

**The effect of *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* on  
cecal motility in feather-pecking laying hens**

by  
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## ABSTRACT

### **THE EFFECT OF LACTOBACILLUS RHAMNOSUS (JB-1) AND SACCHAROMYCES BOULARDII ON CECAL MOTILITY IN FEATHER-PECKING LAYING HENS**

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Feather pecking (FP) is an important behavioral problem in laying hens with welfare and economic implications. It was hypothesized that differences in the microbiome of pecking (P) and non-pecking (NP) birds cause differences in cecal motility and consequently differences in feed passage time observed in peckers. We compared cecal motility in an organ bath system in P and NP birds using different microbial treatments (*Lactobacillus rhamnosus JB-1* and *Saccharomyces boulardii*). Contractions of cecal segments from 30 birds were recorded and analyzed (DMaple©) to measure frequency, velocity, and amplitude of peristalsis. Data were analyzed using GLIMMIX in SAS. This in vitro system successfully measured motility in birds. In P birds, there was a positive correlation between pecking bouts and velocity and the microbe treatments significantly lowered velocity. Microbial treatment decreased frequency of contractions. Future work may include in vivo studies to investigate potential behavioural or welfare implications of these results.

**Keywords:** Laying Hen, Feather Pecking, Cecal Motility, In Vitro, *Lactobacillus rhamnosus JB-1*, *Saccharomyces boulardii*

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## Chapter 1: Literature Review

### 1.1 The Feather Pecking Problem

Feather pecking (FP) is a major problem in the commercial laying hen industry and it has two core symptoms; the pecking at feathers of conspecifics and the pulling out of feathers (Rodenburg et al., 2013). Feather eating is also closely associated with FP as peckers have a high propensity to consume not only feathers pulled from other birds, but also loose feathers (McKeegan & Savory, 2001). FP continues to be a prevalent issue, as it has been estimated that 77% of industry flocks show high rates of FP by week 14 (Huber-Eicher & Sebo, 2001). While gentle feather pecking (GFP), which consists of nibbling or gentle pecks at the feather cover of other birds, causes little damage, severe feather pecking (SFP) involving forceful pecks and pulls of feathers can cause serious tissue damage (van der Eijk, 2018). SFP can lead to reduced welfare of birds and great economic losses resulting from increased mortality, increased feed consumption (Tullett et al., 1980), and reduced egg production (El-Lethey et al., 2000). Addressing this problem is more important than ever, as the European Union has banned the use of conventional cages and Canada plans to phase these out by 2036 (Lambert, 2016). It is more difficult to control FP in group-housing systems as opposed to conventional cages, as it is harder to identify and remove feather peckers and the behaviour may be spread throughout the group via social learning (Rodenburg et al., 2000).

Genetics are known to play a role in the development of this behaviour, as FP is known to be a heritable trait, with heritability ranging from 0.04 to 0.5 in different reports (Cuthbertson, 1980; Bessei, 1984; Kjaer and Sørensen, 1997; Rodenburg et al., 2003). However, tests used to measure and record FP in these studies have differed, thus making it difficult to compare them and come to a consensus.



In a study done in the Netherlands, selection for egg production produced a line of feather peckers incidentally (Korte, 1997). This study found important differences in behavioural, neurobiological, and physiological characteristics in feather peckers compared to non-peckers (Korte, 1997; Hierden et al., 2002). For example, peckers struggled more, exhibited lower plasma corticosterone, higher noradrenaline, lower heart rate, and lower dopamine and serotonin turnover in response to manual restraint than non-peckers (Korte, 1997). When compared with similar studies done in rodents, these results may be viewed in the context of the coping strategy used by these different birds in response to stress (Korte, 1997). The results suggest that peckers are proactive copers who are more internally motivated, while non-peckers are reactive copers who are more externally motivated (Rodenburg et al., 2004; Korte, 1997; Koolhaas et al., 1999). Peckers were also more fearful in open field tests than non-peckers and displayed higher activity and growth (Rodenburg et al., 2004; Jensen et al., 2005). In a separate study done in Denmark in 1997, selection of animals by FP developed two distinct lines of high-feather pecking (HFP) and low-feather pecking (LFP) birds (Kjaer & Sorensen, 1997). HFP birds from this line showed more active responses in behavioural tests (i.e. more vocalizations, more flight attempts, more walking and shorter durations of tonic immobility) than LFP birds which was interpreted as lower levels of fearfulness by the authors and seemed contradict the findings of studies done on the Korte lines (van der Eijk et al., 2018). Conversely, another study with the Kjaer lines showed an increased reaction of the autonomic nervous system in response to physical restraint in HFP birds compared to LFP birds seemingly indicating that HFP birds were more easily stressed (Kjaer & Jorgensen, 2011). In addition, HFP birds are more reactive to handling and restraint than LFP birds indicating greater fearfulness (Kjaer & Guemene, 2009). Collectively, evidence from both the Korte and Kjaer lines has led some to suggest that FP in birds might share similarities with certain mental health

disorders in humans, such as hyperactivity and obsessive-compulsive disorders (Kjaer, 2009; Hierden et al., 2004).

The underlying causes of FP are multifactorial and the gentle and severe forms of it likely have different aetiologies. The most prevalent hypothesis regarding the pathology of SFP is that it stems from an internal frustration in birds which are kept in barren environments (i.e. battery cages) and have no access to foraging substrates (Blokhuys, 1986). In industry, laying hens are fed concentrated low-fibre diets, although high-fibre diets are known to reduce FP and improve gastrointestinal tract (GIT) development in chicks (Krimpen et al., 2005; Hetland et al., 2003). Therefore, the feather eating often associated with FP might represent an attempt to increase insoluble fibre intake in the absence of suitable forage (Harlander-Matauschek et al., 2006). However, some evidence indicates that the “frustrated foraging” hypothesis may not explain all of FP. For example, FP can be observed as early as 1 day after hatching in laying hen chicks (Rodenburg et al., 2004). At this age dustbathing and foraging do not occur, suggesting that there are other motivations at play (Riedstra & Groothuis, 2002). It has also been noted that the incidence of FP increases after housing unfamiliar birds together and that pecks in these scenarios are primarily directed at unfamiliar animals (Rodenburg et al., 2004). Thus, it has been suggested that FP plays a role in social exploration and situations of social stress (McArdie & Keeling, 2002). Additionally, it is known that the removal of loose feathers from the floor during early life reduces the level of FP during the lay period, indicating that FP may be a learned behaviour (Ramadan & Borell, 2008). Peckers housed together had fewer feathers on the floor than non-peckers, implying the peckers were eating a significant number of feathers (McKeegan & Savory, 1999). The link between the GIT and feather eating was further illustrated by Birkl et al. (2018), who found that HFP birds exhibit a decreased presence of *Lactobacillus* bacteria and an increased presence of

*Clostridia* in their ceca compared to LFP birds. Not only this, but LFP birds showed a greater microbial diversity overall in these organs (Birkel et al., 2018). Recently, HFP and LFP birds were also shown to differ significantly in their intestinal microbial metabolism (Meyer et al., 2012). Total amounts of lactate and Short-Chain Fatty Acids (SCFAs) were higher in the ceca of HFP animals and concentrations of acetate, i-butyrate, i-valerate n-valerate and ammonia were higher in the ceca of LFP birds (Meyer et al., 2013). This is logical, as feather intake is known to induce adaption of the intestinal microbiota in chickens, lowering bacterial diversity in the ceca and increasing the concentration of keratinolytic bacteria in both the ceca and ileum (Meyer et al., 2012).

The link between feather eating and FP was further illustrated by Harlander-Matauschek et al. (2007), who showed that HFP birds are more motivated to eat feathers than their LFP counterparts (Harlander-Matauschek et al., 2007). Further research showed that FP may be reduced with the inclusion of feathers in the diet and this indicates that feathers might satisfy some internal need in laying hens by providing a specific substrate not found in their diets (Kriegseis et al., 2012; Meyer et al., 2013). Additionally, Harlander-Matauschek et al. (2006) showed that feathers have a similar effect on the gut as insoluble fibre and that feed passage time is lower in HFP animals allowed to consume feathers (Harlander-Matauschek et al., 2006). This is especially relevant, considering that feeding low-energy high-fibre diets during rearing is known to reduce FP during the laying period, and fasting laying hens leads to increased FP (Krimpen et al., 2009; Webster 1995). This evidence has led to the hypothesis that dietary factors that induce satiety can lead to a reduction in FP and that FP and feather eating is functional and confers a benefit to the bird by promoting feelings of satiety (Krimpen et al., 2009). It is possible this occurs via a positive effect on gut motility through increased grinding activity of the gizzard (Hetland et al., 2003; Krimpen et al., 2011).

Feeding of high-concentrate, low-fibre diets may lead to dietary deficits and unfulfilled preferences in laying hens. However, while feeding high-fibre diets does seem to reduce the motivation to feather peck, it does not eliminate it entirely. It is possible that other factors are at play in the GIT, however, digestive physiology and gastrointestinal function is a neglected field in laying hens (Harlander-Matauschek et al., 2006). While there are many post-hoc hypotheses on the connection between the GIT and feather pecking, there have been few hypothesis-driven experiments carried out. A better physiological understanding of the avian gut is needed with a focus on fibre digestion.

## 1.2 Avian Ceca Biology

A crucial organ with respect to fibre digestion is the ceca. The ceca in the chicken consist of two blind-ended sacs with a mesh-work of long interdigitating villi at their entrance (Clench, 1999). They project from the proximal colon at its junction with the small intestine and are large and well-developed in the domestic chicken (Clench & Mathias, 1995). These sacks are divided into three morphological regions; the *basis ceci*, the *corpus ceci*, and the *apex ceci*. Respectively, these regions consist of well-developed villi near the ileocecal junction, longitudinal folds and small villi at the medial region, and small villi with both longitudinal and transverse folds in the distal region (Ferrando et al., 1987; Sturkie & Benzo, 2014). While the ceca are multi-purpose organs, one of their major roles is in the breakdown of dietary insoluble fibre (Clench, 1999). As feathers are known to mimic insoluble fibre in the chicken GIT, these organs are important in the process of feather digestion (Harlander-Matauschek et al., 2006). During fibre digestion, the ceca may also play a role in triggering propulsive peristalsis of the gut, and thus have a significant impact on intestinal motility in the chicken (Clench, 1999).

Of all the aspects of the avian digestive system, motility of the digestive tract is perhaps the most neglected in research (Duke, 1982). The ceca are filled through peristaltic movements from the proximal to the distal end (Fenna & Boag, 1974). It has been suggested that materials enter the ceca as a result of pressure at the junction of the small and large intestine (Fenna & Boag, 1974). This pressure forces the liquid fraction of the digestive contents into the openings of the ceca and then contents are conveyed to the blind end of the organ by antiperistalsis (Fenna & Boag, 1974). Stimuli from stretch receptors in the walls of the cloaca likely trigger anti-peristaltic contractions in the large intestine which continue into the ceca and mix its contents as well as move them distally (Fenna & Boag, 1974). The ceca are evacuated about once daily in galliformes (Fenna & Boag, 1974) and this seems to occur via a massive peristaltic movement occurring in both ceca simultaneously (Duke, 1982). Two types of contractions appear to occur in the ceca of turkeys, those with “low” amplitude and those with “high” amplitude which have been termed minor and major contractions respectively (Duke, 1982). Major contractions had an average amplitude of 15.7g and an average frequency of 1.2 cycles/min, while minor contractions had an average amplitude of 2.8g and average frequency of 2.6 cycles/min (Duke, 1982).

While there is little understanding of the neural regulation of cecal motility, there is evidence that the mechanism of peristalsis in the chicken gut is different to that found in the mammalian tract (Hodgekiss, 1983). Some intrinsic neurones in the ceca likely inhibit the musculature during distension as local anaesthetics can initiate peristalsis in *in vitro* preparations (Hodgekiss, 1983). In contrast, the opposite effect is observed in mammals wherein peristalsis is neurogenically mediated by the enteric nervous system and is blocked by anaesthesia (Sturkie & Benzo, 2014). Additionally, myogenic peristalsis in the ceca can occur in either direction while peristalsis in mammals has a definite polarity (Hodgekiss, 1983). Peristalsis in general may be

regulated through a variety of pathways, including the enteric nervous system (ENS), vagus nerve, immune system, and through the short-chain fatty acids (SCFAs) produced by gut microorganisms (Zhu et al., 2014).

The ceca are the primary site of microbial fermentation of undigested carbohydrates in the avian GIT (Józefiak et al., 2004). The abundance and concentration of microorganisms is greatest in the ceca, with more than 2200 operational taxonomic units having been reported and bacteria being present at concentrations of  $10^{10} - 10^{11}$  cells/g of cecal material (Danzeisen et al, 2011; Qu et al., 2008). Additionally, microbial species diversity is the highest in these organs and tends to increase with age (Gong et al., 2007).

In general, bacteria are classified taxonomically in descending order by phylum, class, order, family, genus, and species. Of interest are the phyla *Firmicute* - which encompasses the genera of *Lactobacilli* and *Clostridia*, *Bacteroidetes* - which includes the *Bacteroides* and *Prevotella* genera, and *Actinobacteria* - which contains the genus *Bifidobacteria*. These classifications are important because of recent work concluding that there may be important differences in the cecal microbial profiles of birds in HFP compared to LFP lines.

### 1.3 Avian Gut Microorganisms

Recently, 16S rRNA gene sequencing has been able to provide taxonomic information on the composition of bacterial and archaeal populations in a wide variety of environments, allowing for in-depth exploration of the phylogeny of these communities. However, even via the use of modern techniques, reports on the microbiome composition of the chicken gut differ. This may be due to a variety of study-dependent factors affecting microbial populations, such as environment, treatment, feed additive, age, diet, type of chicken, and climate (Qu et al., 2008; Danzeisen et al., 2011).

Singh et al. (2012) found *Proteobacteria* to be the most dominant phylum in the GIT in their study on feed conversion ratios in broiler growers. However, the majority of research is in agreement that *Firmicutes* are the predominant phylum of bacteria colonizing the chicken GIT, representing 50 – 90% of all taxa in the ceca (Wei et al., 2013; Shaufi et al., 2015; Gong et al., 2007; Rehman et al., 2007; Qu et al., 2008; Danzeisen et al., 2011). While only accounting for a small percentage of the microbial population, *Archaea* have also been detected in the chicken GIT and act to remove excess hydrogen ions accumulating during the fermentation process (Saengkerdsub et al., 2007). *Methanobrevibacter* is the most prolific archaeal genus present in the ceca of the chicken (Saengkerdsub et al., 2007).

Studies have also shown some variation in the reported distribution of microbial genera throughout the GIT. Many reports using culture-independent techniques have agreed that *Lactobacillus*, *Clostridium*, and *Bacteroides* are the most dominant types of bacteria found in both the ceca and the ilea (Stanley et al., 2014; Gong et al., 2007; Lu et al., 2003; Torok et al., 2011). *Lactobacillus* appears to be the most abundant, with proportions ranging from 24% to 86% of intestinal bacteria (Stanley et al., 2014; Lu et al., 2003). These results seem to agree with culture-dependent approaches, which have found high proportions of *Bacteroides*, *Clostridia* and *Lactobacilli* in the ceca (Salanitro et al., 1974; Barnes et al., 1972; Bjerrum et al., 2006). However, work done very recently using high-throughput metagenomic analyses disagreed somewhat with these conclusions. While *Clostridium* and *Bacteroides* were prevalent in the ceca (47-70% and 2-20% respectively), *Lactobacillus* bacteria were found only in small amounts in all age groups of birds (<4%; Shaufi et al., 2015). The proportionally lower level of *Lactobacillus* (a bacterium known to promote gut health) in relation to potentially pathogenic bacteria, such as *Clostridium*,

suggests that there may be opportunities to improve the health of commercial birds through modulation of intestinal microbial populations (Shaafi et al., 2015).

1.4 The Action of *Lactobacillus* and *Saccharomyces* on the Mammalian Gut and Behaviour  
Intestinal peristalsis can be regulated by the enteric nervous system (ENS), vagus nerve, immune system, and through the short-chain fatty acids (SCFAs) produced by the metabolic processes of gut microorganisms (Zhu et al., 2014). There is strong evidence that psychoactive gut bacteria may influence animal behaviour via the action of SCFAs on the nervous connection from the gut to the brain. The brain receives information from the gut via the vagus nerve, which is the tenth cranial nerve linking the abdominal cavity to the brain and innervating the pharynx, larynx, and visceral organs (Li & Owyang, 2003). Experimental data has shown that the vagus is associated with emotional well-being and this is thought to occur via sensory vagal inputs from intraganglionic laminar vagal afferent endings (IGLVAEs) which are located between the outer and inner muscle layers of the oesophagus and GIT (Berthoud et al., 2001; Craig, 2009; Crucian et al., 2000). These IGLVAEs play critical roles in visceral sensation which can affect the interpretation of external inputs (Craig, 2009; Crucian et al., 2000).

The vagus plays an important role in sickness behaviour, in which ill individuals change their actions in order to better cope with infection (Dantzer et al., 2000). Vagotomy is known to block the depressing effects of lipopolysaccharide (LPS; a known cytokine inducer found in the cell membranes of pathogenic bacteria) on social exploration and food motivated behaviour in mice (Brettibat et al., 1995). Furthermore, the vagus is also known to modulate behaviours associated with anxiety and depression in rats. Forced-swim tests are well-established methods of evaluating depressive-like behaviour in rats as a model for depression in humans (Lyte & Cryan, 2016). In forced-swim tests taking place over the course of one to four days, rats exposed to vagal



stimulation remained immobile for a significantly lower period of time than unstimulated rats, indicating vagal stimulation had anti-depressant effects (Cryan et al., 2005; Cunningham et al., 2007). However, when vagal stimulation took place over the course of one month, no significant effect was observed on immobility in the forced-swim test, indicating that vagal stimulation may be dose-dependent (Biggio et al., 2009). In humans, vagal stimulation is considered controversial as a method of treating of clinical depression. However, it has been approved by the FDA and anecdotal evidence indicates it is effective in some patients suffering from intractable depression (Martin & Martín-Sánchez, 2012; Rizvi et al., 2011).

There is a growing body of evidence that gut microbes can activate the vagus nerve and subsequently affect behaviour. Pathogenic bacteria, such as *Salmonella* and *Campylobacter* species, cause not only inflammation in the gut when administered orally but also activate areas of the brainstem via the vagal ganglia and have anxiogenic effects on behavior (Wang, 2002; Goehler et al., 2005). Commensal and beneficial bacteria are also known to enhance gastric vagal nerve activity. In particular, *Lactobacillus johnsonii* can increase autonomic nerve transmission and subsequently blood pressure (Tanida et al., 2005). Denervation of vagal nerve fibres eliminate the ability of *Lactobacillus johnsonii* to influence nerve activity and blood pressure (Tanida et al., 2005).

Vagotomy also blocks the effects of *Lactobacillus rhamnosus* on behaviour and mood in adult mice (Bravo et al., 2011). Chronic administration of *Lactobacillus rhamnosus* (JB-1) has anxiolytic effects on adult Balb/c mice in elevated maze tests as well as reducing duration of immobility in forced swim tests with these same mice (Bravo et al., 2011). Overall, reduced anxiety and depression-like behaviour, and increased exploratory behaviour are effects that appear to be induced by *Lactobacillus rhamnosus* administration and these effects are abolished following

vagotomy (Bravo et al., 2011). This highlights a potential role for this microbe in influencing brain chemistry and behaviour by modulating the ENS (Bravo et al., 2011).

Bacteria may affect vagal signalling via IGLVAEs which are vagal mechanoreceptors that might sense any alterations in contractile activity of the intestine caused by the action of bacteria on the ENS (Zagorodnyuk et al., 2001; Phillips & Powley, 2000; Massi et al., 2006; Wang et al., 2010). It is thought that bacteria act on the ENS via intrinsic primary afferent neurons (IPANs) which innervate the intestinal mucosa and may be targeted by neuroactive bacteria causing changes in gut motility (Ekblad et al., 1987; Keast et al., 1984; Lyte & Cryan, 2016). While no explanation exists to sufficiently explain the all of the actions of bacteria on the nervous system, the molecular mechanism may involve the inhibition of calcium-dependent potassium channels (Mao et al., 2013; Wang et al., 2010; Wu et al., 2013; Lyte & Cryan, 2016). This is because bacteria, such as *Lactobacillus reuteri*, are known to mimic the effects of these potassium channel blockers and cause excitability of myenteric IPANs in the rat colon (Kunze et al., 2009). Vagal chemoreceptors may also be activated by substances produced by bacteria that can be transported across the gut epithelium, such as histamine, ATP, serotonin, or SCFAs (Hara et al., 1999; Raybould, 2010; Bertrand, 2009).

Major producers of SCFAs in the gut are *Lactobacilli*-type species and this may contribute to their roles in gut peristalsis in humans (Ojetti et al., 2014). In humans, *Lactobacilli* species are known to increase the humidity of feces, stimulate intestinal peristalsis, and provide relief to patients with constipation-related evacuation disorders (Ojetti et al., 2014; Piano et al., 2010). *Lactobacillus casei* Shirota and *Lactobacillus reuteri* are both known to increase defecation frequency in adults and children suffering from constipation (Koebnick et al., 2003; Wu et al., 2013). Specifically, *Lactobacillus reuteri* and *Lactobacillus acidophilus* are known to increase both the frequency and

velocity of intestinal Migrating Myoelectric Complexes (MMCs), thereby accelerating intestinal transit (Husebye et al., 2001; Barbara et al., 2005). Decreases in populations of commensal bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Bacteroides*, and increases in the amount of potentially pathogenic organisms, such as *Pseudomonas aeruginosa* and *Campylobacter jejuni* have all been connected to bowel disorders in humans (Gerritsen et al., 2011; Kirgizov et al., 2001). There is general consensus that constipation could be caused by intestinal “dysbiosis”; a term for a microbial imbalance or maladaptation inside the body (Zhao & Yu, 2016).

Interestingly, one microbe which reduces populations of pathogenic bacteria in the ceca of birds thus improving dysbiosis is *Saccharomyces boulardii* (Line et al., 1997). While there is little research that has been conducted on this yeast, it is known to reduce the incidence of antibiotic-associated diarrhea and acute diarrhea in humans (Surawicz et al., 1989; Billoo et al., 2006) and to change the neurochemistry of enteric neurones in the jejunum of pigs (Kamm et al., 2004) suggesting it may have similar modes of action as other neuroactive gut microbes. In stressed mice, it has also restored parameters of intestinal motility to unstressed values illustrating its ability to mediate the effects of stress on the small and large intestine (West et al., 2016a).

*Lactobacillus rhamnosus (JB-1)* also has a regulatory effect on stress-induced alterations in the gut motility of mice. In one study, it was found that restraint stress not only reduced MMC frequency and velocity in the jejunum, but also increased these measurements in the colon (West et al., 2016b). In this case, the luminal application of *Lactobacillus rhamnosus (JB-1)* restored motor complex variables to unstressed levels within minutes (West et al., 2016b); thereby demonstrating this microorganisms’ ability to relieve not only depressed motility, but also return accelerated motility to normal levels in a mouse model.

## 1.5 Aims and Objectives

Given the transition to enriched housing or non-cage systems that many Canadian egg farmers will need to make in the near future, there is a critical need for research that determines the causes of FP and identifies potential treatments for this abnormal behaviour. The aetiology of FP continues to be poorly understood, despite the severe economic and welfare problems associated with this behaviour. Birds which exhibit high levels of FP differ in their behavior, neurobiology, physiology, and gut microbiome composition from non-peckers. Considering that the decreased presence of *Lactobacillus* bacteria and increased presence of *Clostridia* found in HFP birds shows some overlap with studies done on human patients suffering from bowel disorders, it is possible that intestinal motility in laying hens is affected by their gut microbiome in a similar way. Gut motility seems to be a promising target as it has strong link to behaviour in other species and is a largely unexplored topic in birds. Understanding how gut motility might impact FP could provide new insights into this prevalent problem and help identify solutions in the form of microbe-based nutraceuticals and therapeutic targeting of the gut microbiome.

It is hypothesized that differences in the microbiome of P and NP birds cause changes in the cecal motility between these two groups leading to the differences in feed passage time seen in live animals. The overall aims of this thesis were to determine if there is a correlation between FP and cecal motility (1) and if luminal microbial stimuli affect P and NP birds differently (2). In order to address these gaps in knowledge, the first objective was to determine if an in vitro system used in mammalian models could successfully measure cecal motility in birds (1). The second objective was to measure the frequency, velocity, and amplitude of cecal movements in P and NP birds and to compare them (2). The third objective was to measure the frequency, velocity, and amplitude of cecal movements in P and NP birds upon perfusion with *Lactobacillus rhamnosus*

(*JB-1*) and *Saccharomyces boulardii* to determine if these measurements differed from baseline values in both groups (3).

This thesis presents results from an experiment in which contractions of cecal tissue from two groups of feather pecking (P) and non-feather pecking (NP) laying hens were measured in an in vitro system previously used in mammalian studies (objective 1-2). It was predicted that the frequency, velocity, and amplitude of contractions would be higher in P than in NP birds. Finally, the tissue was perfused with pure strains of *Lactobacillus rhamnosus* (*JB-1*) and *Saccharomyces boulardii* and it was predicted that administration of these microbes would decrease frequency, velocity, and amplitude of cecal contractions in P birds, thereby restoring them to levels comparable to NP birds.

## Chapter 2: The effect of *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* on cecal motility in feather-pecking laying hens

### 2.1 Introduction

Repetitive behaviour, such as pecking at the feather cover of other birds, is one of the core behavioural symptoms of feather pecking (FP) in domestic birds (McKeegan & Savory, 1999). FP is one of the most important economic and animal welfare problems in commercial laying hens (Rodenburg et al., 2013; Lambton et al., 2010). Despite many years of research and identification of many risk factors, its underlying mechanism is still not fully elucidated but is thought to be triggered by stress (El-Iethy et al., 2000). FP is likely a behavioural symptom of the negative psychological state elicited when an animal is unable to cope with a frustrating situation (Rodenburg et al., 2005). In this way, FP and feather eating is a functional behaviour. A combination of individual sensitivity to frustration (genetic) and a frustrating environment likely leads to stress and triggers this behaviour (Sedlackova et al., 2004; Vestergaard et al., 1997). Recently, Brunberg et al. (2016) reviewed evidence which suggested the brain-gut-microbiota axis as the missing piece which could help explain FP behaviour. The importance of the gut microbiota in relation to stress responses, behavioural disorders and gut motility has been shown in previous studies, particularly in humans and mice (Dinan & Cryan, 2012, Rogers et al., 2016, West et al., 2016b). Behavioural changes resulting from shifts in microbial communities are thought to be mediated by neuroactive substances of microbial origin in the gut which act on the brain (Collins et al., 2012; Lyte, 2011). Motility is coordinated through complex interactions of myogenic smooth muscles and the enteric and central nervous systems, which communicate using the parasympathetic (via the vagus nerve) and sympathetic nervous systems (Kunze & Furness, 1999), but also by regulatory peptides (Gidda & Monkovic, 1985). This could explain the high comorbidity of behavioural problems in patients with hyperactivity (Jia et al., 2008), anxiety and

depression (Dash et al., 2015), and autism-spectrum disorders (Mulle et al., 2013) with intestinal disorders that are known to disrupt the gut microbiome, such as Irritable Bowel Syndrome (IBS; Fadgyas-Stanculete et al., 2014). The use of beneficial gut microbes (e.g. *Lactobacillus rhamnosus*, *Saccharomyces boulardii*) is a prominent field of research in an attempt to tackle behavioural and gastrointestinal disorders in humans (McFarland, 2006). Both *L. rhamnosus* (*JB-1*) and *S. boulardii* have reversed stress-induced gut dysmotility in mice (West et al., 2016b; West et al., 2016a).

FP in birds has been suggested to show similarities with different behavioural disorders as FP birds display a wide range of simultaneously existing, neurological symptoms which include fearfulness (de Haas, 2013) and hyperactive behaviour (Kjaer, 2009). At the same time, FP birds also exhibit non-neurological gastrointestinal symptoms such as consumption of more feathers (McKeegan and Savory, 2001) and an altered feed passage time (Harlander-Matauschek et al., 2006). Furthermore, birds selected to show more or less FP have distinct intestinal microbiota profiles and a different microbial metabolism, especially in the ceca (Meyer et al., 2012, 2013; Birkl et al., 2018). For example, Birkl et al., (2018) showed that the ceca of high FP birds had an increased abundance of *Clostridiales*, while there was a decrease in abundance of *Lactobacillus* compared to low FP birds. These organs also play a role in avian stress (Clench & Mathias, 1995). These findings suggest that the brain-gut-microbiota axis could play a role in the manifestation of FP, however it is not known how gut motility plays into this. Gut motility can be described by contractions and relaxations of smooth muscles in the gut wall (Kunze and Furness, 1999), where contractions can be propagating or non-propagating (standing) depending on frequency, velocity, direction and amplitude (Huizinga & Lammers, 2009). In patients with gastrointestinal disorders such as IBS this is affected, which as previously mentioned is linked to behavioural disorders in

humans (Chey et al., 2001). Similarly, the finding that high FP birds show altered feed passage time (Harlander-Matauschek et al., 2006), together with the different cecal microbiota profiles in these birds (Birkel et al., 2018), and the finding that providing microbes which would balance these microbiota profiles (e.g. *L. rhamnosus JB-1* and *S. boulardii*) reversed stress-induced gut dysmotility in mice (West et al., 2016b; West et al., 2016a) might suggest that gut motility is affected in these birds as well.

It is hypothesized that differences in the microbiome of P and NP birds cause changes in the cecal motility between these two groups leading to the differences in feed passage time seen in live animals. The overall aims of this thesis were to determine if there is a correlation between FP and cecal motility (1) and if luminal microbial stimuli affect P and NP birds differently (2). In order to address these gaps in knowledge, the first objective was to determine if an in vitro system used in mammalian models could successfully measure cecal motility in birds (1). The second objective was to measure the frequency, velocity, and amplitude of cecal movements in P and NP birds and to compare them (2). The third objective was to measure the frequency, velocity, and amplitude of cecal movements in P and NP birds upon perfusion with *Lactobacillus rhamnosus (JB-1)* and *Saccharomyces boulardii* to determine if these measurements differed from baseline values in both groups (3).

## 2.2 Materials and Methods

### 2.2.1 Ethical Approval

This study was approved by the University of Guelph Animal Care Committee (Animal User Protocol Number 3206) prior to the start of these experiments.

### 2.2.2 Animals and Housing

In this study, 30 non-beak trimmed White Leghorn female laying hens at 64 weeks of age were used (total n = 30). Of these, 15 were of the CTL line and 15 were of an HFP line (Kjaer &



Sorensen, 1997). Hens were 64 weeks of age at the onset of the study and were individually identified using wing tags that were attached at hatching, prior to their involvement in this work. They were allocated randomly into 5 identical enriched enclosures (6 hens per pen) at the Ontario Agricultural College Dairy Barn, University of Guelph, Guelph, Ontario, Canada. Each pen (Height = 365cm, Width = 118cm, Length = 118cm) contained 2 perches of lengths 100cm and 110cm, which were mounted at heights of approximately 60cm and 30cm respectively. Pens also contained 1 nest box, 1 ladder, one feeding trough, and one bell drinker.

One Samsung SNO-5080R security camera (Samsung SNO-5080R, IR, Samsung Techwin CO., Gyeonggi-do Korea) was mounted at the top of each pen to record the bird's behaviour twice per week for six weeks. Ten-minute recordings were taken of each pen twice per day. The recordings were taken between 10:00AM and 11:00AM in the mornings and between 2:00PM and 3:00PM in the afternoons on all three days. Birds were provided with a commercial layer mash and water ad libitum (Layer Breeder Mash from the Arkeil Research Station, Guelph, Ontario, Canada). Feed was provided in rectangular metal troughs which were mounted to the sides of the pen. The floor of the pen was covered in a 2-inch layer of wood shavings. Throughout the Ontario Agricultural College Dairy Barn, windows were present which provided natural daylight and darkness for the duration of the experiment.

### 2.2.3 Phenotype Determination

Video recordings were analyzed by one blind observer using Mangold Interact software (v9.7.4, Mangold International GmbH, Arnstorf, Germany, 2014). All birds were observed in each recording at the same time, although the observer had the ability to pause and rewind the video in order to accurately record pecking bouts. During the analysis of these ten-minute sections of video, the first criterion for identification of a pecking bout was a sequence of pecks performed by one

bird directed at the feather cover of another bird. FP bouts per individual were recorded on an all-occurrence basis (Altmann, 1974), with one bout being defined as a sequence of pecks directed at the same bird that is not interrupted for more than 4 s (Zeltner et al., 2000; ex. If a bird pecked 14 times, then stopped for 10 seconds and again pecked 12 times, these would be considered two individual bouts). Total number of pecking bouts were summed for each bird. The sum total of pecking bouts for each bird were then divided by the number of recording days to determine the average number of pecking bouts per day.

From the average number of bouts, the observer then classified each bird into one of two phenotypic classes; either pecking (P) or non-pecking (NP). NP birds were identified as those showing consistently low numbers of pecking bouts over the course of the three days (i.e. sum total was equal to or less than 3.0 pecking bouts or, one or less pecking bouts were performed per day). P birds were identified as those performing consistently high numbers of pecking bouts over the course of the three days (i.e. sum total was greater than 3.0 pecking bouts or, more than one pecking bout was performed per day).

#### 2.2.4 Motility Recordings

Following the behavioural observation period, the gut motility trials commenced. Birds were removed from their pens and sacrificed via cervical dislocation. Following opening of the abdominal cavity, the attached ceca were quickly excised out and placed in a beaker of Krebs buffer solution (West et al., 2016b), which was made fresh daily and continuously bubbled with carbogen gas (95% O<sub>2</sub> and 5% CO<sub>2</sub>). Krebs buffer solution was of the following composition (mmol/L): 118 NaCl, 4.8 KCl, 25 NaHCO<sub>3</sub>, 1.0 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 11.1 glucose, and 2.5 CaCl<sub>2</sub> (Sigma-Aldrich, 2017). Ceca were then separated at their junction and one was placed in the well of a heated tissue bath perfusion system filled with the same oxygenated Krebs buffer

solution (see Figure 1). Mesentery and fat tissue attached to each cecum were removed and 0.5cm incisions were made at the caudal end of each cecum. Silicone tubing was then used to cannulate the cranial end of the tissue. At this end, a manifold was attached to allow for the continuous flow of buffer solution through the lumen of the tissue. After flushing the cecum with Krebs buffer solution, the caudal end was also cannulated with silicone tubing to provide an outflow point which drained into a beaker outside of the system. Tissue bath temperature was maintained at 38°C via the use of an external heat exchanger. Lidocaine hydrochloride monohydrate was added directly into the tissue bath at a concentration of 375 µg/ml (Hodgekiss., 1983) in order to stimulate contractile activity (Sigma-Aldrich, 2017).

For the first half hour of recordings, the tissue was continuously perfused with oxygenated Krebs solution. After this baseline recording term, the tissue was suffused with *Lactobacillus rhamnosus* (*JB-1*) diluted to 1e8 CFU/ml in oxygenated Krebs solution for another 30-minute recording period. Finally, after a 10-minute wash-out period with Krebs solution (West et al., 2016b), the tissue was perfused with the final treatment of  $5 \times 10^8$  lyophilized *Saccharomyces boulardii* for a 30-minute recording.

Contractions of cecal segments were then recorded for thirty minutes using a video camera (Microsoft LifeCam HD-3000) which was mounted 15cm above the bath. A PC captured the videos and transferred them to the program VideoPad Video Editor (version 5.20; NHC Software, Greenwood Village, CO, USA, 2017). Recordings were analyzed using specialized NIH ImageJ software (version 1.51q; NIH, Bethesda, MD, USA, 2017) with a specific plug-in (DMapLE©) in order to generate spatiotemporal maps. These maps depict contractility of a tissue over time and enable the measurement of multiple parameters. They display the long axis of the cecum in the vertical direction (centimetres) and time in the horizontal direction (seconds).

Motility parameters validated in numerous other studies were determined for each isolated cecum (West et al., 2016b; Wu et al., 2013; Wang et al., 2010). These included frequency, velocity, and amplitude. Mean contraction frequency (Hz) for each cecum was calculated by taking the inverse of intervals between 8-10 successive contractions and averaging these values. The mean contraction velocity (cm/s) of a cecum was found by averaging the slope of propagating contractions. Finally, mean amplitude (cm) of contractions for ceca were determined by averaging the change in cecal diameter before and during peak contractions.

#### 2.2.5 Statistical Analysis: Determination of Pecking Differences, Ceca Differentiation, and Comparison of Motility Variables by Pecking Group and Treatment

As a first step, we used a generalized linear mixed model with pecking phenotype as the class variable in order to determine if true differences in feather pecking existed between the two phenotypic classes to which we assigned our birds (P or NP). The Glimmix procedure of SAS version 9.4 was used to perform all statistical analyses (version 9.4, SAS Institute Inc., Cary, NC, USA, 2016). As average number of pecking bouts per bird per day was used in order to assign a phenotype, pecking bouts were count data and thus, a Gaussian model was inappropriate. A Poisson distribution was used and no random effects were partitioned. The model was transformed with a log link and Least Square Means and standard errors on the data scale were obtained using the ilink option. Differences between the means were compared using a Tukey-Kramer adjustment. The confidence interval of the Least Square Means for both classes was requested in order to determine if there was any overlap. Should the confidence intervals have overlapped, we could state with certainty that the two classes did not significantly differ from one another in their pecking scores. The studentized residuals were requested in order to determine if any outliers were present. In order to confirm the assumptions of the variance analysis, scatterplots of the studentized

residuals against the predicted values and the phenotype (treatment), as well as a Shapiro-Wilk test of normality were also requested. For all statistical tests, a Type 1 error rate of 0.05% was used.

Next, the average frequency, velocity and amplitude of contractions in each cecum overall were compared in order to determine whether results for each cecum should be analyzed separately. A generalized linear mixed model using the ceca number as a factor was used to perform this analysis. A Gaussian model was assumed to be most appropriate, and a normal distribution model was used. The variance of each dependent variable (i.e. velocity, frequency, and amplitude) was partitioned into a model with ceca number as the fixed effect. The same requests were made as above (i.e. Least Square Means, standard errors, confidence intervals, studentized residuals, Shapiro-Wilk test of normality).

Finally, the data was sorted by phenotype and a Spearman rank correlation was carried out using the baseline motility data and the pecking data to determine if any correlation was present between average number of pecking bouts per day and the dependent variables (i.e. velocity, frequency, and amplitude). Then, the frequency, velocity and amplitude of cecal contractions for H and L birds and for each treatment (Baseline, *Lactobacillus rhamnosus JB-1*, *Saccharomyces boulardii*) were compared. As there was found to be no difference in contraction frequency, velocity, and amplitude between the first and second ceca to be run in the bath, an average of each of these variables for each bird was used when both ceca contracted in the bath. When only one ceca contracted, then only this value was used. A generalized linear mixed model with a repeated measure covariance structure using the class variables treatment, phenotype and wing tag ID was used to analyze the data. For velocity and amplitude, a Gaussian model was most appropriate, and a normal distribution model was used. For frequency, a lognormal distribution with an identity link was used and the data was back-transformed following the model. The variance of each

dependent variable (i.e. velocity, frequency, and amplitude) was partitioned into the fixed effects of phenotype, treatment, and the phenotype\*treatment interaction. The random effect of treatment nested by ID was partitioned and an autoregressive covariance structure was selected based on a plot of the studentized residuals and fit statistics. The same requests were made as above (i.e. Least Square Means, standard errors, confidence intervals, studentized residuals, Shapiro-Wilk test of normality).

## 2.3 Results

### 2.3.1 Pecking Phenotype

The two phenotypic classes to which we assigned our birds differed significantly in the number of pecking bouts they performed ( $F_{1,15} = 70.19$ ,  $P < .0001$ ). The P group showed a mean number of pecking bouts per day of  $15.9 \pm 1.44$  (95%CI 13.1;19.3) while the NP group had a mean number of bouts of  $1.1 \pm 0.33$  (95%CI 0.5;2.0). This result was also emphasized by the 95% confidence intervals of these classes which did not overlap, illustrating that the P group had a significantly higher number of pecking bouts per day than the NP group. However, no significant differences in pecking were seen between the CTL birds and the HFP birds ( $F_{1,15} = 1.15$ ,  $P = 0.3011$ ). The CTL birds showed a mean number of pecking bouts per day of  $3.4 \pm 0.81$  (95%CI 2.1;5.7) while the HFP birds had a mean number of bouts of  $4.87 \pm 1.084$  (95%CI 3.03;7.82). This result was also emphasized by the 95% confidence intervals of the lines, which overlapped. The line\*phenotype interaction was not statistically significant ( $F_{1,15} = 0.13$ ,  $P = 0.7256$ ).

### 2.3.2 Differences in Motility Between the Paired Ceca

As a second step, we determined whether the first and second ceca from each bird differed in their velocity, frequency and amplitude. No significant differences between the two ceca were found in any of our three variables. For 30 birds with two ceca each, 60 observations were used for each variable in these “Ceca Difference” calculations. The baseline velocity, frequency, and amplitude

were used as variables in this analysis. Overall, the average velocity of contractions in the first ceca was  $0.17\text{cm/s} \pm 0.040\text{cm/s}$  (95%CI 0.09;0.25) while in the second ceca average velocity was  $0.22\text{cm/s} \pm 0.040\text{cm/s}$  (95%CI 0.14;0.30). The average frequency of contractions in the first ceca was  $0.043\text{Hz} \pm 0.0078\text{Hz}$  (95%CI 0.027;0.058) while in the second ceca average frequency was  $0.049\text{Hz} \pm 0.0078\text{Hz}$  (95%CI 0.034;0.065). Finally, the average amplitude of contractions in the first ceca was  $0.04\text{cm} \pm 0.012\text{cm}$  (95%CI 0.01;0.06) while in the second ceca average amplitude was  $0.05\text{cm} \pm 0.012\text{cm}$  (95%CI 0.02;0.07). Between the two ceca, no significant differences in the velocity ( $F_{1,58} = 0.95$ ,  $P = 0.3336$ ), frequency ( $F_{1,58} = 0.36$ ,  $P = 0.5529$ ), or amplitude ( $F_{1,58} = 0.47$ ,  $P = 0.4963$ ) of cecal contractions in the baseline were found.

Thus, for birds where both ceca worked in the tissue bath ( $n = 12$ ), an average of both measurements for each variable was taken and used in later analyses. For birds from which only one ceca worked in the tissue bath ( $n = 9$ ), only that measurement was used in later analyses.

### 2.3.3 Velocity

In our exploratory Spearman correlation statistics, a strong positive correlation was found between average number of pecking bouts per day and velocity in the P group (Spearman's  $\rho = 0.81172$ ,  $P = 0.0079$ ; See **Table 7**). This means that if the average number of daily pecking bouts exhibited by a given bird is high within this group, then the velocity is also likely to be high. In the NP group, no such correlation was found (Spearman's  $\rho = -0.38988$ ,  $P = 0.2359$ ; See **Table 7**). Velocity measures differed among P and NP birds with the highest velocity being observed in the baseline P group ( $0.49\text{cm/s} \pm 0.049\text{cm/s}$ ) to the lowest velocity being found in the baseline NP birds ( $0.34\text{cm/s} \pm 0.032$ ), mirroring the results of the Spearman correlation. In the model itself, the treatment (baseline, *Lactobacillus*, *Saccharomyces*)\*phenotype (P, NP) interaction was significant ( $F_{2,31} = 4.98$ ,  $P = 0.0134$ ; See **Table 4**), indicating that treatment effects were not generalized

across both groups (H and L) and that each group should be examined individually within each treatment for the velocity variable. Based on p values adjusted for a multiple means comparison, velocity of contractions in the baseline of the P group tended to differ from the velocity measures recorded for this group during luminal perfusion of both *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* ( $P = 0.1$ ). This was not the case in the NP group. Neither velocity measured during perfusion of *Lactobacillus rhamnosus* (JB-1) or *Saccharomyces boulardii* differed from baseline measures of velocity in the NP group ( $P = 0.6$ ). This interaction illustrates that the luminal microbial stimuli seemed only to cause a response in the P group.

There were no significant differences found in the velocity of cecal contractions between the three different treatments of this experiment ( $F_{2,31} = 2.35$ ,  $P = 0.1126$ ; See **Table 4**) illustrating that there was no general treatment effect on velocity. In the baseline, the average contraction velocity of the P group was  $0.49\text{cm/s} \pm 0.049\text{cm/s}$  (95%CI 0.39;0.59) while in the NP group the average velocity was  $0.34\text{cm/s} \pm 0.032\text{cm/s}$  (95%CI 0.27;0.40). When the tissue was perfused with *Lactobacillus rhamnosus*, the average velocity in the P group was  $0.32\text{cm/s} \pm 0.042\text{cm/s}$  (95%CI 0.24;0.41) while in the NP group the average velocity was  $0.34\text{cm/s} \pm 0.031\text{cm/s}$  (95%CI 0.28;0.41). Upon perfusion with *Saccharomyces boulardii*, the average velocity in the P group was  $0.34\text{cm/s} \pm 0.042\text{cm/s}$  (95%CI 0.26;0.43) while in the NP group it was  $0.40\text{cm/s} \pm 0.032\text{cm/s}$  (95%CI 0.34;0.47). See **Table 1/Figure 1**.

No significant differences in the velocity of cecal smooth muscle contractions between the P group and NP group were found overall ( $F_{1,19} = 0.42$ ,  $P = 0.5227$ ; See **Table 4**). The P group had an overall average velocity of  $0.39\text{cm/s} \pm 0.030\text{cm/s}$  (95%CI 0.32;0.45) while the NP group had an average velocity of  $0.36\text{cm/s} \pm 0.021\text{cm/s}$  (95%CI 0.32;0.45). See **Table 1/Figure 4**.



#### 2.3.4 Frequency

In our exploratory Spearman correlations, no significant correlations were found between frequency and average number of pecking bouts per day in either the P or NP groups (P: Spearman's  $\rho = -0.42678$ ,  $P = 0.2520$ ; NP: Spearman's  $\rho = 0.12213$ ,  $P = 0.7205$ ; See **Table 7**). However, a significant treatment effect was found ( $F_{2,31} = 6.52$ ,  $P = 0.0043$ ; See **Table 5**). The treatment\*phenotype interaction was not significant ( $F_{2,31} = 1.37$ ,  $P = 0.2679$ ; See **Table 5**), indicating that the treatment effects were generalized across the two groups and thus these effects should be examined generally and not be partitioned into the groups.

To reiterate, a significant effect of the treatments on the frequency was found and was generalized across both phenotype groups ( $F_{2,31} = 6.52$ ,  $P = 0.0043$ ). In both groups, the baseline frequency of contractions was the highest followed by frequency during the *Saccharomyces boulardii* treatment and then the *Lactobacillus rhamnosus* treatment. In the baseline, the average overall contraction frequency was  $0.07\text{Hz} \pm 0.024\text{Hz}$ . When the tissue was perfused with *Lactobacillus rhamnosus*, the average frequency was  $0.019\text{Hz} \pm 0.0057\text{Hz}$ . Upon perfusion with *Saccharomyces boulardii*, the average frequency was  $0.028\text{Hz} \pm 0.0087\text{Hz}$ . See **Table 2/Figure 2**.

No significant difference in the frequency of cecal smooth muscle contractions between the P and NP groups was found ( $F_{1,19} = 0.22$ ,  $P = 0.6434$ ; See **Table 5**). The P group had an average frequency of  $0.04\text{Hz} \pm 0.016\text{Hz}$  while the NP group had an average frequency of  $0.030\text{Hz} \pm 0.0086\text{Hz}$ . See **Table 2/Figure 5**.

#### 2.3.5 Amplitude

In our exploratory Spearman correlations, a strong positive correlation was found between average number of pecking bouts per day and amplitude in the P group (Spearman's  $\rho = 0.73641$ ,  $P = 0.0237$ ; See **Table 7**). This means that if the average number of daily pecking bouts exhibited by

a given bird is high within this group, then the amplitude is also likely to be high. In the NP group, no such correlation was found (Spearman's  $\rho = -0.30063$ ,  $P = 0.3690$ ; See **Table 7**). The treatment effect approached significance ( $F_{2,31} = 2.71$ ,  $P = 0.0820$ ; See **Table 6**). The treatment\*phenotype interaction was not significant ( $F_{2,31} = 0.51$ ,  $P = 0.6064$ ; See **Table 6**), indicating that the treatment effects were generalized across the two groups and thus these effects should be examined generally and not be partitioned into the groups.

To reiterate, the treatment effect tended towards significance ( $F_{2,31} = 2.71$ ,  $P = 0.0820$ ). In the baseline, the average contraction amplitude was  $0.06\text{cm} \pm 0.019\text{cm}$  (95%CI 0.02;0.10). When the tissue was perfused with *Lactobacillus rhamnosus*, the average amplitude was  $0.10\text{cm} \pm 0.018\text{cm}$  (95%CI 0.06;0.13). Upon perfusion with *Saccharomyces boulardii*, the average amplitude was  $0.10\text{cm} \pm 0.018\text{cm}$  (95%CI 0.06;0.14). To summarize, amplitude was highest during the treatment with *Saccharomyces boulardii* followed by the *Lactobacillus rhamnosus* (JB-1) treatment. The lowest amplitude was found in the baseline. See **Table 3/Figure 3**.

No significant difference in the amplitude of cecal smooth muscle contractions between the P group and the NP group was found ( $F_{1,19} = 0.35$ ,  $P = 0.5609$ ; See **Table 6**). The P group had an average amplitude of  $0.08\text{cm} \pm 0.025\text{cm}$  (95%CI 0.02;0.13) while the NP group had an average amplitude of  $0.10\text{cm} \pm 0.018\text{cm}$  (95%CI 0.06;0.13). See **Table 3/Figure 6**.

## 2.4 Discussion

Some human psychological disorders have been associated with gastrointestinal diseases (Buie et al., 2010), including alterations of gut motility. Additionally, the motility of mammalian gut segments can be modified by probiotic strains such as *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* (West et al., 2016a; West et al., 2016b). which offers new therapeutic perspectives in both psychological and gastrointestinal disorders (Aizawa et al., 2016; Ojetti et al.,

2014). However, whether psychological disorders cause or are a consequence of gastrointestinal disorders is unknown. In addition, the precise interplay of underlying molecular mechanisms leading to changes in psychological and gastrointestinal well-being by probiotics are largely unknown.

Similar associations could be hypothesized in domestic birds kept for egg laying, since movement of ingesta through the gastrointestinal tract was shorter (Harlander-Matauschek et al., 2006) and *Lactobacillus* manifestation in the ceca was lower (Birkel et al., 2018) in birds performing abnormally high levels of repetitive pecking (behavioural disorder) at the feather cover of other birds than in non-peckers. Yet, in order to establish an association between behavioural and gastrointestinal disorders in domestic birds, the following three key questions need to be answered: Firstly, can we measure movements of the gut wall in peckers and non-peckers in an ex-vivo model used in mammalian research? Secondly, if we can measure movement of the gut wall, is there a motility response evoked by bacteria, such as *Lactobacillus rhamnosus (JB-1)* and *Saccharomyces boulardii*. Thirdly, do peckers and non-peckers differ in their response?

**A mammalian ex-vivo organ bath can be used to measure avian ceca motility.** In total, 33 ceca out of 60 were still alive at the time of baseline recordings. Ceca that did not contract during the first hour of recording were considered dead. For 9 birds, only one ceca contracted in the bath. For 12 birds, both ceca contracted. For 9 birds, neither ceca contracted (6 P, 6 NP). In summary, tissue from 7 P birds and 14 NP birds survived (Total N = 21 birds). These results illustrate the variation in survivability of the tissue between birds in this in vitro system. However, on average this system was able to keep the cecal tissue alive for 1 – 4 hours. Variation in survivability might result from slight individual differences in the duration of dissection and excision of the tissue from the time of death of the bird.

An important difference between mammalian and avian tissue bath systems is that an extra stimulus is needed to elicit peristalsis in avian models. While intraluminal pressure is sufficient to stimulate contractile activity in mammalian models (West et al., 2018b), intraluminal pressure alone is not sufficient to initiate peristalsis in the avian ceca (Hodgekiss, 1983). However, a combination of raising intraluminal pressure and the presence of local anaesthetics such as procaine have elicited contractions in the avian ceca in in vitro systems in past studies, suggesting that the mechanism of peristalsis in the chicken gut is quite different to that found in mammalian species (Hodgekiss, 1983). It was for this reason that lidocaine hydrochloride monohydrate was added to this in vitro system at the same concentration as procaine used in past avian studies (Hodgekiss, 1983).

**The paired ceca do not differ in velocity, amplitude, frequency.** Our findings that the two ceca did not differ in the velocity, frequency or amplitude of contractions in each respective bird are in line with observations made by Duke in 1989 in their study on cecal contractions in turkeys. They found that there was coordination between the ceca 84% of the time during evacuation events (Duke, 1989).

In addition, our results were comparable to those of other studies. For example, in 1982 Duke measured the frequency of cecal contractions in turkeys and found an average frequency of 2.6 cycles/min  $\pm$  0.3 cycles/min (Duke, 1982). This translates to 0.04 cycles/sec  $\pm$  0.005 cycles/sec. Average frequency of contractions measured in the present study 0.07 cycles/sec  $\pm$  0.024 cycles/sec. In one other study conducted on contractile activity in the avian ceca, velocities ranging from 1.9 mm/sec to 3.3 mm/sec were found (Janssen et al., 2009) and this is comparable to the average values of 0.49cm/s (4.9 mm/s) and 0.34cm/s (3.4 mm/sec) found in our two groups of birds.

**There was a positive correlation between a high number of pecking bouts and high baseline ceca velocity in feather peckers (P).** Interestingly, in mammals there is evidence that acute and chronic exercise increases colonic motility (Gisolfi et al., 2000). In humans, acute strenuous exercise causes gastrointestinal stress in the form of abdominal pain, diarrhea and even gastrointestinal bleeding (de Oliveira & Burini, 2009). Distance runners are susceptible to intestinal motility disorders that include diarrhea and irritable bowel syndrome (van Nieuwenhoven et al., 2004; Peters et al., 1999). Although the precise pathophysiology remains largely undefined, the possible causative factors include enteric fluid and electrolyte balance, mesenteric ischemia, increased mucosal permeability, and mechanical trauma (Casey et al., 2005). Similar considerations could be assumed in domestic birds. Peckers perform significantly higher levels of locomotor activity in general than non-peckers and travel greater distances within their environments via walking and running (Kjaer, 2009). These findings have led to the suggestion of a hyperactivity disorder model for feather pecking (Kjaer., 2009). It is possible that the higher activity exhibited by feather peckers affects their gastrointestinal motility in the form of the higher contraction velocity found in the peckers.

**Result 4: There was a significant interaction between pecking phenotype and bacteria/yeast treatment, where the luminal stimulus of both *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces* significantly lowered velocity in feather peckers (P).** This result showed that *Lactobacillus* and *Saccharomyces* affect the enteric nervous system and the cecal musculature only in peckers. *Lactobacillus* and *Saccharomyces* induced a relaxation response (i.e. lower velocity) in the peckers. Whether or not this effect is positive for the well-being of the birds remains to be seen and should be examined in future hypothesis-driven experiments.

**The frequency of ceca movements was significantly lower under *Lactobacillus* and *Saccharomyces* treatment compared to baseline measurements in peckers and non-peckers.**

This result mirrors the findings of other experiments in which both *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* were able to lower the frequencies of colonic contractions in stressed mice (West et al., 2016a; 2016b). This finding demonstrates a potential for certain microbes to alter intestinal motility for the first time in an avian species.

## Chapter 3: Limitations and Conclusions

### 3.1 Limitations

As a preliminary study, this experiment provided a good starting point for the use of this *in vitro* system in studies of avian gut motility. However, there are a number of limitations to this model. Firstly, given that only isolated segments of tissue can be used in this system, it is impossible to make conclusive interpretations about these results in the context of the whole body and to state whether these effects would be replicated in live animals. Drawing conclusions about the welfare implications of these findings is also out of the question. To this end, future experiments should measure the *in vivo* effects of these treatments on feather pecking behaviour and the well-being of the live animal.

While the use of the cecum as the tissue of choice for this experiment was logical given this organ's link to both fibre digestion and stress physiology in birds, it needed to be modified in order to fit to the apparatus. An incision of 0.5cm in length was made at the distal, blind end of this organ to provide an outflow point for the physiological buffer solution and it is possible that this modification had unintended consequences on the contractility of the tissue. In future studies, other sections of the avian GIT that are not blind-ended and are thus more suited to the design of this apparatus might be used instead, such as segments of the jejunum and colon. In addition, other parameters of motility such as direction of propagation of contractions and changes in the length of the tissue might have provided additional important information that was not included in this study.

### 3.2 Conclusions

In summary, this study examined a possible connection between cecal motility and feather pecking in laying hens from a genetic and phenotypic standpoint. The primary objective was to investigate whether differences in feed passage time observed in feather pecking birds might be attributable

to intrinsic differences in gut smooth muscle contraction. This study represents the first attempt to use an in vitro apparatus to correlate intestinal motility with behaviour in chickens, a connection that has been established in humans. An in vitro tissue bath system used in studies of gut motility in mammals was successfully used to measure motility in birds with some minor modifications. There were no differences in parameters of motility (i.e. velocity, frequency, and amplitude of contractions) between the two ceca in each bird. In the feather pecker group, if the average number of daily pecking bouts exhibited by a given bird was high, then the velocity was also likely to be high. The *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* treatments were able to significantly lower velocity but only in the feather pecking group. Across both groups, the frequency of ceca movements was lower under *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* treatments.



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## Tables

**Table 1.** Least square means of the average velocity (cm/s) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution (Baseline), *Lactobacillus rhamnosus* (JB-1) solution, and *Saccharomyces boulardii* yeast in a tissue bath system at the University of Guelph, Ontario, Canada in 2017

Group	Mean*	Tukey Means Comparison**	Standard Error
Baseline: P	0.49	A	0.049
Baseline: NP	0.34	A	0.032
<i>Lactobacillus rhamnosus</i> : P	0.32	A	0.042
<i>Lactobacillus rhamnosus</i> : NP	0.34	A	0.031
<i>Saccharomyces boulardii</i> : P	0.34	A	0.042
<i>Saccharomyces boulardii</i> : NP	0.40	A	0.032
P	0.39	a	0.030
NP	0.36	a	0.021

\*Means followed by the same letter are not significantly different according to a Tukey's test ( $\alpha=0.05$ ,  $n=18$ )

\*\*Lowercase and uppercase letters denote separate comparisons

**Table 2.** Least square means of the average frequency (Hz) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution (Baseline), *Lactobacillus rhamnosus* (JB-1) solution, and *Saccharomyces boulardii* yeast in a tissue bath system at the University of Guelph, Ontario, Canada in 2017

Group	Mean*	Tukey Means Comparison**	Standard Error
Baseline	0.07	A	0.024
<i>Lactobacillus rhamnosus</i>	0.019	B	0.0057
<i>Saccharomyces boulardii</i>	0.028	B	0.0087
P	0.04	a	0.016
NP	0.030	a	0.0086

\*Means followed by the same letter are not significantly different according to a Tukey's test ( $\alpha=0.05$ ,  $n=18$ )

\*\*Lowercase and uppercase letters denote separate comparisons

**Table 3.** Least square means of the average amplitude (cm) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution (Baseline), *Lactobacillus rhamnosus* (JB-1) solution, and *Saccharomyces boulardii* yeast in a tissue bath system at the University of Guelph, Ontario, Canada in 2017

Group	Mean*	Tukey Means Comparison**	Standard Error
Baseline	0.06	A	0.019
<i>Lactobacillus rhamnosus</i>	0.10	A	0.018
<i>Saccharomyces boulardii</i>	0.10	A	0.018
P	0.08	a	0.025
NP	0.10	a	0.018

\*Means followed by the same letter are not significantly different according to a Tukey's test ( $\alpha=0.05$ ,  $n=18$ )

\*\*Lowercase and uppercase letters denote separate comparisons

**Table 4.** Variance analysis summary of the average velocity (cm/s) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution, *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* (treatments) through the lumen of the ceca in a tissue bath system and average pecking bouts per day directed at conspecifics used as a covariate in the model at the University of Guelph, Ontario, Canada in 2017

Covariance Parameters	Estimate	SE		
AR(1)	0.2723	0.1870		
Residual	0.01231	0.002586		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
treatment	2	31	2.35	0.1126
phenotype	1	19	0.42	0.5227
treatment*phenotype	2	31	4.98	0.0134

**Table 5.** Variance analysis summary of the average frequency (Hz) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution, *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* (treatments) through the lumen of the ceca in a tissue bath system and average pecking bouts per day directed at conspecifics used as a covariate in the model at the University of Guelph, Ontario, Canada in 2017

Covariance Parameters	Estimate	SE		
AR(1)	0.5849	0.1356		
Residual	1.7130	0.4230		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
treatment	2	31	6.52	0.0043
phenotype	1	19	0.22	0.6434
treatment*phenotype	2	31	1.37	0.2679

**Table 6.** Variance analysis summary of the average amplitude (cm) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution, *Lactobacillus rhamnosus* (JB-1) and *Saccharomyces boulardii* (treatments) through the lumen of the ceca in a tissue bath system and average pecking bouts per day directed at conspecifics used as a covariate in the model at the University of Guelph, Ontario, Canada in 2017

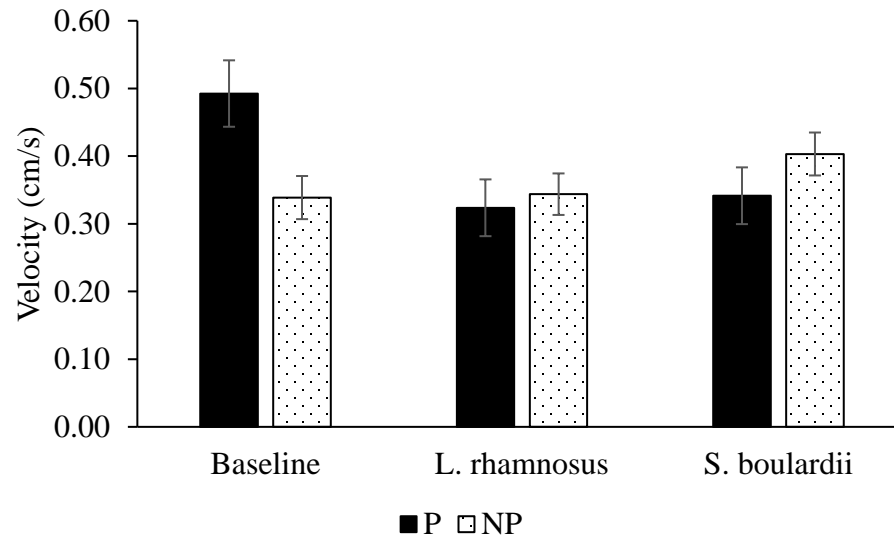
Covariance Parameters	Estimate	SE		
AR(1)	0.6795	0.09963		
Residual	0.005880	0.001478		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
treatment	2	31	2.71	0.0820
phenotype	1	19	0.35	0.5609
treatment*phenotype	2	31	0.51	0.6064



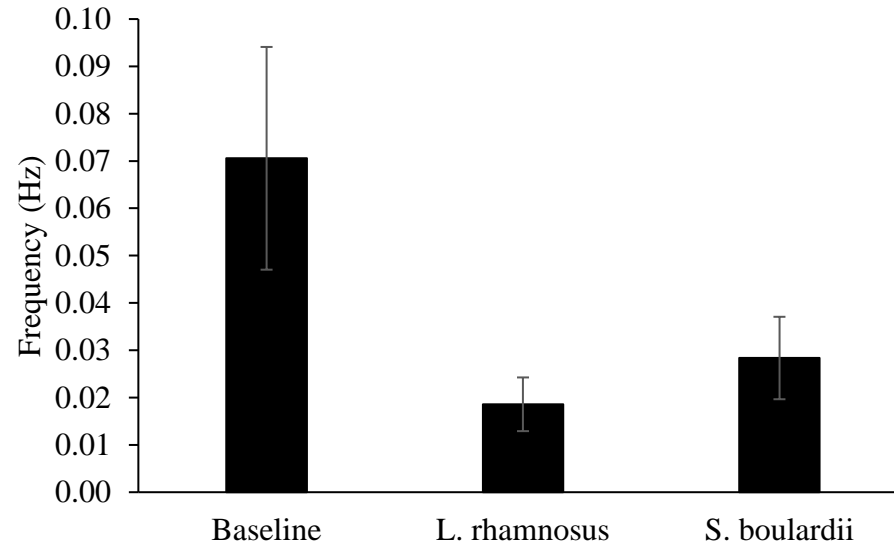
**Table 7.** A summary of Spearman rank correlations between the baseline velocity (cm/s), frequency (Hz), and amplitude (cm) of in vitro cecal contractions and the average number of pecking bouts per day directed at conspecifics in a Pecking (P) and Non-Pecking (NP) group laying hens at the University of Guelph, Ontario, Canada in 2017

Group	Velocity		Frequency		Amplitude	
	Spearman's rho	P Value	Spearman's rho	P Value	Spearman's rho	P Value
P	0.81172	0.0079	-0.42678	0.2520	0.73641	0.0237
NP	-0.38988	0.2359	0.12213	0.7205	-0.30063	0.3690

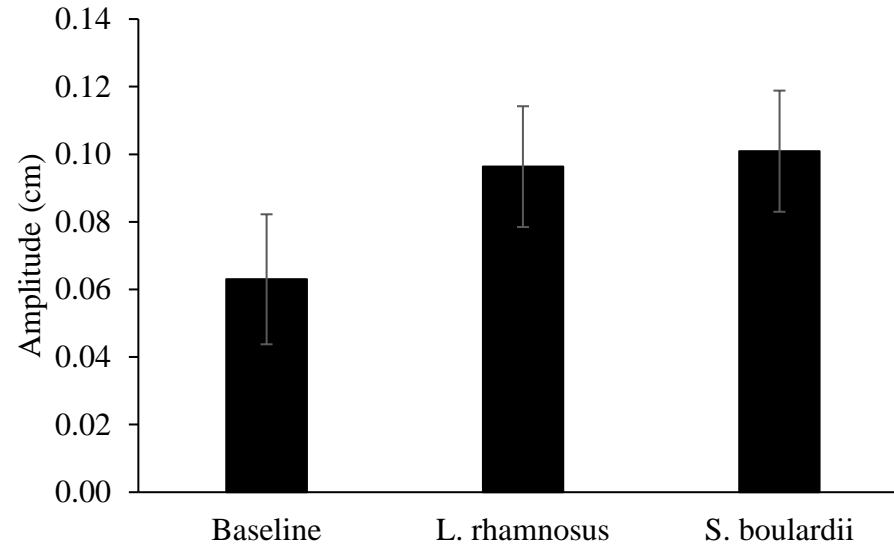
## Figures



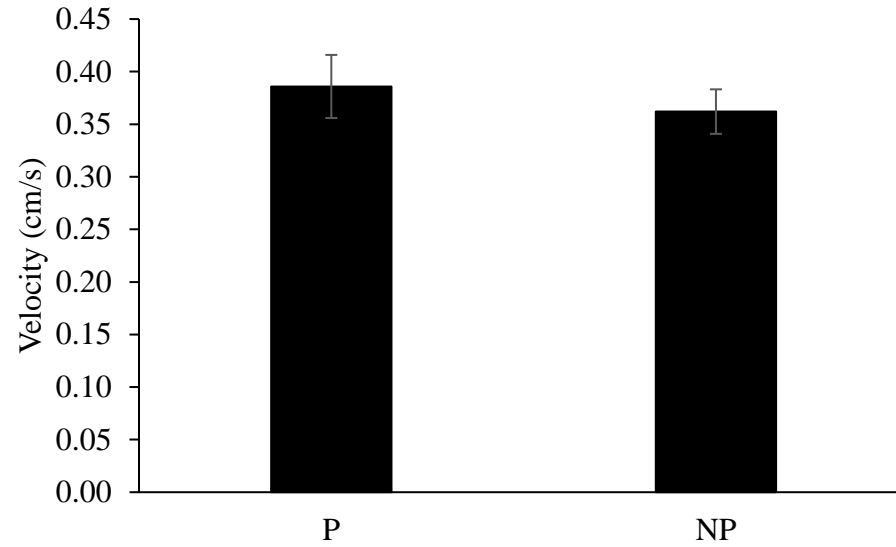
**Figure 1.** The least square means of the average velocity (cm/s) of in vitro cecal contractions from a Pecking (P) and Non-Pecking (NP) group of laying hens when perfused with Krebs solution (Baseline), *Lactobacillus rhamnosus* (JB-1) solution, and *Saccharomyces boulardii* yeast in a tissue bath system at the University of Guelph, Ontario, Canada in 2017



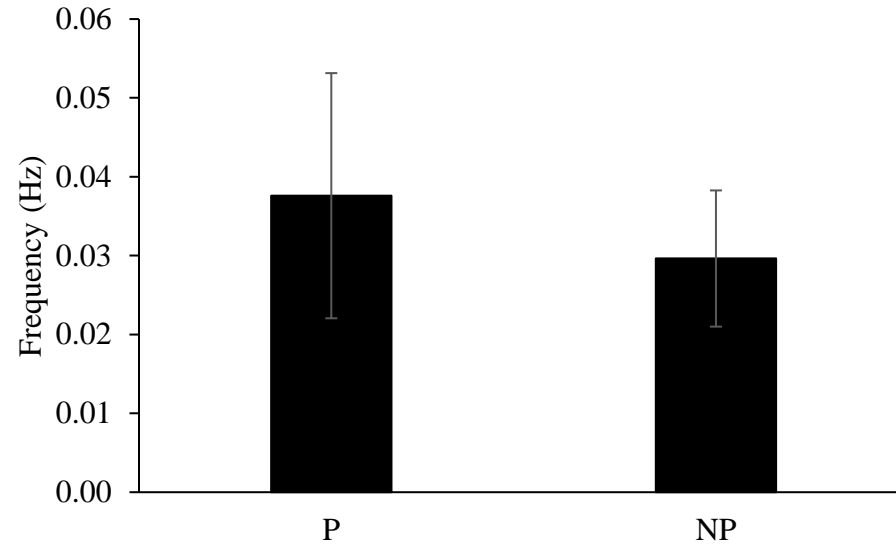
**Figure 2.** The least square means of the average frequency (Hz) of in vitro contractions of laying hens' ceca when perfused with Krebs solution (Baseline), *Lactobacillus rhamnosus* (JB-1) solution, and *Saccharomyces boulardii* yeast in a tissue bath system at the University of Guelph, Ontario, Canada in 2017



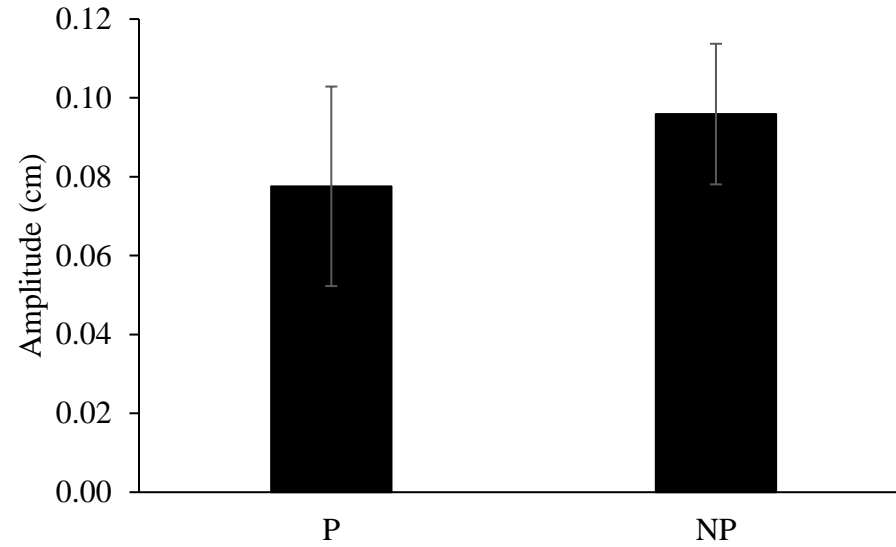
**Figure 3.** The least square means of the average amplitude (cm) of in vitro contractions of laying hens' ceca when perfused with Krebs solution (Baseline), *Lactobacillus rhamnosus* (*JB-1*) solution, and *Saccharomyces boulardii* yeast in a tissue bath system at the University of Guelph, Ontario, Canada in 2017



**Figure 4.** The least square means of the average velocity (cm/s) of in vitro contractions of ceca from a Pecking (P) and Non-Pecking (NP) group laying hens in a tissue bath system at the University of Guelph, Ontario, Canada in 2017



**Figure 5.** The least square means of the average frequency (Hz) of in vitro contractions of ceca from a Pecking (P) and Non-Pecking (NP) group laying hens in a tissue bath system at the University of Guelph, Ontario, Canada in 2017



**Figure 6.** The least square means of the average amplitude (cm) of in vitro contractions of ceca from a Pecking (P) and Non-Pecking (NP) group laying hens in a tissue bath system at the University of Guelph, Ontario, Canada in 2017