or a pure culture of Salmonella were subjected to increasing levels of allicin and a lysozyme-based product. This study evaluated the bactericidal effect of allicin and lysozyme at increasing levels on bacteria generated from digesta from broilers fed maltodextrin or a *Salmonella enterica* serovar *Heidelberg* pure culture.

MATERIALS AND METHODS

The maltodextrin supplement consisted of 78.5% corn maltodextrin (Grain Processing Corporation, Muscatine, IA, U.S.A) with a dextrose equivalent of 11.0, 19.1% broiler finisher diet (Table 3.1), 0.4% NaCl and 2.0% water. To ensure pelleting, water was added to the finisher feed component before mixing with maltodextrin.

Market-aged male broilers (Ross 508×Ross 508) were supplied the maltodextrin diet for 9h as described by Asante et al. (2019). Birds were euthanized by cervical dislocation and the entire jejunum and the ileum was aseptically harvested and digesta was stored at -80°C. The activities with birds subjected to the pre-shipping preparation feed treatments for the study were approved by the Local Animal Care and Use Committee following the guidelines of the Canadian Council of Animal Care (CCAC, 2009). In preparation for this study, digesta was thawed for 3 h at room temperature. A loop full of digesta was removed and placed in 100 ml Luria Berthani (LB) broth (Lot No. 0082283). Similarly, a loop full of *Salmonella enterica* serovar *Heidelberg* (Strain No. S-8; CFIA, Canada) pure culture was inoculated in 5 ml of LB broth. The bacteria culture generated from maltodextrin fed market-aged broiler digesta and *Salmonella enterica* serovar *Heidelberg* pure culture were incubated at 35±1°C overnight. After incubation, both cultures were diluted to McFarland's standard of 1.5×10^8 cells per ml, determined by dilution to an optical density of 0.08-0.1. These cultures were further diluted so that 100 µl delivered approximately 10,000 cells.

Preparation and Sampling of the Antimicrobials

A commercially available lysozyme product (Inovapure), extracted from hen egg white (Neova Technologies Inc., Abbotsford, BC, Canada) and mixed with EDTA at a ratio of 1:4 (lysozyme: EDTA) was one of the tested antimicrobials. This product has an enzymatic activity of 24,000 units/mg. Allicin from garlic was the other tested product. The stock solutions used for this experiment were prepared by adding 1 g of lysozyme product or allicin to 20 ml of buffered peptone water (BPW) and stomached until completely dissolved. The solution was used to deliver 50, 100 and 150 mg of allicin or lysozyme product. As positive control, 0.105 g of tetracycline (Sigma Aldrich, St. Louis, MO) was dissolved in 13 ml of BPW for a solution of 80 $mg.ml^{-1}$. The Salmonella strain used in the culture preparation was resistant to this level of tetracycline. There were 10 treatments in the first experiment including 80 $mg.ml^{-1}$ tetracycline), 0, 50, 100 and 150 mg of lysozyme for *Salmonella enterica* serovar *Heidelberg* or culture from maltodextrin fed broiler digesta each one replicated 6 times. These treatments were added to 9 ml of BPW and 100 µl of the prepared culture was added to each of the respective treatments.

In the second trial, 0, 50, 100 and 150 mg of allicin or lysozyme were evaluated in a similar fashion. Since the antimicrobials were delivered into 9 ml of BPW in the form of liquid, the total volume for the 150 mg treatment tube was 12 ml. The volume for all the other tubes were brought up to 12 ml with additional BPW. The first three replicates of the treatments represented block 1 and the last three replicates represented block 2, with 30 m between preparation of the two blocks. All samples were incubated aerobically at 35±1°C for 6 h. Following incubation, 1 ml from each sample was diluted 5 times and dilutions 2 to 5 were plated on aerobic 3M petrifilm plates in duplicate. The plates were incubated for 24 h at 35±1°C. All red colonies were enumerated as aerobic bacteria according to the AOAC 940.37, 2005, method #940.37.

Experimental Design

The results from these trials were analyzed as a completely randomized experiment as a 2×5 and 2×4 for trials 1 and 2 respectively for cultures and levels of antimicrobials. All bacterial counts were transformed into $\log_{10}$ values and analyzed using Proc Mixed of SAS (SAS Institute Inc. 2003). The model for the analysis was $\gamma_{ijk=}\mu + \tau_i + \beta_j + \tau\beta_{ij} + \varepsilon_{ijk}$. In trial 1, $\gamma_{ijk}$was for the aerobic bacteria numbers, μ was for the overall mean, τ_i was for the treatment effect of the cultures (i=1,2), β_j was for the treatment effect of the antimicrobials levels (j=1,2,3,4,5), $\tau\beta_{ij}$was for the interaction effect of the treatments (digesta or Salmonella cultures) and the antimicrobials levels. ε_{ijk} was for the error term. All too numerous to count (TNTC) plates were replaced with 168 at the 5th dilution which were equivalent to 7.23 $\log_{10}$ cfu before the data were analyzed.

In trial 2, $\gamma_{ijk}$was for the aerobic bacteria numbers,μ was for the overall mean, τ_i for the treatment effect of the antimicrobials (i=1,2), β_j was for the treatment effect of the antimicrobials levels (j=1,2,3,4), $\tau\beta_{ij}$ was for the interaction effect of the treatments (bacteria culture from feed containing maltodextrin fed to market-aged broilers and Salmonella pure culture) and the antimicrobial levels, $\varepsilon_{ijk}$was for the error term. All Too Numerous To Count (TNTC) plates were replaced with the preferable upper count limit of aerobic bacteria which was 250 at the highest dilution which was 5. This was transformed to $\log_{10}$cfu value of 7.40. Significant differences among treatments were separated using the mean separation test of Tukey-Kramer ($P \leq 0.05$).

RESULTS AND DISCUSSION

Results from bacteria enumeration of the *Salmonella enterica* serovar *Heidelberg* pure culture and the maltodextrin digesta culture, treatments without antimicrobial were not different ($P>0.05$). Tetracycline was added to prevent the growth of bacteria other than the Salmonella *enterica* serovar *Heidelberg* however, the tetracycline treatment of the maltodextrin digesta culture resulted in the same bacterial count as the pure culture (Table 1). Market-aged broilers have a diverse microbial community in their GIT (Thompson et al., 2008). Similarly, Shang et al. (2018) reported that chicken harbor a very complex and diverse microbiota in their digestive tract. The diversity of microbiota in the gastrointestinal tract is influenced by the age of the birds, the part of the digestive tract and diet supplied to the chicken (Shang et al., 2018). In this research, the digesta was harvested from the entire jejunum and ileum of market-aged broilers. For digesta culture

samples treated with lysozyme at 50, 100 and 150 mg/9ml, the aerobic bacteria numbers decreased with increasing lysozyme levels ($P<0.05$). Lysozyme reduced *Salmonella enterica* serovar *Heidelberg* at 50, 100 and 150 mg per 9 ml ($P<0.05$). *Salmonella enterica serovar Heidelberg* was susceptible to lysozyme in vitro. In a recent in vivo research by Asante et al. (2019), the addition of lysozyme in a feed containing maltodextrin for market aged-broilers reduced bacilli in the ileal content of market-aged broilers. The presence of lysozyme in the maltodextrin feed did not affect the entire population of bacteria when analyzed using Next Generation sequencing. Lysozyme reduced *Clostridium perfringens*, *Escherichia coli* and Lactobacillus in the ileum of chickens in a study by Liu et al. (2010). Lysozyme functions as an antimicrobial agent by cleaving the peptidoglycan component of bacterial cell walls, which causes cell death (Oliver and Wells, 2015). Ellison and Giehl (1991) reported using lysozyme at the level of 0.05 $mg.ml^{-1}$ had a bactericidal effect on *V. cholerae*, *S. typhimurium* and *E. coli* in 1% bactopeptone.

Allicin had no effect on bacterial growth ($P>0.05$). All the allicin treatments were overgrown and assigned the upper limit for bacterial numbers (Table 2). Cellini et al. (1996) observed 90% inhibition of *Helicobacter pylori* isolated from antral mucosal biopsies of patients at a garlic extract concentration of 5 $mg.ml^{-1}$. Abiy and Berhe (2016) reported that the crude extract of allicin had antimicrobial effect on *Staphylococcus aureus* and *Escherichia coli*. Differences in garlic products, concentrations used, and bacteria strains present may account for the variation in the results. In this research, the powdered form of garlic was used which is different from crude extracts of garlic used by Abiy and Berhe (2016). The result from Abiy and Berhe (2016) made it clear that crude extract of garlic can be more effective in controlling bacteria than the processed powder. Drenkard (2003) demonstrated that some bacteria strains are resistant to certain antimicrobials. It is possible that most bacteria present in the solution were resistant to allicin.

Lysozyme treatments of 100 and 150 mg/9ml were equal for the extent of bacterial inhibition and were more effective than 50 mg/9ml ($P<0.05$). Lysozyme levels of 50, 100 and 150 mg/9ml reduced bacteria growth by 1.65, 4.15 and 3.91 log_{10} cfu respectively. Others have reported inhibitory effects of lysozyme in a nutrient broth. Zhang et al. (2006) reported that lysozyme from hen egg white had an inhibitory effect against *Clostridium perfringens* in nutrient broth.

In conclusion, the higher levels of lysozyme evaluated were effective in controlling aerobic bacteria numbers in digesta culture generated from maltodextrin fed market-aged broiler and *Salmonella enterica serovar Heidelberg* pure culture. Allicin did not have any measurable effect on bacteria. Therefore, the lysozyme appeared more promising to use in broiler chicken feed containing maltodextrin to control bacteria population than allicin.

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CONFLICT OF INTEREST
There is no conflict of interest with regards to this research.

Apendix

Tables

Table 1. The effect of lysozyme on bacteria generated from maltodextrin fed broiler digesta and *Salmonella enterica* serovar *Heidelberg* pure culture (Trial 1).

Treatments (culture)	Antimicrobials	Levels (mg/9ml)	Aerobes ($log_{10}cfu.ml^{-1}$)
Digesta culture	Lysozyme	0	$7.23\pm0.22^{a\dagger *}$
		50	6.61 ± 0.22^{b}
		100	5.70 ± 0.22^{c}
		150	4.62 ± 0.22^{d}
	Tetracycline	150	6.88 ± 0.22^{ab}
Salmonella pure culture	Lysozyme	0	$7.23\pm0.22^{a*}$
		50	5.66 ± 0.22^{c}
		100	4.27 ± 0.22^{d}
		150	4.04 ± 0.22^{d}
	Tetracycline	150	$7.23\pm0.22^{a*}$

Anova	P-value
Treatments	0.001
Levels	<0.001
Treatments×levels	0.002

Means with different superscripts are significantly different ($p\leq0.05$).
† Mean ± Standard error of the mean.
*TNTC- too numerous to count (this was replaced with upper limit is 168 on the 5^{th} dilution which was equivalent to 7.23 log_{10}cfu).

Table 2. The in vitro effects of allicin and lysozyme on bacteria generated from maltodextrin fed market-aged broiler's digesta (Trial 2).

Treatments (Antimicrobials)	**Levels of antimicrobials (mg/9ml)**	**Aerobes ($log_{10}cfu.ml^{-1}$)**
Allicin	0	$7.40\pm0.11^{a\dagger*}$
	50	$7.40\pm0.11^{a*}$
	100	$7.40\pm0.11^{a*}$
	150	$7.40\pm0.11^{a*}$
Lysozyme	0	$7.40\pm0.11^{a*}$
	50	5.69 ± 0.11^{b}
	100	3.40 ± 0.11^{c}
	150	3.60 ± 0.11^{c}

Anova	**P-value**
Treatments	<0.001
Levels	<0.001
Treatments×levels	<0.001

Means with the same superscript are not significantly different (P≥0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.
*TNTC- too numerous to count (this was replaced with 250 at the 5th dilution and was equivalent to 7.40 log_{10}cfu).