# MICROBIOLOGY AND FOOD SAFETY

## Use of a maltodextrin-based feed with a lysozyme product to alter bacterial in the ileum of market-aged broilers

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**ABSTRACT** Poultry meats can become contaminated with pathogenic bacteria through digesta leakage during processing. Reducing the bacteria load in digesta of market-aged broilers prior to processing reduces the incidence of fecal contamination at the processing plant. A lysozyme product was incorporated in a maltodextrin-based feed offered during the pre-shipping feed withdrawal period to reduce bacteria in ileal contents of market-aged broilers. Twenty 36-day-old broilers were randomly allocated to each of 16 pens. For a 9 h period each pen was randomly assigned to one of the following treatments: no feed, maltodextrin-based feed with a lysozyme product (Inovapure) added at 0, 10, or 20 g per kg of feed. Feed consumption was determined and a minimum of 3 birds were randomly selected from each pen and euthanized. The ileal contents were removed and weighed. Samples were analyzed for *Clostridium perfringens*, aerobic bacteria, Enterobacteriaceae, *E. coli*, and coliform numbers using standard culturing techniques and next generation sequencing was performed to determine population shifts. Bacteria counts were transformed to $\log_{10}$ colony forming units (cfu) and analyzed as a completely randomized design. The data from next generation sequencing was analyzed as a 3 × 5 factorial design using Proc Mixed of SAS. Lysozyme did not affect feed consumption nor were the weight of ileal contents different for birds fed maltodextrin-based feeds compared to birds on traditional feed withdrawal. *E. coli*/coliforms and Enterobacteriaceae plates had no signs of bacterial growth. The number of *Clostridium perfringens* and aerobic bacteria in the ileal contents of market-aged broilers was not different between treatments using the traditional culturing techniques. Next generation sequencing was a useful alternative to traditional culture techniques as results revealed that bacilli were reduced and clostridia increased for the 20 g lysozyme treatment. Addition of lysozyme to a maltodextrin based feed did not change overall numbers of bacteria but was effective in altering the participants in the bacteria community in ileal contents of market-aged broilers.

**Key words:** lysozyme, maltodextrin, feed withdrawal, market-aged broiler, next generation sequencing

2019 Poultry Science 98:6897–6902
http://dx.doi.org/10.3382/ps/pez426

## INTRODUCTION

The poultry industry is fully aware of the association of bacteria from poultry products with foodborne illness. Some of the more prominent pathogens include *Salmonella* (Crump et al., 2002) and *Clostridium perfringens* (Sinosh et al., 2010). One of the most likely points of contamination of poultry meat is the processing plant (Berrang and Bailey, 2009). During processing, digesta from the gastrointestinal tract (**GIT**) can become a source of bacteria for contamination of the carcass (Chao et al., 2008). This is often referred to as fecal contamination. Broilers are fed low doses of antibiotics to control pathogenic bacteria and improve growth performance (Apajalahti et al., 2004). However, long term use of antibiotics may lead to the emergence of antibiotic resistant bacteria (Butaye et al., 2003; Mayrhofer et al., 2004). Antibiotic resistant bacteria can be transferred to humans through the food chain when contaminated meats are consumed (Hurd et al., 2004). Due to these concerns, poultry researchers have investigated the use of alternatives to antibiotics from plant and animal sources (Kamysz, 2005; Abdalla, 2011). Alternatives to antimicrobials include prebiotics, probiotics, organic acids, and animal and plant extracts (La Ragione et al., 2001). An example of an alternative antimicrobial is lysozyme from hen egg white (Mine et al., 2004). Lysozyme has been incorporated in broiler feed in a study by Liu et al. (2010) and was found to inhibit growth of *Clostridium perfringens* in chickens. They supplemented broiler feed with 40 mg of lysozyme per kg of feed and reported improved intestinal barrier


Received October 19, 2018.
Accepted July 30, 2019.
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function and growth performance of broilers compared to the control. The barrier function was evaluated by the indirect method of bacterial translocation to the liver and spleen. In our study, lysozyme from hen egg white was evaluated in a maltodextrin feed offered to market-age broilers for a short time prior to shipping. This was performed to evaluate the effect of lysozyme added to maltodextrin feed on bacterial numbers in the ileal contents of market-aged broilers prior to shipping. Maltodextrin is a highly digestible feed supplement that was developed to feed poultry prior to slaughter to ensure the evacuation of the GIT and to maintain body weight of broilers (Farhat et al., 2002; Rathgeber et al., 2007). Bacteria populations were evaluated using both Next Generation Sequencing (**NGS**) and traditional culturing methods. The traditional culturing method of enumerating bacteria provides information on the number of bacteria (Zengler, 2009). The NGS effectively allows the evaluation of the broad group of bacterial diversity and the manipulation of the bacteria community (Metzker, 2010).

## MATERIALS AND METHODS

### Maltodextrin Feed Formulation

A maltodextrin-based feed was produced by modifying a formulation used by Farhat et al. (2002). The feed withdrawal supplement in this study consisted of 70% maltodextrin (Grain Processing Corporation, Muscatine, IA), 3% tallow, 2% corn germ (Corn Products International, Westchester, IL), 2% mono and diglycerides (Univar, Quebec, BC, 86 Canada), 0.33% caramel color (Sethness Caramel Color, W. Touhy Avenue, Lincolnwood, IL), and 0.36% salt. To ensure that the feed supplement formed pellets, 22.31% finisher diet was added. Melted tallow was mixed with the finisher feed in a mixer (Hobart Mixer, Closter, NJ) and added to the other ingredients. A total of 3 treatments were formulated to contain 0, 10, or 20 g of a lysozyme product (Neova Technologies, Abbotsford BC, Canada) per kg of feed. This product was a commercially prepared mixture of lysozyme and EDTA (1:4) with an enzymatic activity of 24,000 units/mg. The finisher feed component was reduced by the amount of lysozyme added. Rate of pellet formation in the pellet mill (California Pellet Mill Co. CPM, Crawfordsville, IN) was adjusted to facilitate to control pellet temperature (72 ± 1°C) at the exit from the 3 mm die.

### Feeding and Allocation of Market-Aged Broilers

Male, Ross broilers were randomly allocated to 16 pens with 20 birds per pen. There were 4 pens in each of 4 rooms. The birds were managed with the same diets and environment settings until 37 D of age. On day 37, 1 pen per room was randomly assigned one of the following treatments, conventional feed withdrawal (no feed) or maltodextrin-based feed with 0, 10, or 20 g of lysozyme product per kg. The treatments were provided after synchronizing the feeding behavior of the broilers by removing finisher feed for 4 h followed by access to finisher feed for 2 h (Zuidhof et al., 2004). Feed synchronization was carried out to ensure minimized variation in the volume of GIT contents. All animal use was approved by the Local Animal Care and Use Committee (Dalhousie University) following the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

### Sample Collection

After 9 h of access to maltodextrin feed, the remaining feed was weighed and recorded to determine feed consumption. A minimum of three birds were selected at random from each pen. The birds were euthanized by cervical dislocation and ileal contents were harvested aseptically and pooled to produce two 30 g samples per pen. The ileum was identified as the tract distal to the Meckel's diverticulum up to the junction with the ceca. The number of birds required to produce a 30 g sample of digesta was recorded.

### Laboratory Analysis

***Microbial Plating*** From each of the 30 g digesta samples, 25 ± 0.05 g was placed in a blender bag. Two 125 ml of buffered peptone water (**BPW**) was used as a diluent for blending in a stomacher for 60 s. A total of 8 serial dilutions were performed. The first 3 dilutions were plated on *Clostridium perfringens* agar pour plates, the 4th to the 6th dilutions were plated on 3 M aerobic count petrifilm. The 7th to 8th dilutions were plated on 3 M Enterobacteriaceae and *E.coli*/coliform petrifilm. The pour plates of *Clostridium perfringens* agar plates were incubated in a controlled atmosphere incubator (Bactron IV, Shel Lab, Cornelius, OR) for 48 h at 35 ± 1°C under anaerobic conditions (supply gas mixture, 90% Nitrogen, 5% Carbon Dioxide, and 5% Hydrogen). All black colonies characteristic of *Clostridium perfringens* were enumerated as *Clostridium perfringens* (Bolder et al., 1999). All petrifilm plates were incubated in a standard aerobic incubator. The aerobic plates were incubated at a temperature of 35 ± 1°C for 24 h and all colonies were counted (AOAC 940.36, 2005). Enterobacteriaceae plates were incubated for 24 h and red colonies with yellow zones, with or without gas production were enumerated as Enterobacteriaceae (AOAC 940.37, 2005). All red colonies were enumerated as coliforms after 24 h incubation at 35 ± 1°C. The plates were reincubated for another 24 h at 35 ± 1°C and all blue colonies with gas production were counted as *E. coli* (AOAC 989.10, 2005).

***DNA Extraction from Digesta for Bacteria Detection*** Subsamples of 1 g from each digesta sample were taken and stored at −80°C for DNA extraction. Samples were thawed at 20°C and processed according to manufacturer's instructions (QIAGEN, Toronto, ON—stool kit). The quantity of DNA was determined

using a spectrophotometer at 260 nm and the quality of the samples were verified with agarose gels. The DNA was amplified by using PCR. The samples were sent to the Research and Testing Laboratory LLC (Lubbock, TX) for NGS analysis. The 454 NGS technique (Roche 454 sequencer) was used. The sequencing was performed using a universal primer (preparatory primer used by the NGS group, Lubbock, TX) on a ¼ slide with 5,000 reads per plate.

### Statistical Analysis

The data were analyzed as a completely randomized design. There were 4 treatments and 4 replicates. All bacteria counts were transformed into $Log_{10}$ colony forming units (**cfu**) values before subjecting them to ANOVA using Proc Mixed of SAS model (SAS Institute Inc. 2003). Significant differences among treatments were separated using the mean separation test of Tukey-Kramer ($P \leq 0.05$).

The results from NGS were analyzed using a denoising program to obtain the classification summary spread sheet. Following that, the sequences were trimmed to keep them with a minimum length of 200 bp. The data was aligned against the silva 16S backbone. This 16S backbone is software used for NGS sequenced read cleaning, assembling and explanation of transcriptome, read mapping, and single nucleotide polymorphism selection (Blanca et al., 2011). The data were screened to ensure that chimeric sequences were removed as it was tested against the silva gold database (Quince et al., 2011). One of the NGS data points was found to be an outlier. The outlier was removed and the rest of the data were subsampled to obtain the bacteria classifications. The classifications were then run with silva database and the percentages of same sequences in the sample were calculated. The files were merged by selecting those classifications where the taxa had ≥0.5% representation in at least 1 sample. The data files were merged into the final file (Quince et al., 2011). The data were run as 3 × 5 factorial design using Proc Mixed of SAS (SAS Institute Inc. 2003). The model was $\gamma_{ijk} = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \varepsilon_{ijk}$. $\gamma_{ijk}$ was for the bacteria groups, $\mu$ was for the overall mean, $\tau_i$ was for lysozyme levels ($i = 1$, 2, and 3). $\beta_j$ was for bacteria groups (Gram-negative and Gram-positive), $j = 1$, 2, 3, 4, and 5. $\tau\beta ij$ was for the interaction effects of lysozyme levels and bacteria groups, and $\varepsilon ijk$ was for the error term. Significant differences among treatments were separated using the mean separation test of Tukey-Kramer ($P \leq 0.05$).

## RESULTS AND DISCUSSION

### Feed Consumption

There were no differences ($P > 0.05$) (Table 1) observed for feed consumption for all maltodextrin treatments (0, 10, and 20 g of lysozyme). Therefore, lysozyme levels in the diet had no influence on feed intake.

**Table 1.** Feed consumption of maltodextrin feed with or without lysozyme for broilers.

| Feed treatments | Lysozyme levels (g/kg of feed) | Feed consumption (g/bird) |
|---|---|---|
| Maltodextrin-based diet | 0 | 30.11 ± 3.06[†] |
| | 10 | 28.89 ± 3.06 |
| | 30 | 30.28 ± 3.06 |
| Anova | *P*-value | |
| Feed treatments | 0.94 | |

[†]Mean ± Standard error of the mean.

**Table 2.** Digesta weight in the ileum of 36 day old broilers after 9 h of feeding maltodextrin or feed withdrawal.

| Feed treatments | Lysozyme levels (g/kg of feed) | Digesta weight (g/bird) |
|---|---|---|
| Maltodextrin-based feed | 0 | 9.00 ± 1.15[†] |
| | 10 | 11.07 ± 1.15 |
| | 20 | 11.86 ± 1.15 |
| Feed withdrawal | - | 10.62 ± 1.23 |
| Anova | *P*-value | |
| Feed treatments | 0.36 | |

[†]Mean ± Standard error of the mean.

### Digesta Weight

The amount of digesta in the ileum of the treated birds was not different ($P > 0.05$) from the feed withdrawal birds (Table 2). These results were different from research performed by Farhat et al. (2002). They observed lower digesta volume from birds offered the maltodextrin-based feed compared to birds on standard feed withdrawal. The purpose of the feed withdrawal period is to ensure that the birds emptied their GIT prior to slaughter (Farhat et al., 2002; Northcutt et al., 2003; Rathgeber et al., 2007). Farhat et al. (2002) found maltodextrin feed more useful for GIT emptying than withdrawing feed from birds. However, providing maltodextrin feed to market-aged broilers did not result in further emptying of the GIT of broilers in this study.

### Clostridium Perfringens

The consumption of lysozyme-enriched maltodextrin feed did not influence the number of *Clostridium perfringens* in the digesta of the ileum of market-aged broilers in this study ($P > 0.05$) (Table 3). Other researchers have reported that lysozyme is effective against *Clostridium perfringen* in some conditions. Zhang et al. (2006) indicated that lysozyme at 100 μg/mL inhibited α-toxin production and that 156 μg/mL completely inhibited the growth of *Clostridium perfringens* type A in a nutrient broth. At these levels it caused massive damage to the cell wall of *Clostridium perfringens* due to *N*-acetylmuramoylhydrolase enzymatic activity of lysozyme. This results in lysis of the peptidoglycan layer of the cell wall of *Clostridium*

**Table 3.** *Clostridium perfringens* in ileal contents of market-aged broilers on feed withdrawal or fed a maltodextrin-based feed with or without lysozyme.

| Feed treatments | Lysozyme (g/kg of feed) | *Clostridium perfringens* $\log_{10}$ (cfu/g) |
|---|---|---|
| Maltodextrin-based feed | 0 | 2.21 ± 0.48[†] |
| | 10 | 2.56 ± 0.35 |
| | 20 | 2.43 ± 0.35 |
| Feed withdrawal | | 2.50 ± 0.35 |
| Anova | *P*-value | |
| Feed treatments | 0.92 | |

[†]Mean ± Standard error of the mean. cfu- colony forming units.

**Table 4.** The in vivo effect of maltodextrin feed with or without lysozyme on the aerobic bacteria numbers in the ileum of market-aged broilers.

| Feed treatments | Lysozyme (g/kg of feed) | Aerobes (cfu/g) |
|---|---|---|
| Maltodextrin-based feed | 0 | 6.36 ± 0.38[†] |
| | 10 | 5.71 ± 0.34 |
| | 20 | 5.83 ± 0.34 |
| Feed withdrawal | - | 6.38 ± 0.34 |
| Anova | *P*-value | |
| Feed treatments | 0.24 | |

[†]Mean ± Standard error of the mean. cfu- colony forming units.

*perfringens* (Masschalck and Michiels, 2003). This indicates that, lysozyme is effective on *Clostridium perfringens* even at low levels. Liu et al. (2010) fed a diet with lysozyme at 40 mg/kg to broilers for 28 D and found that lysozyme reduced *Clostridium perfringens* in the ileum of broilers. The time exposure for the lysozyme to act on *Clostridium perfringens* may have been too short in this study.

## Aerobic Bacteria

There were no differences ($P > 0.05$) in the aerobic bacteria numbers among treatments (Table 4). The maltodextrin-based feed (control) did not affect overall bacterial numbers in the ileum of market-aged broilers. Others have investigated the effects of maltodextrin on bacterial numbers in the upper GIT and in carcass rinses. Rathgeber et al. (2007) found no differences in aerobic bacteria in the crop after subjecting turkeys to the maltodextrin feed for 5 h. Northcutt et al. (2003) found no difference for numbers of *Campylobacter*, *E. coli*, and coliforms in whole carcass rinses in BPW after subjecting the birds to 8 h of maltodextrin feed followed by 0, 4, 8, and 12 h of feed withdrawal. It could be that the carcasses were not contaminated with the digesta from the market-aged broilers used in their study. The addition of lysozyme to maltodextrin feed did not have an inhibitory effect on aerobic bacteria ($P > 0.05$).

## NGS

This is the first report to describe the intestinal bacterial profile from broilers fed lysozyme-enriched maltodextrin-based feed using NGS. Gram-negative and Gram-positive bacteria identified in the GIT of the market-aged broilers were approximately ≤0.7% and ≤99.2% of the total, respectively. Gram-positive bacteria were reduced by 20 g of lysozyme per kg of maltodextrin feed but it did not affect the number Gram-negative bacteria (Table 5). This confirms the results from Cunningham et al. (1991) and Liu et al. (2010) that lysozyme is more effective against Gram-positive than Gram-negative bacteria. Among the class of Gram-positive bacteria group, Actinobacteria and Firmicutes were identified. The sequencing results showed that Firmicutes bacteria that exist in the GIT of broilers used in this trial had 2 subgroups of bacteria, these include bacilli and clostridia (Table 5). Bacilli were found to have the highest population followed by the clostridia group. The clostridia found in the digesta were non-harmful clostridia species. It is possible that the use of the universal primer in this study was not sensitive enough to detect the small numbers of those clostridia species that are of clinical importance such as *Clostridium perfringens* (Bjerrum et al., 2006). Clostridia levels were reduced in maltodextrin digesta more so than lysozyme-enriched maltodextrin feed, however, it increased with an increase in lysozyme levels. Perhaps the maltodextrin feed itself influenced the clostridia species. Bacilli decreased with an increase in lysozyme levels which demonstrated the antimicrobial activity of lysozyme. Similarly, lysozyme was indicated to have bactericidal effect on 4 spoilage lactic acid bacteria (*Lactobacillus kunkeei, Lactobacillus brevis, Pediococcus parvulus, and Pediococcus damnosus*) at a concentration of 125 and 250 mg/L in grape juice at 20 ± 0.5°C (Gao et al., 2002). This indicates that lysozyme is effective against Gram-positive bacteria. The proportion of data for bacteria are expressed in percentages (%). clostridia increased with increase in lysozyme levels relative to the bacilli. Research has indicated that lysozyme was effective on *Clostridium perfringens* when delivered through a broiler soy bean-based diet or in a micro-broth. Liu et al. (2010) reported that lysozyme inhibited *Clostridium perfringens* in the ileum of older birds challenged with *Clostridium perfringens*. Similarly, Zhang et al. (2006) found that lysozyme had inhibitory effect on *Clostridium perfringens* in a micro-broth. It is possible that some bacteria may be sensitive to higher levels of lysozyme. Lysozyme-enriched maltodextrin shows promise in reducing Gram-positive bacteria but as to whether its effects will be extended to the Gram-negative bacteria should be investigated with higher levels of lysozyme. In general pathogenic bacteria such as *Salmonella, Clostridium perfringens*, *Campylobacter*, *Escherichia coli*, and *Shigella* were not found in the sequencing results in this research.

## CONCLUSION

In this study, enriching maltodextrin feed with lysozyme had no effect on feed consumption and ileal weight contents. Lysozyme levels used did not affect

**Table 5.** Next generation sequencing information and diversity of bacteria in the digesta of market-aged broilers.

| Bacteria class | | Level of lysozyme | | |
|---|---|---|---|---|
| Gram sensitivity | Subgroups | 0g | 10g | 20g |
| Actinobacteria(+) | | 0.13 ± 9.71[c] | 0.00 ± 9.71[c] | 0.43 ± 9.71[c,†] |
| Firmicutes (+) | | 99.47 ± 9.71[a] | 99.70 ± 9.71[a] | 93.10 ± 9.71[a] |
| | Bacilli | 98.43 ± 9.71[a] | 98.17 ± 9.71[a] | 58.37 ± 9.71[b] |
| | Clostridia | 0.57 ± 9.71[c] | 1.53 ± 9.71[c] | 34.87 ± 9.71[b] |
| Proteobacteria (-) | | 0.37 ± 9.71[c] | 0.23 ± 9.71[c] | 4.57 ± 9.71[c] |
| Anova | *P*-value | | | |
| Group (Bacteria class) | <0.00 | | | |
| Treatment | 0.95 | | | |
| Group × treatment | 0.04 | | | |

Means with the same superscript are not significantly different ($P \geq 0.05$).
[†]Mean ± Standard error of the mean.

*Clostridium perfringens* and aerobic bacterial numbers in ileal contents using the traditional culturing methods but it was found to reduce Gram-positive bacteria (bacilli) when measured with NGS. NGS is an ideal tool for determining bacterial populations in complex matrices such as digesta.

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