OREGANO: A POTENTIAL ALTERNATIVE TO ANTIBIOTICS AS GROWTH PROMOTER WITHIN THE BROILER INDUSTRY

by

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Abstract

This thesis presents the investigation of the effects of oregano supplementation on broiler microbiota and intestinal health presented as literature review (Chapter 1) followed by four published manuscripts (Chapters 2 to 5) and final conclusions (Chapter 6). The initial *in vitro* assay uses specially designed microbiological media that could sustain a large proportion of chicken intestinal ileal and cecal bacteria. 16S rRNA gene sequencing was used to investigate the overall changes to the microbial community as well as individual taxa.

This was followed by *in vivo* effects of different concentrations of oregano on microbiota structure of broilers, including alpha and beta diversity, pathogen presence and abundance, effects on beneficial microbiota and dosage effects. Different sections of the gut were investigated for a complete understanding of where the oregano has significant effects due to the fast absorption of phytogens. Short-chain fatty acids and histological analysis of the different gut sections were also investigated. The investigation of the influence oregano on the development and maturation of intestinal microbiota followed. Surprisingly, even a high concentration of oregano did not result in very different mature microbiota; the differences were small and variable from week to week.

Lastly, the change of functional abilities of microbiota from the gut of broilers supplemented with 2% oregano powder was investigated. The 16S amplicon sequencing was used in combination with PiCrust algorithm to predict the genes and functions present in microbiota of control and oregano. The data suggested significant changes in function rather than in microbiota. Oregano reduced the abundance of genes involved in the ability of bacteria to invade epithelia, make toxins and move. It also reduced a range of infection and disease-related genes and reduced abundance of genes involved in carbohydrate and lipid metabolism and steroid synthesis.

Finally, in the last chapter, a discussion of the outcome of the thesis and the possibility of oregano and other phytogens causing and selecting for antimicrobial resistance on farms, which was absurdly the very reason the antibiotics were replaced with phytogens. And a proposition of future work and directions recommended from the outcomes of this thesis.

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Peer-reviewed publications

Bauer, B.W., Gangadoo, S., Bajagai, Y.S., Van, T.T.H., Moore, R.J., and Stanley, D. Oregano powder reduces *Streptococcus* and increases SCFA concentration in a mixed bacterial culture assay of chicken, PloS One (2019).

Bauer, B.W., Radovanovic, A., Willson, N., Bajagai, Y.S., Van, T.T.H., Moore, R.J., and Stanley D. Oregano: A potential prophylactic treatment for the intestinal microbiota, Heliyon 5 (10), e02625, (2019)

Bauer, B.W., Bajagai, Y.S*., Alsemgeest, J., Willson, N.L., Van, T.T.H., Moore, R.J., and Stanley, D. Functional analysis of changes in gut microbiota genetic potential in broilers supplemented with 2% oregano. Australian Poultry Science Symposium. 2020

Bajagai, Y.S., **Bauer, B.W*.,** Alsemgeest, J., Willson, N.L., Van, T.T.H., Moore, R.J., and Stanley, D. Effects of supplemented oregano on the maturation of broiler microbiota. Australian Poultry Science Symposium. 2020

***Equal Contribution**

Publications that are not part of this thesis

Gangadoo, S., **Bauer, B. W.,** Bajagai, Y. S., Van, T. T. H., Moore, R. J., & Stanley, D. (2019). In vitro growth of gut microbiota with selenium nanoparticles. Animal Nutrition. https://doi.org/10.1016/j.aninu.2019.06.004

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Chapter 1

Oregano: a potential alternative to antibiotics as growth promoters within the broiler industry

Abstract

Antimicrobial Growth Promoters (AGPs) have been scrutinised for increasing microbial resistance. The removal of AGPs in the EU has added global pressure to their total abolition. Phytoadditives of plants that naturally produce antimicrobials have progressed as a replacement to AGPs, as they have shown to have similar antimicrobial effects to antibiotics. Oregano has been widely studied for the antimicrobial attributes of its two main volatile constituents, carvacrol and thymol. Oregano essential oil (OEO) has been the focus of the vast majority of previous studies. This study aims to investigate the efficacy of oregano in pathogen control, compare it activity to currently used AGPs and optimize the concentration of dried oregano for use in organic and other AGP free animal production. Delivering oregano as a dry spice with optimized particle size will resolve problems of volatility and rapid absorption that prevented the use of essential oils in the feed. Using whole oregano will also add dietary fibre which is known to promote the growth of beneficial bacteria. Here we provide a review of the literature supporting the benefits of oregano and phytogens generally, in livestock pathogen control.

Introduction

There is increasing global pressure for the complete ban on antibiotic (AB) growth promoters. Media often release stories that enhance public support of the ban (Economist, 2017, Harvey, 2017, Øistein, 2014) but fail to report that hospitals are the major source of AB resistance (Corbella X, 2000, Shlaes D M, 2016) and that the use of AGPs not only controls zoonotic and host pathogens but also assists in the increased feed conversion ratio (FCR) and body weight gain (BWG) of animals. The only poultry AGP that is also used in human health is the zinc bacitracin (ZnBac). The Australian poultry industry has voluntarily removed AGPs from the production. Despite the expectation that AGPs would devastate a healthy microbial community, their concentrations used in broiler feed were sub-therapeutic, and minimal disturbance to intestinal microbiota of birds was reported (Crisol-Martinez 2015). The industry is acting preemptively by creating organic and antibiotic-free products for pathogen control. A natural/organic products, capable of control of the host and zoonotic pathogens to the same degree as AGPs and without adverse effects on birds health and welfare are needed. This study aims to investigate oregano powder as a potential alternative to AGPs.

The intestinal tract of any animal is home to diverse and dynamic transient populations of microbes. Antibiotic growth promoters controlled these microbial communities by reducing the chance of pathogen and zoonotic species presence (Phillips et al., 2004, Davies and Davies, 2010, Theuretzbacher, 2013). After the ban of AGPs, the use of therapeutic drugs in Denmark increased by 21% between 1988-1994, due to their use in the agricultural industry. Farmers and the Danish government agreed to the removal of antimicrobial products in 1994. Between 1994 and 2003 AGP use declined by 47% (Grave et al., 2006). A study conducted in Denmark indicated that the removal of AGPs in 1998 did not have any significant impact on weight gain or bird deaths; however, the feed conversion ratio increased marginally (Emborg et al., 2000). Another study also indicated that when selective pressure is restricted, microbial resistance becomes less prevalent (Aarestrup et al., 2001, Wegener, 2003). While these studies indicate that there is little need for AGPs to be used in the agricultural system, in the context presented, it does not highlight the costs to farmers/government (Jensen and Hayes, 2014). It is not clear how successfully other countries could replicate such changes. There are strong arguments that challenge the evidence that AGP use is a major cause of microbial resistance (Phillips et al., 2004) and that the hospitals are the main source of antibiotic resistance (McGowan, 1983). It is also believed that necrotic enteritis has increased since the removal of AGPs in Europe (Timbermont et al., 2011, Du et al., 2016). Furthermore, resistance occurs naturally both by

organisms producing the antimicrobial agent and by the targeted organisms (Phillips et al., 2004, Davies and Davies, 2010). The above demonstrates the need to efficiently replace AGPs with a viable alternative.

There are several potential alternatives to AGPs that are being studied (Joerger, 2003, Bontempo et al., 2014, Puvaca et al., 2013). Probiotics are one such potential alternative to AGPs. They are thought to function by exclusion, reduction of luminal pH and production of bacteriocins (Abdelrahman and Mohnl, 2014). Prebiotics alone or in combination with probiotics have been used to control pathogenic populations with moderate success (Ferket, 2004, Park and Floch, 2007, Schrezenmeir and de Verse, 2001). Other AGP alternatives include phytochemicals that contain phytobiotics which are most commonly found in dry herbs, spices and essential oils (EOs) (Brenes and Roura, 2010). Phytobiotics have shown to be of the broad-spectrum activity having anti-fungal, anti-parasitic and anti-bacterial properties. These alternatives aim to remove or control pathogens comparatively to AGPs. Most research has not investigated the total microbial communities and populations of their application *in vivo*.

The Lamiaceae family include antimicrobial herbs such as mint, sage, thyme, marjoram, and oregano. Oregano EO (OEO) consists of over 30 compounds, of which carvacrol and thymol and their precursors equate to 80% of contents (Brenes and Roura, 2010, Giannenas et al., 2003). Carvacrol and thymol, phytobiotics, are the known antimicrobial compounds in oregano and have shown to have high interaction with the cell membrane (Anjana Rao, 2010). Phytobiotics share characteristics with AGPs they affect a broad spectrum of organisms not only pathogens but probiotics, including some species of *Lactobacillus* (Horosova et al., 2006). With such strong peer-reviewed literature, we propose a hypothesis that oregano powder could influence microbial community structure, control pathogens and have an impact on bird performance.

The objectives of this project are to investigate if oregano can represent a viable alternative to AGPs, the following questions should be answered:

i) Have the microbial communities been disturbed or otherwise affected?
ii) Are the pathogens reduced in vitro and *in vivo*?
iii) Are the probiotic and other known beneficial bacteria reduced by oregano?
iv) Is there a difference in intestinal morphology?
v) Are there variations in host epithelial gene immune expression?
vi) Is there a difference in bird welfare and performance?

Notable factors

Environment

The environment that a broiler is exposed to will greatly impact on growth parameters (Ferket P R, 2004). Most important variables that must be considered include population density, feed type and adequate nutrition, exposure to the faecal waste, biosecurity and hygiene (van der Most et al., 2011, Mennerat et al., 2010). Farmers control the environment of their premises to maximise animal productivity. Some forms of animal husbandry can be referred to as industrialised farming. When industrialised farming first began, there were immediate problems with animal health, due to the high density of populations resulting in increased exposure to animal waste, enabling pathogen overgrowth. In the 1950's, scientists observed that prophylactic sub-therapeutic antibiotic use improved animal health and farms' yield (Emborg H. D, 2000). This was followed by improvements in biosecurity management strategies that have helped to elevate hygiene levels. Such management strategies decrease the stress that an animal endures and the chances of pathogen exposure rates. The role of AGPs is decreased as the environmental factors are optimised.

Dietary influence

During the process of consumption, nutrients, non-nutrients, beneficial or non-beneficial microorganisms are consumed (Yegani and Korver, 2008). The diet significantly impacts on intestinal flora and therefore the morphology of the intestinal tract. An animal that is undernourished will be more susceptible to disease and infection. Providing animals with appropriate levels of nutrients with the feed that is free of pathogens is essential. In the words of Anthelme Brillat-Savarin '*Dis-moi ce que tu manges, je te dirai ce que tu es'* [tell me what you eat, and I will tell you what you are] (Brillat-Savarin, 1826)

Key indicators to intestinal health

In recent years the value and understanding of intestinal health has been growing and is now a major area of research (Stanley et al., 2012). The gut is the largest endocrine organ in an animal's body system (Ahlman and Nilsson, 2001). There are many indicators to intestinal health; some key factors are located in the mucosa, which is the innermost layer of the intestinal tract. Intestinal health can be evaluated by examining microbial communities, the mucosa layer, mucus gel layer, epithelium layer, villi, crypt, and the cell types at specific locations along the gastrointestinal tract (GIT) such as the duodenum, jejunum, ileum and ceca.

Mucus Gel layer

The epithelium of the GIT is covered with a mucus gel layer. The mucus gel layer has the first contact with the intestinal lumen as such; it facilitates the line of defence against lumen content. Goblet cells produce mucin glycoproteins which are the main component of the mucus gel layer (Klasing, 1999). Therefore, there is a consistent presence of antimicrobial peptides and secretory immunoglobulin A (SIgA) (Dharmani et al., 2009).

The mucus gel layer can be produced by enterocytes; however, most of the mucin is produced by goblet cells (Tarabova et al., 2016). The mucus gel layer is comprised of approximately 95% water and 5% mucin. Goblet cells are located throughout the entirety of the gastrointestinal tract; however, the morphology and function of the mucus gel layer differ dependent on location and exposure (Deplancke and Gaskins, 2001). The morphology of mucus is comprised of two layers, one tightly and the other loosely bound to the epithelia. The tightly bound layer proximal to the epithelial layer functions in preventing bacterial access to some epithelial receptors (Shokryazdan et al., 2017, Slomiany et al., 2001, Johansson et al., 2013). The mucus gel layer is normally thinner in the small intestine, at times it may even not be present, and slowly thickens posteriorly being at its thickest throughout the large intestines (Slomiany, 2001, Atuma et al., 2001).

Mucins are gel-forming glycoproteins. The mucin backbone contains alternating O-linked glycans and nonglycosylated domains (Frostner et al., 1995), as the term “glyco” indicates they are a source of carbohydrate; as such it provides a food source for commensal and pathogenic bacteria and the attachment sites which facilitate niche environments (Sonnenburg et al., 2004). There are four main mucins associated in the gel layer being N-Acetylglucosamine, N-acetylgalactosamine, fucose, and galactose (Frostner J F, 1995, Deplancke B, 2001). These niche communities of commensal and pathogenic bacteria are carried away from the GIT (Abdelrahman W H A (Editor-in-Chief), 2014). The production of mucins from the goblet cells fluctuates. This fluctuation may be induced by direct bacterial interaction or by host bioactive factors after interaction with GIT associated bacteria (Slomiany, 2001, Deplancke B, 2001).These niche communities ofThere are many innate immune functions and defenses present within the mucus gel layer.

Villi

The villi are numerous slender projections of the epithelium of the GIT. Villi are covered by the mucus gel layer and extend into the intestinal lumen. The structure of villi and its increased height allows for the greater intestinal surface area, which is associated with increased

absorption (Yegani and Korver, 2008). When the lumen has toxins present the villi shorten and thin, which decreases surface area and therefore, absorption of nutrients from the intestinal lumen is lower (Xu et al., 2015). Deeper crypts indicate faster tissue turnover, so longer villi and shallower crypts ratio are desirable (Awad et al., 2009). The villi height is measured by taking the measurement from its tip to the crypt-villus junction; villi width can be taken, using crosshair, at the midpoint of the villi length from the epithelial layer to its midpoint (Sarica et al., 2014). The epithelium that comprises the villi contains intraepithelial lymphocytes (IELs) (Vervelde and Jeurissen, 1993), natural killer cells (NK) and goblet cells. Below the epithelial layer of the villi is an area called the lamina propria. The lamina propria contains T cells, B cells, heterophils (Kogut et al., 2005), NK cells and dendritic cells. Heterophils produce cytokines which among many other functions is associated with decreasing muscle growth. The villi:crypt ratio and gene expression of the mucosa is an extremely important indicator of the hosts' immune responses and stress the host has been encountering as seen in many studies (Du et al., 2016).

s'Tight Junctions

The epithelial layer of the intestinal tract is a layer of consecutive cells mostly consisting of enterocytes which are considerably surrounded by tight junction (TJ) proteins within the cellular lipid bilayer (Anderson and Van Itallie, 2009, Furuse, 2010). Tight junctions are composed of cytoplasmic actin-binding proteins and adhesive transmembrane proteins most specifically claudins (comprising a multigene family) and occludins (Anderson and Van Itallie, 2009, Applegate and Troche, 2014, Sonoda et al., 1999). According to Furuse (Furuse, 2010) a network of paired TJ strands generates a continuous belt that that established the diffusion barrier and controls the solute flow. The paracellular pathway is controlled by TJ’s and the pH of the extracellular environment, and the pathway accounts for 90% of the absorption of ions and nutrients (Furuse, 2010, Applegate T J, 2014).

A study conducted by Zeissig et al., (2007) analysed TJ structure be freeze-fracture electron microscopy of intestinal biopsy samples of patients with active and inactive Chron’s disease; the results showed reduced and discontinuous TJ strands. Furthermore, occludin and sealing TJ proteins claudin 5 and claudin 8 were downregulated and redistributed off the TJ, whereas the pore-forming tight junction protein claudin 2 was upregulated (Zeissig et al., 2007). CPE is a protein that consists of a single ~35kDa polypeptide with a C-terminal receptor-binding region and N-terminal toxicity (McClane, 2001). The ~35kDa polypeptide is reported to connect claudin-3 and claudin-4 at their COOH terminal half (Sonoda N and Horiguchi Y,

1999). In 2000, *C. perfringens* was the third most commonly identified foodborne disease in the USA (McClane B A, 2001). Sonoda (Sonoda N and Horiguchi Y, 1999) study the effects of *C. perfringens* enterotoxin on TJ's via a multitude of assays, including fluorescein isothiocyanate-dextran (FIT-C) dextran and freeze-fracture electron microscopy. The disruption of TJ formation is clearly visible in electron microscopy images taken time 0h, 4h and 8h. Likewise, the permeability of monolayers is evident by the FITC dextran assay (Sonnenburg et al., 2004, Sonoda N and Horiguchi Y, 1999). When the tight junction proteins are significantly disrupted the host can suffer from the leaky gut syndrome. If the leaky gut condition occurs in the host, large molecules and microbes can pass through the epithelial layer of the intestinal tract. This may lead to sever health issues/death of the host.

Necrotic enteritis (NE) caused by *C. perfringens* is a major pathogen within the broiler industry that has a significant global economic impact (Lee et al., 2013, Du et al., 2016). The zoonotic nature of *C. perfringens* adds to the cost and impact, which increases the desire to control its prevalence. In another study (Du et al., 2016), *C. perfringens* challenged birds had downregulated claudin-1 and occludin mRNA expression and gut lesions increased.

There are many other causes for leaky gut such as *E. coli* causing dephosphorylation of occludin via changes in intracellular [Ca^{2+}] (Applegate T J, 2014) ions and restricted feed intake (Kuttappan et al., 2015). Evaluating the integrity of genes associated with TJ's, cellular morphology, trans-epithelial electrical resistance (TEER) and FITC Dextran is a respectable assay that can be used to evaluate the permeability of the GIT.

Microbiota

The term microbiota includes all the microorganisms including bacteria, protozoa, fungi, archaea and arguably viruses. Gong et al. (2008) argued that intestinal microbiota plays an important role in the health and growth of animals through its effects on gut morphology/development, nutrition and immune functions. It is thought that the next step in the success of animal husbandry is no longer the selection of desirable genetic traits, but the selection of the microbial communities (Stanley et al., 2014). It was predicted that there are approximately 10^{12} microbes/ml of luminal content in the distal gut (Sonnenburg et al., 2004). The intestinal microbiota is collectively considered as a major organ in the body, capable of digesting otherwise indigestible nutrients and supporting the host wellbeing (Hooper et al., 2002). Currently, plant compounds such as carvacrol and thymol are being studied by analysing their effects of microbiota modulation throughout the GIT (Du et al., 2016, Corduk et al., 2013,

Alali et al., 2013). In order to manage broiler gut health, it is important to manage their exposure to microbes, nutrients and water.

Microbial Resistance

It has been reported that animals and workers at farms using AGPs have a higher rate of resistant bacteria present in their intestines when compared to intestinal flora of similar establishments not using AGPs (Marshall and Levy, 2011, Turnidge, 1999). Resistance has been found in areas of the world that have not been exposed to antibiotics; however, those resistant genes are more prevalent in areas where antibiotics are in use (Chattopadhyay, 2014, Davies and Davies, 2010, Theuretzbacher, 2013). Bacteria with resistant genes will survive and proliferate during AB administration (Marshall and Levy, 2011). Bacteria in the presence of subtherapeutic antibiotic dose are more likely to have an optimal mutation for resistance (Hong et al., 2016, Kohanski et al., 2010a). Bacteria can also share the gene with other bacterial species via horizontal gene transfer. The bacteria carrying the gene is the facilitator to a potential uncontrollable outbreak via horizontal gene transfer (Kohanski et al., 2010a, Wegener, 2003). The modern phenomenon of global travel also allows microbial resistant genes to travel freely (Theuretzbacher, 2013). For that reason, regulations of antibiotics need to be accepted and implemented by all countries and their states.

The role of AGPs

Antibiotic growth promoters are used in industry to prevent host/zoonotic disease, control microbial populations and promote the growth of animals (Marshall and Levy, 2011). Common diseases controlled by AGPs are considered to be necrotic enteritis (NE) and *Salmonella* (Timbermont et al., 2011). Growth promotion is also aided by improved FCR, meaning that the animal needs less feed per 1kg weight gain (Chattopadhyay, 2014). This could be due to reduced immune response caused by lack of pathogens. Animals fed AGPs are also observed to have a higher protein and lower fat percentage (Chattopadhyay, 2014), with lower disease rate measures such as morbidity, mortality and inflammation reduced, which, evidentially all increase farm productivity (Chattopadhyay, 2014).

Alternatives to Antibiotics

The term antibiotics was originally used to describe a naturally occurring chemical substance that is produced by microorganisms' that suppresses the growth of bacteria. Antibiotic growth promoters The modern use of the term antibiotics encompasses synthetic agents alongside naturally occurring substances (Turnidge J, 1999).

Herbal antimicrobial active ingredients such as carvacrol and thymol are not identified as antibiotics; they are referred to as phytobiotics and various other terms. The original definition of an antibiotic was a compound produced by a microorganism that acts against another microorganism (Waksman, 1947). One of the reasons that probiotics are administrated to animals is in the hope that they will produce bacteriocins. Bacteriocins inhibit the growth of similar or closely related microorganisms. Probiotics produce bacteriocins that have a narrow spectrum of antimicrobial activity (Dobson et al., 2012). Carvacrol and thymol are secondary plant metabolites, and in contradiction to probiotics they have a broad spectrum of antimicrobial, fungal and parasitic activity (Rao et al., 2010, Rosado-Aguilar et al., 2017, Abbaszadeh et al., 2014, Alali et al., 2013, Bajpai et al., 2012b, Betancourt et al., 2014). An added potential benefit for the use of these compounds is revealed in research where the presence of carvacrol and thymol increased the populations of *L. crispatus* and *L. agilis* and sustained *Lactobacillus* (Yin et al., 2017, Mohiti-Asli and Ghanaatparast-Rashti, 2017).

Antibiotics have different modes of action; many of them work by disrupting cell wall formation or by the inhibitory process which affects bacterial cells growth (Brotz-Oesterhelt and Brunner, 2008, Kohanski et al., 2010b, Molitor and Graessle, 1950). Eukaryotic cells do not have a cell wall and the inhibitory factors mentioned are specific to bacteria. This means that mainstream antibiotics are presumed to be non-toxic to animal health (Molitor H, 1950). Antibiotics that interact with cell membranes have minimal use (topical application only) due to potential toxic effects (Molitor H, 1950, Neu and Gootz, 1996). Carvacrol and thymol function by disrupting the cell membrane. This allows the compounds to have an interaction with eukaryotic animal cells. Consumption of the natural concentrations of carvacrol and thymol found in oregano have shown to be non-toxic to animal health over the centuries that humans and animals have consumed the plant. It is, however, unknown what reaction may occur if their concentrations are increased over a prolonged period or animal's entire productive life. The attributes above suggest that using phytobiotics as a low dose prophylactic treatment is optimal, saving the use of antibiotics as a high-dose curative treatment when outbreak occurs.

Microbial Management

Hygienic management strategies such as pest control manure management, biosecurity protocols, hazard control at critical control points and quality assurance (Turnidge J, 1999, Ferket P R, 2004). The quality of feed, significant changes in nutrients and feeding mistakes can compromise the normal healthy microflora (Abdelrahman W H A (Editor-in-Chief), 2014).

These management systems aim to have broilers achieve a state of eubiosis. Eubiosis refers to the state when the host and its microflora are living in a symbiotic relationship. Dysbiosis is a state when the host and its microflora are living in an antagonistic relationship (Abdelrahman W H A (Editor-in-Chief), 2014). While farmers do their best to ensure the best possible conditions for their animal's health creating a sterile environment is not possible, and dysbiosis can occur followed by even minimal disturbance (Zhuang et al., 2015, McDougal, 2018). AGP's are the current defence against potential dysbiosis; however, phytobiotics appear to be strong potential candidates, and further research is warranted.

Feed Additives

Feed additives such as enzymes, probiotics, minerals, biochar and phytoadditives have been trialled in broilers with evidence of beneficial results. Enzymes have shown to increase digestion of protein, fat, cholesterol and alter microbial populations in the small intestine and ceca (Ferket P R, 2004, Chen et al., 2016). Dietary fibres are polysaccharides and present a mixture of soluble and insoluble fibre. The soluble component is completely fermented by bacteria to produce fatty acids, while the insoluble portion is only partially fermented and excreted in the stool (Park and Floch, 2007). Dietary fibre was shown to improve nutrient digestion and gizzard function, however, this is highly dependent on the fibre source (Mateos et al., 2012). There is potential for symbiosis between dietary fibre and probiotics. Probiotics are live microorganisms added to feed, which are known to beneficially assist a healthy microbial gut flora (Boostani et al., 2013). The term probiotic is from the Greek pro (for) bio (life) was first used by Lilly and Stillwell in 1965 who observed growth promotion produced by microorganisms (Ghadban, 2001). Probiotics have many modes of action and are still being widely studied. Current evidence indicating that they are beneficial as feed additive include the ability of competitive pathogen exclusion, improving feed digestion, improving histomorphology of GIT, altering bacterial metabolism, reduced cholesterol and reduced immune modulation (Ghadban G S, 2001, Boostani et al., 2013, Shokryazdan et al., 2017, Karimi Torshizi et al., 2010, Yang et al., 2009)

Probiotics

Probiotics have been associated with human health for centuries. Health benefits of consuming fermented milk products are recorded in the Book of Genesis and by Roman historian Plinius, who recommended it for the treatment of gastroenteritis (Schrezenmeir J, 2001). There have been many definitions for what the term probiotics means, the most recent of which is given by Schrezenmeir and de Verse "a viable mono-or mixed culture of microorganisms which

applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (Abdelrahman W H A (Editor-in-Chief), 2014, Schrezenmeir J, 2001).

The functions of probiotics are diverse and complexed. There have been many studies that suggest that microflora play key roles in the GIT and immune development (Abdelrahman W H A (Editor-in-Chief), 2014). It is hypothesised that probiotics are able to maintain a healthy GIT by filling niches, therefore excluding other bacterial species and by actively producing antimicrobial compounds known as bacteriocins (Stanley et al., 2012). Improved FCR, BWG, immune system function can be expected, and current research endeavours to find the most elite forms of probiotics (Stanley et al., 2014).,

Probiotics are often considered a potential replacement to AGPs, either on their own or in combination with other potentially pathogen-controlling agents (Park and Floch, 2007). Although there is an abundance of published investigations of the ability of probiotics to control pathogens *in vitro*, and *in vivo* animal trial settings, general use in birds in a farming environment has not always lived up to expectations (Abdelrahman W H A (Editor-in-Chief), 2014, Dobson et al., 2012, Lilly and Stillwell, 1965, Park and Floch, 2007).

Phytoadditives

Phytoadditives, also known as phytobiotics, may be considered as alternatives to the use of prophylactic sub-therapeutic use of AGPs. Many plants produce secondary substances that are used for either attracting or deterring foreign organisms (Fraenkel, 1959). Plants that produce compounds that defend against pathogenic bacteria, fungi and parasites could have a benefit from natural selection. Oregano produces the highest concentrations of carvacrol and thymol in the flowering time (Grevsen K, 2009, Ozkan et al., 2010). Carvacrol and thymol are naturally produced compounds that reduce or inhibit the growth of bacteria (Bajpai et al., 2012a). Phytobiotics are produced by the plant as antibacterial, antiviral, antifungal and insecticides (Bakkali et al., 2008, Hyldgaard et al., 2012, Bajpai et al., 2012a). Phytoadditives have almost exclusively been used as essential oils and oleoresins and studied for their antimicrobial activities by in vitro and in vivo assays (Viuda-Martos et al., 2011, Lee et al., 2013). EOs are extremely variable in compound concentration (Bokov et al., 2015, Toncer et al., 2009, Alloui et al., 2014). Essential oils are generally comprised of terpenes, terpenoids, phenol derived aromatic and aliphatic components (Bakkali et al., 2008, Bajpai et al., 2012a). The volatile nature of these compounds leads to evaporation, and further inconsistencies of dosage may be encountered. The lipophilic nature of essential oils allows them to readily interact with the cellular membrane (Knobloch et al., 1989, Burt et al., 2007). That can lead to various further

interaction such as disrupting cell membrane potential due to loss of ions, the collapse of the proton pump and depletion of the ATP pool (Bakkali et al., 2008, Burt et al., 2007).

Oregano

Origanum *vulgare* L. essential oil (OEO) has been widely studied as a phytoadditive. OEO generally contains monoterpenes carvacrol and thymol and their biosynthetic precursors *p*-cymene and *y*-terpinene as the dominant components at approximately 80% (Lagouri et al., 1993, Grevsen K, 2009). Currently, research is conclusive that carvacrol and thymol have antimicrobial effects against bacteria and fungi. Research has also shown an additive effect of the two compounds (Nostro et al., 2007). However, the application has not been optimised, and in vivo, results have been inconsistent (Puvača et al., 2013). A reason for such inconsistencies could be the variability of carvacrol and thymol. It is possible to predict harvest quality by harvesting at times that have shown to have higher oil, carvacrol and thymol content (Ozkan et al., 2010). In the future hand-held NIR devices may also be used by farmers so as they can ensure they harvest at a time that provides a quality product (Camps et al., 2014). In vivo trials may also be affected by the lack of knowledge within the scientific community of how this molecule interacts with the host. Some projects show microbial shifts and others have shown no effect on microbial communities. A hypothesis for this divergence is that the molecules are absorbed early within the digestive tract limiting microbial interaction (Michiels et al., 2010, Mohiti-Asli and Ghanaatparast-Rashti, 2017).

Mediterranean (mostly Greek and Turkish) oregano oil concentration varies and is highest after flowering at the beginning of the fruiting stage. The antioxidant activities of OEO are mainly due to flavonoids and phenolic acids. Carvacrol has shown to have the highest values at July harvest (Grevsen K, 2009). Oregano contains the highest oil content, while the plant is budding (Ozkan et al., 2010).

Currently, research is focused on using oregano essential oil (OEO) and its photoactive ingredients as the feed additive. There is considerable in vivo research using OEO to modulate the growth of bacteria and improve intestinal health (Du et al., 2016, Mohiti-Asli and Ghanaatparast-Rashti, 2017). However, there have been inconsistencies in vivo application noted in reviews (Thacker, 2013, Zeng et al., 2015). Some examples of this are discrepancies in FCR where some (Roofchaee et al., 2011) noted an increase and others (Kırkpınar et al., 2011) observed decreased FCR.

Carvacrol and thymol modes of action have been widely studied and have shown to be a potential replacement to AGPs due to their antimicrobial activities (Nostro et al., 2007, Burt et al., 2014, Michiels et al., 2010, Lambert et al., 2000). The hydroxyl group found on both compounds is believed to be the mechanism that causes a decrease in the cytoplasmic membrane potential (ΔpH), the collapse of the membrane potential and the inhibition of ATP synthesis (Ultee et al., 2002). Carvacrol and thymol have shown an additive effect (Du et al., 2015, Rivas et al., 2010), while *p*-cymene has shown to have a synergistic relationship with carvacrol. It is assumed that *p*-cymene does not have antimicrobial activity when used alone due to the lack of a hydroxyl group (Ultee et al., 2002).

The anti-oxidant abilities of carvacrol and thymol have been widely studied for their preservative (Olmedo et al., 2014, Llana-Ruiz-Cabello et al., 2015, Lagouri et al., 1993, Bajpai et al., 2012a, Hyldgaard et al., 2012) and inflammatory benefits (Silva et al., 2012, Ocana-Fuentes et al., 2010, Arigesavan and Sudhandiran, 2015). Pro-oxidant and antioxidant properties of carvacrol, thymol and a representative of the natural carvacrol thymol mixture (10:1), found in oregano, were shown in several assays conducted by Llana-Ruiz-Cabello (Llana-Ruiz-Cabello M, 2015). 2,2-diphenyl-1-picrylhydrazyl DPPH and 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) ABTS assay was used to show antioxidant activity and dichlorofluorescein (measuring reactive oxygen species) and fluorescent probe monochlorobimane (measuring glutathione levels).

These assays showed that a mixture of both carvacrol and thymol 10:1 at high concentrations induced oxidative stress. At lower concentrations, the mixture had a protective role against induced oxidative stress. Dagli Gul (Dagli Gul et al., 2013) demonstrated that 20mg/kg/day was more effective at reducing oxidative stress than doses of 40mg and 80mg in protecting pancrease isolates from H_2O_2 induced cellular injury, where the mechanisms of inhibition of the ATP cycle were revealed. This disruption in the electron chain can produce free radicals which oxidise and damage lipids, proteins and DNA (Bakkali et al., 2008) dependent on the metal ions present (Stadler et al., 1995). This could explain why carvacrol and thymol are stronger antioxidants at lower concentrations. Thymol is the stronger antioxidant in lipids due to steric hindrance of its phenolic group (Yanishlieva et al., 1999). The antioxidant activity of carvacrol has also shown to decrease inflammation response in colitis-associated colon cancer induced by 1,2 dimethylhydrazine (DMH) and dextran sodium sulphate (DSS) in male Fischer 344 rat model (Arigesavan and Sudhandiran, 2015). The study revealed that carvacrol induces the endogenous antioxidant system by significantly increasing the presence of enzymes such

as GSH. Suppression of pro-inflammatory mediators such as nitric oxide synthase (iNOS) and interlukin-1 beta (IL-Iβ) was observed, which led to the restoration of histological lesions. Lima et al., (2013), also found a reduction in the production of pro-inflammatory mediators and attributed that to carvacrol possibly by inducing the release of interleukin-10 (IL10). Others (Ocana-Fuentes et al., 2010), also revealed a decrease in pro-inflammatory cytokines and increase of anti-inflammatory cytokines using an enzyme-linked immunosorbent assay (ELISA). The study also demonstrated that a concentration of OEO higher than 30µg/ml decreased THP-1 derivated macrophages viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay.

Oregano essential oil has been studied as a potential replacement to AGPs in various studies. OEO increased weight gain (Peng et al., 2016) compared to avilamycin (broiler AGP) and a non-supplemented diet. The jejunum morphology of OEO treatment had significantly lower crypt depths and improved crypt to villus height ratios.

Yin et al., (2017), conducted a challenge experiment, using *Clostridium perfringes* to induce NE disease and blend of 25% thymol and 25% carvacrol as a prophylactic treatment. The manuscript observed decreased mortality, gut lesions and virulence factors of pathogenic bacteria (VF 0073-ClpE, VF0124-LPS, andVF0350-BSH). Thymol-carvacrol blend modulated ileum microbial populations by increasing *L. agilis* and *L. crispatus*, and decreasing *L. salivarius* and *L. johnsonii.* A study (Zou et al., 2016) revealed that pigs treated with OEO had significantly lower *TNF-α, IL-Iβ, IL-6,* and *INF-γ,* and the villi height was significantly higher.

Mathlouthi et al. (2012), compared supplementary effects of rosemary essential oil, OEO, thymol-carvacrol blend and avilamycin. Rosemary essential oil had an antibacterial effect on *Escheria coli*, *Salmonella Indiana* and *Listeria innocua* shown by disk diffusion. OEO had an increased antibacterial effect compared to rosemary essential oil effect on pathogens, *Staphylococcus aureus* and *Bacillus subtilis*. In this study, all treatments out-performed the control, and there were no significant differences in growth between treatments. These findings highlight the need to clarify in vivo efficacy, as in vitro results are indicative but not necessarily a true reflection of activity in a complex microbial community. To improve the bioavailability of carvacrol and thymol when using whole herb, it is recommended to reduce particle size, thereby increasing surface area.

Based on the literature above, in this thesis, we present the investigation that aims to answer the following scope:

- Can oregano herb control pathogens while sparing beneficial bacteria
- Is there already existing natural resistance to oregano among pathogenic or beneficial genera
- What is the minimum concentration that provides microbial inhibition, and maximum concentration before the oregano starts casing intestinal inflammation
- Can oregano, if given from hatch, influence the development of microbiota
- How is microbiota changed in different gut sections and what functional capabilities microbiota gained or lost

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No.

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure

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(Original signature of Candidate) Date 10/03/2020

Chapter 2

Oregano powder reduces *Streptococcus* and increases SCFA concentration in a mixed bacterial culture assay

This chapter presents a manuscript that describes the first steps of our oregano investigation. The experiments were performed using in-vitro essay and specially designed microbiological media that could sustain, under anaerobic conditions, a large proportion of chicken intestinal, both ileal and cecal bacteria. Oregano was added to the treatment media and performed the first essay that allowed us to observe the effects oregano has on pathogens and beneficial strains to allow optimizing of the next steps of the investigation. Sequencing of 16S rRNA gene was used to investigate the overall changes to the microbial community as well as individual taxa.

PLOS ONE

RESEARCH ARTICLE

Oregano powder reduces *Streptococcus* and increases SCFA concentration in a mixed bacterial culture assay

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Abstract

Food borne illnesses have a world-wide economic impact and industries are continuously developing technologies to reduce the spread of disease caused by microorganisms. Antimicrobial growth promoters (AGPs) have been used to decrease microbiological infections in animals and their potential transfer to humans. In recent years there has been a global trend to remove AGPs from animal feed in an attempt to reduce the spread of antimicrobial resistant genes into the human population. Phytobiotics, such as oregano powder, are one of the potential replacements for AGPs due to their well-established antimicrobial components. 16S rRNA gene amplicons were used to determine the effect of oregano powder (1% w/v) on the microbiota of mixed bacterial cell cultures, which were obtained from the ceca of traditionally grown meat chickens (broilers). Oregano powder had a mild effect on the microbial cell cultures increasing *Enterococcus faecium*, rearranging ratios of members in the genus *Lactobacillus* and significantly reducing the genus *Streptococcus* ($p = 1.6e\text{-}3$). Beneficial short chain fatty acids (SCFA), acetic and butyric acid, were also significantly increased in oregano powder supplemented cultures. These results suggest that oregano powder at a concentration of 1% (w/v) may have beneficial influences on mixed microbial communities and SCFA production.

Introduction

Illness caused by the consumption of contaminated foods has a wide economic and public health impact worldwide [1]. In recent years, broiler producers have begun searching for alternatives to low dose sub-therapeutic AGPs that can maintain a healthy microbial gut flora without affecting the cost to poultry producers, the consumer or the environment. AGPs (e.g. avilamycin and zinc bacitracin) have been shown to reduce the abundance of some pathogenic groups of microorganisms, without having significant impacts on other members of the indigenous microbiota [2]. The ideal AGP alternatives would display similar influences over the microbial populations, while avoiding unforeseen problems with the health and performance

of animals [3]. AGPs added to livestock and poultry feed have been shown to decrease zoonosis whilst improving animal health and performance [4, 5]. The occurrence of bacterial resistance in animal production facilities has led to the fear of the resistance genes spreading into the human population [4, 6–8], driving the European Union in banning AGPs, which has resulted in the global decrease of AGP usage [9–11]. However, reports have indicated that the sudden removal of AGPs may result in higher incidence of diseases in poultry farms, consequently increasing the use of therapeutic antibiotics [12, 13]. The efficiency of AGP alternatives need to be thoroughly investigated to ensure that the benefits to animals, consumers and producers are not lost. One such AGP alternative could be the use of oregano powder, replacing the system while maintaining similar benefits.

Oregano (*Origanum vulgare*) is a phytobiotic and is known to contain antimicrobial compounds such as carvacrol (CAR), thymol and their precursors, *p*-cymene and γ-terpinene, generally equating to 80% of essential oil contents [14, 15]. CAR and thymol have consistently demonstrated antimicrobial properties at low doses [16–22], and reduced cell membrane potential which can lead to cell death [23]. This antimicrobial activity may have the potential to protect broilers from enteric pathogens and consumers from food borne diseases. Oregano powder has the potential to be used as a low dose subtherapeutic feed additive, preserving antibiotics for situations when high dose therapeutic treatments are required.

An assay utilising oregano, showed its superior influence with broiler weight gain and intestinal morphology when compared to avilamycin [24]. In another study, oregano, as a blend essential oil (25% thymol and 25% CAR), had a reduction in mortality, gut lesions and necrotic enteritis in broilers challenged with *Clostridium perfringens* [25], which cost the broiler industry billions of dollars per year [26]. Oregano essential oil has superior activity against pathogens compared to other herbs such as rosemary [27] and is a highly anti-oxidant [28], preservative [29–32] and anti-inflammatory substance [17, 19, 33].

In Australia, organic and layer chicken farmers restricted by the use of AGPs, are routinely adding oregano and oregano-based products to poultry feed as an antibiotic alternative. However, the range of effects of dry rubbed oregano on pathogens and beneficial intestinal bacteria are unknown. Most of the previously mentioned studies were performed using classic culturing methods: plates, liquid cultures and disc diffusion assays. These methods targeted selected poultry pathogens in single cultures, showing that some bacterial species behave differently in the complexed microbial communities of the gastrointestinal tract [34]. Culturing a mixed bacterial culture could allow for the growth of unculturable strains due to cross-feeding of metabolic products and other complex community interactions (Lagier et al., 2012). The increased understanding of intestinal microbiota and its complex interactions have ensued different approaches towards microbiological media design, resulting in the successful cultivation of novel species, giving birth to a new area of microbiology—microbial culturomics [35].

Chickens have a short gastrointestinal feed retention time of approximately 3–4 h, and variable excreta microbiota due to periodic emptying of different gut sections [36]. Chicken caeca empty several times a day, continually sampling both ascending and descending microbiota through peristalsis and intestinal movement [37]. Thus, chicken caeca are considered as a reservoir of gut microbial diversity of all gastrointestinal sections. In this study we used modified and enriched LYHBHI microbiological media, capable of supporting a wide range of the caecal microbial community in the presence and absence of 1% oregano. The analysis of this complex bacterial community was performed using 16S rRNA gene sequencing. We present the first comprehensive study of the effects of oregano supplementation on caecal microbiota cultured in an in vitro model system.

Materials and methods

Animal ethics statement

The animal-based work in this study was approved and monitored by Central Queensland University Animal Ethics Committee under approval number A1409-318.

LYHBHI media preparation and enrichment

The LYHBHI media [Brain-heart infusion (37 g/L, BD), yeast extract (5g/L, Alfa Aesar), cellobiose (1g/L, BD), hemin (0.005 g/L, BD), L-cysteine (0.5 g/L, Alfa Aesar), resazurin sodium salt (5 mg/L, Alfa Aesar)], was initially diluted in 798 ml of water and autoclaved. After cooling down the media, 100 ml of filter sterilised bacterial ferment, 100 ml of chicken feed extract, 1 ml of vitamin and 1 ml of trace element mix was added to make up the volume up to 1 L of enhanced LYHBHI media. The media was then purged with (nitrogen) before use in an anaerobic chamber.

Bacterial ferment was prepared by culturing *Lactobacillus plantarum* (ATCC® BAA-793™) and *Lactobacillus rhamnosus* (ATCC® 53103™) in 60 ml of LYHBHI each in an aerobic incubator at 37˚C, until stationery phase. The cultures were centrifuged and 50 ml of supernatant from both cultures was mixed and filter sterilised to enrich the LYHBHI media.

Feed extract was prepared by grinding on full power (1500 W, Nutri Ninja Auto iQ Duo, SharkNinja, USA) 100 g of chick starter crumble (Red Hen Chick premium micro starter crumbles antibiotic and hormone free, Lauke Mills, Daveystone SA, Australia) for 3 min. One litre of water was added to the powdered feed and blended again for an additional 3 min. The mixture was then autoclaved, allowed to cool overnight then centrifuged (3220 rcf, 5 min) in 50 ml falcon tubes. The clear supernatant was then pooled, filter sterilised and 100 ml of filtrate added to lukewarm LYHBHI.

Vitamin mix was prepared by dissolving a capsule of vitamin mix (Multivitamins and Minerals, Cenovis, Aust) and a capsule of vitamin K2 (Caruso's Natural Health, Australia) in 10 ml of water separately and filter sterilising 1 ml of resulting solutions was added to 1 L of LYHBHI media. Trace elements mix (Youngevity, California, USA) was filter sterilised (1 ml) and added to the enriched media. Both vitamin and trace mineral mix concentrations in final 1 L of enriched LYHBHI is provided in Table A in S1 File.

Caecum starter cultures

The complete caeca from three roosters (6 months old) were donated to the laboratory by a local organic heritage breeder (Rockhampton, QLD, AUS). The caeca were delivered frozen and in anaerobic conditions. Before culturing caeca were placed in the anaerobic work station (A35, Whitley, Shipley, UK) at 37˚C using mixed gas (CO_2 10%, H_2 10%, N_2 80% BOC, QLD, Australia) until they were completely thawed. The caecal contents were aseptically emptied into Erlenmeyer flasks containing 50 ml of enhanced LYHBHI media inside the anaerobic work station. To ensure good transfer of mucosa-associated bacteria, the whole caeca were then opened laterally its contents and tissue transferred into the prepared Erlenmeyer flasks. Cotton stoppers were placed in the mouth of each flask to allow for gas exchange. The cultures from all three rooster's caeca were incubated for 2 hrs in the anaerobic chamber at 37 °C on the orbital shaker (89 rpm). After 2 hrs the cultures were aliquoted into Cryo-tubes with 700 μl culture and 300 μl sterile glycerol in each tube and stored at -80˚C.

Oregano powder preparation

The dried aerial parts of oregano were used to make the powder. The oregano was processed by blending (100 g, 1.5 min/max, 1500 W, Nutri Ninja Auto iQ Duo) to reduce particle size.

That powder was then processed in a Planetary Ball Mill Machine (speed no. 5, 2 hrs, 40 g*each run, Changsha Yonglekang Equipment, China). A representative sample of each stage of processing was kept in a cool dark place. The oregano was then placed in an electric sieve machine (Changsha Yonglekang Equipment, China) to collect material that passed through the 75μm, sieve.

Inoculation of caecal microbial culture with oregano

One aliquot of caecum starter culture of each of the three roosters was gradually thawed by slowly increasing temperature. Enhanced LYHBHI media was purged using nitrogen in an anaerobic workstation. The media was aliquoted (20 ml) into twelve 50 ml Erlenmeyer flasks. Oregano powder was added to each of the six treatment flasks at 1% w/v. The flasks were plugged with cotton stoppers to allow gas exchange and incubated at 37 °C under a CO_2 10%, H_2 10%, N_2 80% gas mix in an orbital shaker (100 rpm) in the anaerobic workstation. In a separate experiment we established that the cultures reached stationary phase before 24 hours. The cultures were incubated for 24 hrs and then centrifuged at 1300 rpm for 10 min. The supernatant was removed, and stored at -80 °C for short chain fatty acid (SCFA) analysis. The pellet was processed for 16S rRNA amplicon sequencing.

DNA extraction

The pellet obtained from centrifuged cultures was used to extract total DNA for 16S rRNA sequencing. The pellets were resuspended, in the remaining media, using a vortex and transferred into tubes containing 0.2 g of glass beads (0.1 mm, diameter) and 0.7 ml of lysis buffer (500 mM NaCl, 50 mM EDTA, 50 mM TrisHCl (pH8), 4% SDS). Samples were homogenised at maximum speed for 5 min using a beadbeater (Mini-Beadbeater, Biospec products). The samples were then incubated at 75˚C for 15 min vortex at 5 min intervals. Samples were then centrifuged (16,000 rcf, 5 min) and 0.4 ml of the supernatant was transferred to a 1.5 ml tubes that contained 500 μl of binding buffer (5 M Gu-HCl, 30% isopropanol). The samples were vortexed and then centrifuged (16,000 rcf, 1 min). All of the supernatant was transferred into a DNA spin column with collection tube (Enzymax LLC, Cat# EZC101, Kentucky, US). The spin column was centrifuged (8,000 rcf, 1min) and the contents of collection tube discarded. The spin column was then washed twice with 800 μl of wash buffer (10 mM Tris-HCl, 80% ethanol (pH = 7.5) centrifuging at 8,000 rcf for 1 min. The spin columns were dried by centrifugation (8,000 rcf, 1min) and placed in new collection tubes. and eluted with 50 μl of elution buffer (10 mM Tris-HCl). The DNAs were analysed in a NanoDrop spectrophotometer to determine concentration and quality.

16S rRNA sequencing

The primers to amplify the V3-V4 region of 16S rRNA genes were: forward `ACTCCTACGG GAGGCAGCAG` and reverse `GGACTACHVGGGTWTCTAAT`. The primers contained barcodes, spacers and Illumina sequencing linkers were designed and used as previously described. The sequencing was conducted on an Illumina MiSeq instrument using 2x300 bp paired-end sequencing.

Microbial communities were initially analysed using Quantitative Insights Into Microbial Ecology (QIIME v.1.9.1) [38]. Paired end sequences were joined using Fastq-Join algorithm and with no mismatches allowed within the region of overlap. Phred quality threshold was minimum 20. OTUs were picked at 97% similarity using Uclust [39] and inspected for chimeric sequences using Pintail [40]. All taxonomic assignments were performed in QIIME against the GreenGenes database and QIIME default arguments [41]. Unifrac matrix was calculated in

QIIME and on the rarefied OTU table. OTUs with less than 0.01% abundance were removed. Statistical analysis including Spearman correlations, alpha and beta diversity were done on Hellinger transformed data [42, 43]. Significantly differentially abundant taxa were analysed using negative binomial distribution based DeSeq2 [44] method for differential analysis of sequencing count data. DESeq is performed on raw sequence counts.

Further data exploration was done in Calypso [45]. Sequencing data is publicly available on MG-RAST database under library accession number mgl745313.

Metabolite extractions for SCFA analysis

Culture supernatant samples were thawed on ice and 1 ml of each SCFA sample was transferred into a capped 2 ml tube containing 1 ml of 70% ethanol. The sample was then filtered through a 0.45μm cellulose syringe filter into a 2 ml vial. Standards of acetic acid, butyric acid, propanoic, valeric acid, isobutyric acid were prepared in 70% ethanol at concentrations of 1 ppm, 10 ppm, 50 ppm, 100 ppm, 500 ppm, 700 ppm, 1000 ppm. CAR was prepared in the same manner with concentrations of 10 ppm, 20 ppm, 40 ppm, 80 ppm and 100 ppm.

GC-MS systems and methods

The samples and standards were then placed in a GC-MS (GC-MS-QP2010 Ultra fitted with an AOC-20s Shimadzu auto sampler and a Shimadzu AOC-20i auto injector) with a polar column (Agilent J&W GC, 30m, 0.250 diam (mm), film 0.25 (μm) temperature limits form 40˚C to 260˚C). SCFA was determined by injecting 1 μl of sample at 250˚C with helium (1.97 ml/min) as the carrier gas with a 5.0 split injection mode. Pressure was maintained at 143.3 kPa and helium flow of 103.4 ml/min. The mass spectrometer operated in the electron ionization mode at 0.2kV, the source temperature was 220˚C with scan mode between 33 to 150*m/z*.

CAR was detected by injecting 1 μl of sample at 250˚C with helium (1.7 ml/min, 5.0) as the carrier gas with a 50.0 split injection mode. Pressure was maintained at 161.1 kPa and helium flow of 89.7 ml/min. The mass spectrometer operated in the electron ionization mode at 0.2 kV, the source temperature was 230˚C with scan mode between 33 to 280 *m/z*. Total program time was 5.88 min. The National Institute of Standards and Technology (NIST) library was used to identify and match peaks.

Results

Microbiota supported by enhanced LYHBHI

The caecal phyla supported by the media included (in order of abundance) Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Tenericutes and Spirochaetes. These phyla were represented by a total of 50 genera, 23 of which are uncultured. The cultured genera included: *Arthrobacter*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Collinsella*, *Coprobacillus*, *Dehalobacterium*, *Dorea*, *Enterococcus*, *Epulopiscium*, *Erwinia*, *Eubacterium*, *Faecalibacterium*, *Lactobacillus*, *Lactococcus*, *Ochrobactrum*, *Oscillospira*, *Pediococcus*, *Proteus*, *RFN20*, *Ruminococcus*, *Slackia*, *Sphaerochaeta*, *Streptococcus*, *Sutterella* and *Trichococcus* with unclassified genera from families Streptococcaceae, Enterobacteriaceae and Lactobacillales among the most abundant. Fig 1 shows the 20 most abundant genera supported by enhanced LYHBHI media. The high number of unclassified genera members in the original caeca is a consequence of the impact of non-industrialised traditional housing and a very different environmental conditions [46] such as fully ranging outdoor roosting birds, high live plant and insect food content, sharing yard with other poultry species and exposure to wild birds and animals.

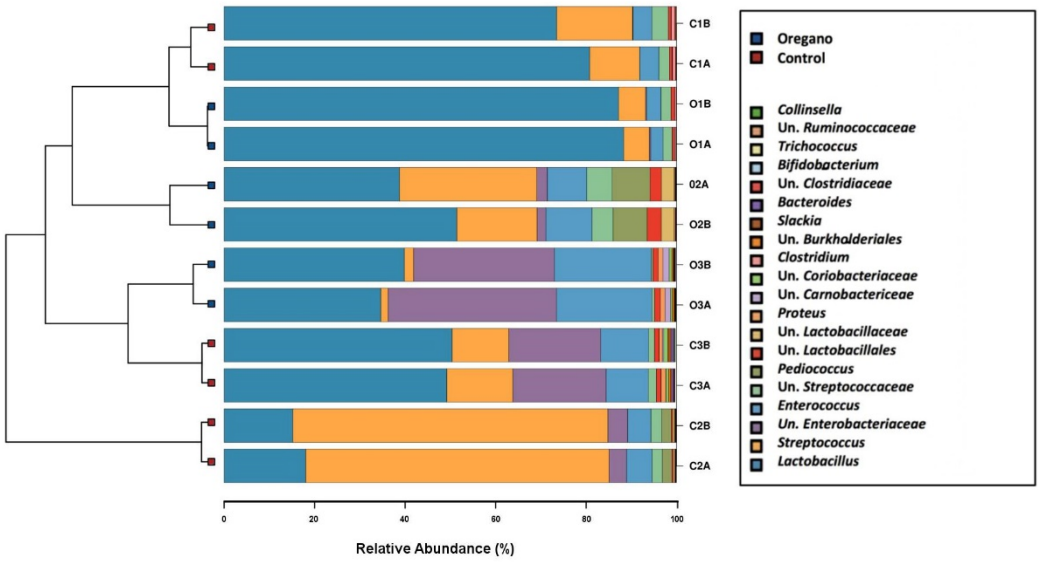

Fig 1. Hierarchical sample clustering bar-chart showing 20 most abundant genera. Sample names start with C or O (control or oregano), number (rooster 1, 2 or 3) and replicate code (A or B for 2 replicates).

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Alpha and beta diversity

Alpha diversity was not affected by the addition of 1% oregano based on Richness ($p = 0.96$), Evenness ($p = 0.54$), Shannon index ($p = 0.58$) or Simpson index ($p = 0.43$). Beta diversity indicators did not suggest major community perturbations by oregano with OTU level permutational multivariate analysis of variance (PERMANOVA) (Bray-Curtis) showing no significant oregano effect ($p = 0.166$). However, the microbiota biological donor had substantial influence on microbial community with rooster impact $p = 3.3E^{-4}$ (Fig 2A and 2B).

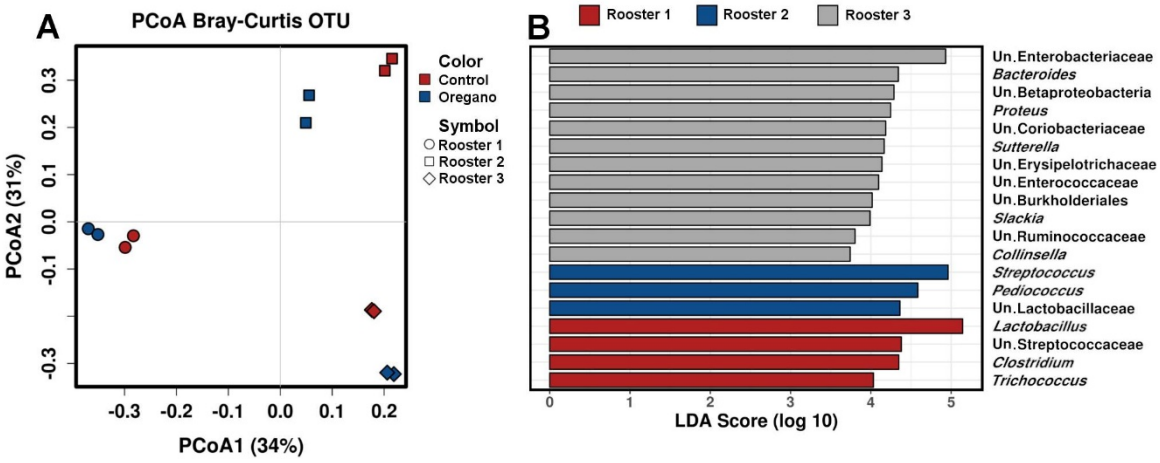

Fig 2. High effect of biological microbiota donor. Panel A shows Bray-Curtis PCoA plot separating samples by the rooster donor rather than oregano; panel B shows linear discriminant analysis (LDA) effect size method showing genera likely responsible for difference between the rooster's microbiotas.

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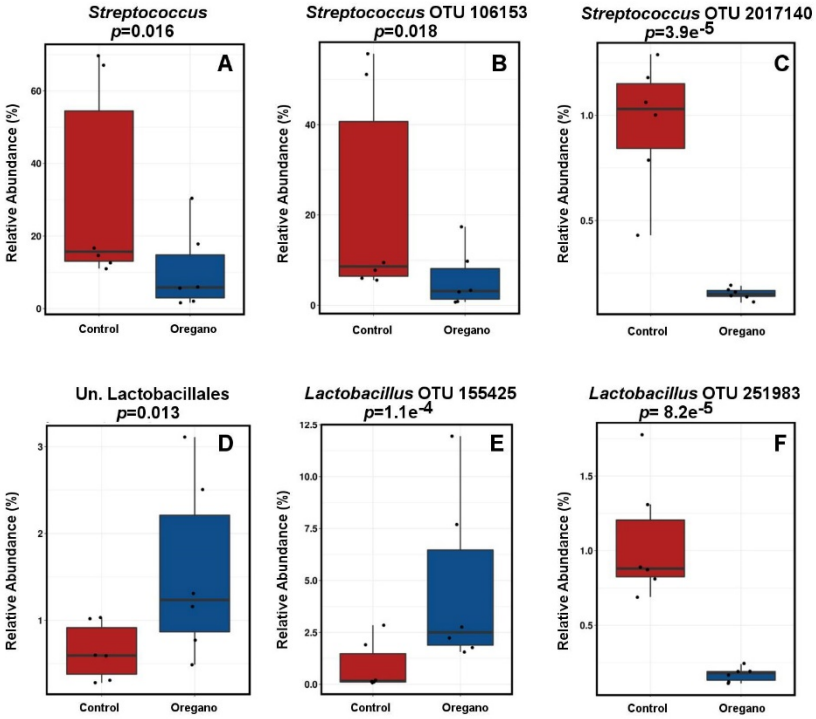

Fig 3. DeSeq2 differentially abundant taxa. Table with all significant OTUs is provided in S1 File.pdf, Table B.

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We used DeSeq2 negative binomial analysis to identify differentially abundant features. The biggest effect was seen in: *Streptococcus* ($p = 0.016$), was 60% reduced and an unclassified genus of Lactobacillales ($p = 0.013$) 2.4 fold increased in oregano treatment group (Fig 3A–3D). 96 OTUs were significantly different in abundance between the treatments. Among a number of those belonging to unclassified genera, there were also 62 differentially abundant OTUs from *Lactobacillus*, 15 from *Streptococcus* and 5 from *Enterococcus* genera. Both *Streptococcus* and *Enterococcus* OTUs were consistently changed in the same direction, all 15 *Streptococcus* OTUs were reduced (Fig 3B and 3C) while all 5 *Enterococcus* OTUs were increased by oregano. Blastn analysis identified the most abundant *Streptococcus* OTU (Fig 3B) to be highly similar to *Streptococcus gallolyticus* strain St5, with 100% sequence identity and an e-value = 0. Oregano treatment reduced this OTU in rooster 2 caecal community, where it was the most abundant, from 55.7% to 17.4% in one and from 51.1% to 9.7% reads in another replicate. Most of the other *Streptococcus* OTUs aligned with uncultured *Streptococcus*. The *Enterococcus* OTUs were either 100% identical to *Enterococcus faecium* strain HBUAS54015 or to uncultured bacterial clone database targets.

The *Lactobacillus* genus was not significantly changed ($p = 0.46$), however, 62 *Lactobacillus* OTUs were significantly altered by oregano treatment, some significantly increased (Fig 3E) and others reduced (Fig 3F). Based on blastn analysis OTUs 100% similar to *Lactobacillus crispatus* (shown in Fig 3E) or *Lactobacillus ingluviei* were significantly increased and other OTUs similar to uncultured *Lactobacillus* bacterial clone database targets (shown in Fig 3F) were reduced by oregano treatment. All significant OTUs with their closest blast hit IDs are given in Table B in S1 File.

Oregano influenced SCFA production

The main SCFA produced in cultures were acetate, butyrate, proponate, valerate and isobutyrate. There were significantly increased amounts of acetate ($p = 6.6e^{-4}$) and butyrate ($p = 6.3e^{-3}$) in oregano treatment groups (Fig 4). Overall on average oregano groups produced 61% more of the total 5 SCFA analysed ($p = 0.022$).

SCFA production correlated with some microbial taxa

We used PERMANOVA (on Bray-Curtis matrix) to evaluate if concentrations of produced SCFA, namely acetate, butyrate, isobutyrate, proponate and valerate had significant interactions with microbial communities. Only acetate ($p = 6.6e^{-4}$) and butyrate ($p = 6.3e^{-3}$) showed significant interaction with microbiota. Since we did not supplement SCFA to the culture, it is not possible to distinguish if oregano increased the abundance of high SCFA-producing microbiota, or if oregano stimulated existing bacteria to make more SCFA. It is also impossible to infer if microbiota influenced increase of SCFA or increased SCFA influenced microbiota structure. Spearman's correlation test showed a number of significant interactions between concentrations of AA (Table C in S1 File, Fig 5A) and of BA (Table D in S1 File, Fig 5B) with bacterial genera.

Acetate significantly ($p < 0.05$) positively correlated with *Enterococcus*, *Ruminococcus*, *Proteus*, *Coprobacillus*, *Bacteroides*, *Sutterella*, *Bifidobacterium* and *Collinsella* and negatively

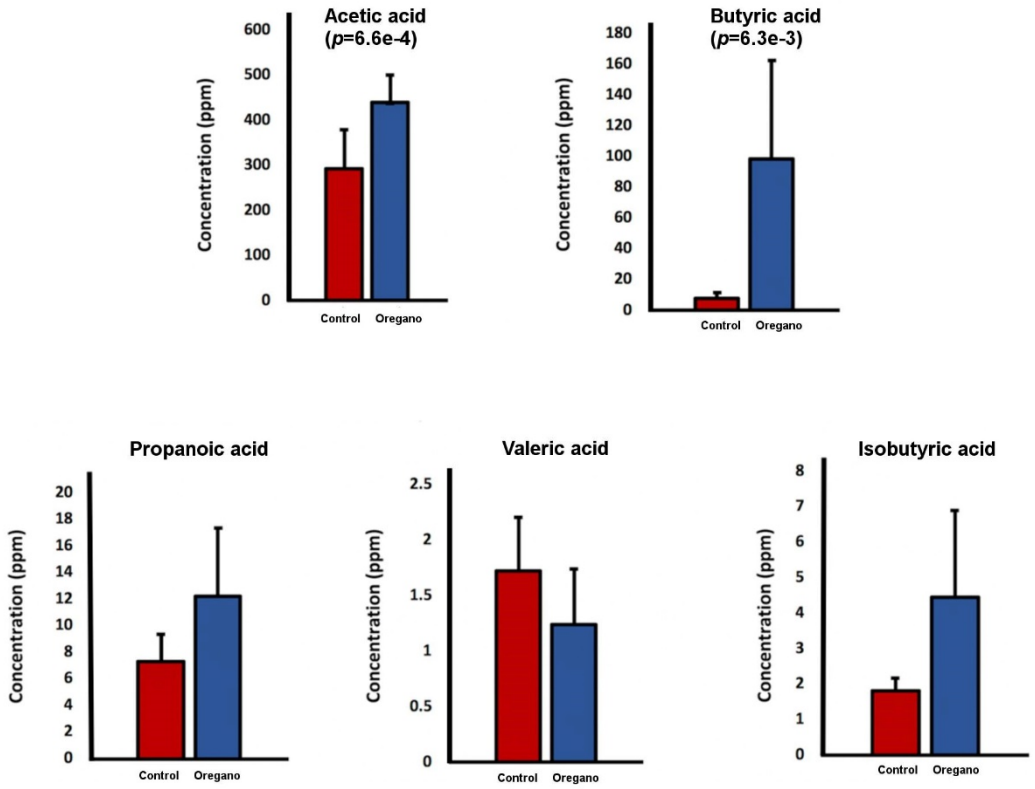

Fig 4. Concentrations of SCFA in control and oregano supplemented cultures. One-way Anova was used to determine significance. There were two SCFA significantly increased, which were AA ($p = 6.6e^{-4}$) and BA ($p = 6.3e^{-3}$).

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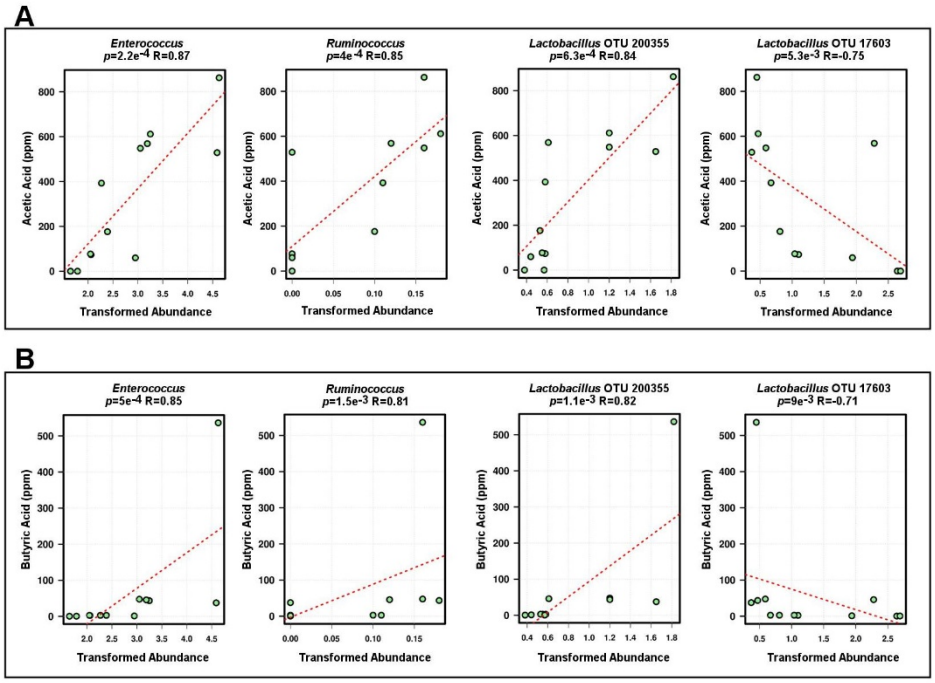

Fig 5. SCFA: AA and BA spearman correlations of concentration and abundance.

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correlated with *Clostridium* while butyric had very similar interaction profile to acetate, with positive interactions with *Enterococcus*, *Ruminococcus*, *Proteus*, *Coprobacillus*, *Sutterella* and *Bacteroides* and negative correlation with *Clostridium*. Among the top 20 most abundant OTUs the significant correlations with acetate and butyrate were almost identical, with *Lactobacillus* OTU 200355 positively correlated with both acetate (p = 6.3E-4, R = 0.84) and with butyrate ($p = 1.1e^{-3}$, R = 0.82) while another *Lactobacillus* OTU 17603 showed significant negative correlations with both acetate ($p = 5.3e^{-3}$, R = -0.75) and BA (p = 0.009, R = -0.71). The other significantly correlated OTUs, especially the most abundant ones, showed the same pattern, with very similar response to acetate and butyrate (Fig 6). Other SCFAs also had a number of highly significant correlations, however, did not have significant influence on microbial community structure (Fig 6).

CAR and microbial community

As expected CAR was not detected in the control group (Table 1), and based on PERMANOVA performed on Bray-Curtis matrix at an OTU level, CAR had marginally significant influence on microbial communities (p = 0.044). There were no genera significantly correlated with concentration of carvacrol in the microbial cultures and overall effect of acetate and butyrate appeared to overpower borderline significance of carvacrol influence. OTUs that correlated with CAR concentrations are provided in Table E in S1 File.

Discussion

The gastrointestinal tract (GIT) microbiota has been extensively studied because of the significant role it plays in the health and performance of animals, including poultry [47–49]. Earlier

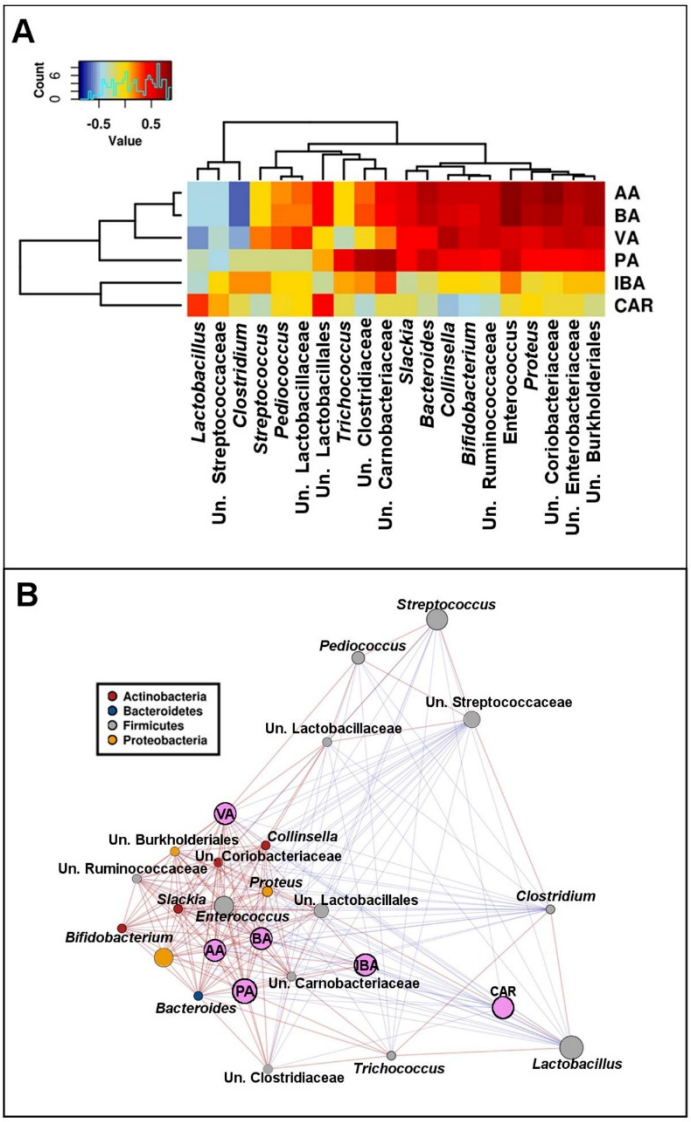

Fig 6. Heatmap (A) and a network (B) showing 20 most abundant genera relationship with SCFA and CAR. Both figures are based on Spearman correlation.

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studies on GIT microbiota were mostly, invitro, culture-based techniques. However, traditional methods are unable to cultivate the majority of the GIT microbiota and thus had limitations to unravel the complexity of this ecosystem. It has been estimated that only 10–50% of

Table 1. The concentration of CAR in cultures shown in mmol/L. CAR concentrations were determined by GCMS analysis.

Condition	η	1A	1B	2A	2B	3A	3B
Control	3	0	0	0	0	0	0
Oregano	3	0.2	0.21	0.2	0.19	0.19	0.19

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chicken caecal bacteria can be cultured [37]. In this assay, both control and treatment cultures had a wide range of unknown bacteria, this could be an indication that the enhanced LYHBHI media may be beneficial in culturing fastidious bacteria. The co-culture of diverse caecal microbial metabolic products can cross-feed bacteria facilitating their growth[50]. This fact could also explain the presence of unidentified bacteria within the cultures.

Oregano, at 1% concentration, did not have a destructive effect on the caecal microbiota, in fact, its effects were largely overpowered by the biological variation originating in the caeca of the different roosters. Instead, oregano selectively targeted certain bacterial groups, especially reducing *Streptococcus*, increasing *Enterococcus* and re-arranged ratios of *Lactobacillus* species, without affecting the total *Lactobacillus* abundance.

Reduction of *Streptococcus* (p = 1.6e-3) species by 1% oregano media supplementation was not surprising as CAR has been shown in numerous assays, to inhibit the growth of various *Streptococcus* species, such as *S. pyogenes*, *S. mitis*, *S. mutans*, *S. sanguis*, *S. milleri* and *S. pneumoniae [51–53]*. However, the heterogeneity of oregano, particularly the concentrations of other antimicrobial phytochemicals, such as thymol, *p*-cymene and *y*-terpinene, may have contributed to the reduction of *Streptococcus*, alongside variations in the complexed microbiota. In the cultures, the most abundant *Streptococcus* OTU identified with 100% sequence identity across the amplified region was *Streptococcus gallolyticus*, it was reduced in one rooster's caecal community, where it was the most abundant, from 55.7% to 17.4% and from 51.1% to 9.7% reads in another replicate. *Streptococcus gallolyticus* is known to cause septic bacteraemia in preterm neonates [54] as it has been strongly associated with intestinal damage and colorectal cancer [55, 56] as well as colonising colorectal tumours [57], endocarditis [58] and other associated health issues. This indicated possible benefit of oregano for individuals genetically predisposed to colorectal cancer. In poultry production this could protect against zoonosis of pathogenic *Streptococcus* strains to consumers.

The Enterococcus OTUs promoted by oregano were related (100% sequence id) to *Enterococcus faecium*, a well-known intestinal coloniser. *Enterococcus faecium* strains can be either alpha-haemolytic or non-haemolytic, extremely pathogenic or probiotic. Pathogenic strains represent a major health issue as a Vancomycin-resistant *E. faecium*, referred to as VRE [59] Other strains are marketed as probiotics improving intestinal health [60] and immunity [61]. In chickens probiotic strains of *E. faecium* show a positive effect in birds infected with *Salmonella* sp. [62]. An *E. faecium* probiotic strain was shown to promote growth performance, improve intestinal morphology, and improve the caecal microflora in *Escherichia coli* challenged broilers [63].

Rearrangements in representatives of the *Lactobacillus* genus can have either positive or negative influence on the bird performance. In human research, *Lactobacillus* are becoming well known as obesogenic [64] despite being beneficial on other health fronts. On the other hand, other *Lactobacillus* strains were shown as weight loss promoting [65]. The same discrepancy was reported in chicken feed efficiency, with some *Lactobacillus* strains positively correlated and others negatively correlated with weight gain or productivity [66]. Moreover, it was shown that the outcome of *Lactobacillus*, either as a probiotic or in weight loss and productivity, is strictly strain level specific. Fak and Backhed have shown that two different strains of *L. reuteri* had opposite effect on weight loss in mice [67]. Thus, changes in the abundance of different *Lactobacillus* species may promote or reduce more beneficial *Lactobacillus* strains and influence bird heath and productivity.

Despite the influence of oregano on *Lactobacillus* sp. and *E. faecium* being strain specific, restricting definite inference of beneficial effects using the 16S-based methodology, the SCFAs were quantitatively measured and shown to be increased by 1% of oregano. The health promoting effects are well documented. Butyrate has been shown to be the preferred energy

source for colonic cells, and increases the production of tight junction proteins [68], improves maintenance and structure of villi [12], and reduces inflammation [69, 70], which may improve the growth and performance of broilers experiencing stress [71]. Acetate has shown to be a potent antimicrobial [72] against *Streptococcus* and *Salmonella* [73] and additionally can cross-feed butyrate production. SCFA production was 61% higher in treatment groups, in live birds these increases would translate to healthier microbial communities, intestinal morphology and immune systems of broilers that receive oregano powder as a feed supplement. The observed increased levels of acetate and butyrate in treatment groups suggests that in vivo application of oregano powder at 1%, could benefit the host by providing energy to intestinal cells, anti-inflammation and antimicrobial activities resulting in assistance with competitive exclusion.

Supporting information

S1 File.
(PDF)

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Software: Dragana Stanley.

Supervision: Dragana Stanley.

Validation: Dragana Stanley.

Visualization: Dragana Stanley.

Writing – original draft: Benjamin W. Bauer.

Writing – review & editing: Benjamin W. Bauer, Sheeana Gangadoo, Yadav Sharma Bajagai, Robert J. Moore, Dragana Stanley.

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All co-authors assisted with the conception and revision of the article. Their combined contribution is estimated at 20%.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No.

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure

…….. ……

(Original signature of Candidate) Date 10/03/2020

Chapter 3

Oregano: A potential prophylactic treatment for the intestinal microbiota

This chapter presents a manuscript on in vivo effects of different concentrations of oregano on microbiota structure of broilers, including alpha and beta diversity, pathogen presence and abundance, effects on beneficial microbiota and dosage effects. Different sections of the gut are investigated for a complete understanding of where the oregano has major effects due to the fast absorption of phytogens. Additionally, short-chain fatty acids and histological analysis of the different gut sections were investigated.

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Research article

Oregano: A potential prophylactic treatment for the intestinal microbiota

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ABSTRACT

Prophylactic use of antibiotics in poultry diets has been identified as a problematic practice because of its potential to exacerbate the spread of antibiotic resistance to human pathogens. A range of countries have opted to completely ban the use of antibiotics in animal feed. The animal production industries are looking for alternative ways to effectively control pathogens while providing the performance benefits previously secured by antibiotics in feed. Here, we present evidence that oregano (*Origanum vulgare*) could be a potential alternative for pathogen control in the poultry industry. Broiler diets were supplemented with oregano powder (0%, 0.5%, 1%, and 2%) for six weeks. The capacity for pathogen control was estimated by microbiota profiling of the jejunum, ileum, and caecum content, and in the faeces, by 16S rRNA gene amplicon sequencing. The concentrations of short-chain fatty acids in the caecal content were also measured, as were villus/crypt parameters in the ileum. There were no differences among treatments in weight gain, feed intake, or the concentration of short-chain fatty acids. The height, width, and the surface area of villi in the ileum were not influenced by oregano addition. However, 1% and 2% of oregano produced a significant increase in the villus height to crypt depth ratio. There were no visible histopathological changes in the liver in control and treated groups. Although oregano had no significant effect on overall microbial diversity and gross composition, some specific genera, like *Proteus*, *Klebsiella* and *Staphylococcus*, which include known pathogens, were reduced in relative abundance by oregano treatment. *Bifidobacterium*, recognized as a beneficial and probiotic genus, was also suppressed by the oregano treatment.

1. Introduction

The poultry industry has to deal with high pathogen load environments, influenced by high bird stocking density and faecal loaded surroundings, requiring control measures to reduce the incidence and risk of disease outbreaks. For many years the industry has used in-feed supplementation of antibiotic growth promoters (AGPs) to control poultry pathogens. The beneficial effects of sub-therapeutic use of AGPs on bird health have been particularly evident on farms where biosecurity and bird living conditions were the poorest. This suggests that improving the living conditions and animal husbandry techniques could be an effective way to reduce the requirement for AGPs. However, it is prudent to maintain multiple lines of defence against pathogens to prevent disease outbreaks. A major challenge for pathogen control in broiler sheds is the high excreta pathogen load that can be found in the litter, particularly as coprophagous activity is normal amongst broilers. Interactions with the litter have been implicated in the bird to bird transmission of pathogens such as *Campylobacter jejuni* [1]. The use of AGPs and their ability to control pathogens and improve the overall health and performance of birds has contributed to the immense growth of the poultry industry. However, the use of AGPs in production has been gradually overshadowed by the public concern of antibiotics in the human food chain as well as the potential for farms to become breeding grounds for antibiotic-resistant bacteria.

Phytobiotics, such as oregano's volatile compounds - carvacrol, thymol, and their pre-cursors *p*-cymene and γ-terpinene, have been demonstrated to have synergistic/additive effects, such as antifungal, antiparasitic, antioxidant and antimicrobial activities. This suggests that

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oregano could be a viable alternative to AGPs provided there are no negative effects on the birds' health and that the efficiency of controlling the pathogen load in the gastrointestinal tract community is confirmed *in vivo*. Carvacrol and other oregano compounds can be cytotoxic [2, 3] at high doses indicating the need for optimising the concentration of oregano in feed to achieve maximum positive effects without cytotoxic effects. Additionally, there is a valid concern that phytobiotics may induce microbial resistance [4]. Use of phytobiotics in the form of essential oil is complicated by the volatility of compounds, which leads to rapid absorption in the upper gastrointestinal tract and difficulty in delivering sufficient dosages to the lower regions of the gut.

In the current study, chicken feed was supplemented with a range of oregano powder concentrations (0%, 0.5%. 1% and 2%) to investigate the effects on the pathogen load in the gastrointestinal tract and on short-chain fatty acid (SCFA) production in the caecum of broiler chickens. We evaluated liver cytotoxicity and ileal digestive capacity using histological assessment of these sections. Our results demonstrate the ability of oregano to control some poultry and human pathogens without visible effect on ileal and liver morphology.

2. Materials and methods

2.1. Oregano powder preparation

The dried aerial parts of oregano (Turkish, Saucy Spice Company, NSW) were used to make the powder. We have previously evaluated the effect of the oregano spice particle size on the growth of poultry pathogens in-vitro and concluded that the oregano powder with particle size lower than 80μm are the most efficient in pathogen control. Oregano was processed by blending (100g, 1.5min/max, 1500W, Nutri Ninja Auto iQ Duo, SharkNinja, USA) to reduce particle size. That powder was then processed in a Planetary Ball Mill Machine (speed no. 5, 2 hrs, 40g*each run; Changsha Yonglekang Equipment, China). The oregano was then placed in an electric sieve machine (Changsha Yonglekang Equipment, China); the powder used in the trial was the material that passed through a 75μm sieve. The particle size of the final oregano powder product was determined by laser diffraction (Mastersizer 2000, Malvern, ATA scientific, Australia) to have an average diameter of 10μm.

2.2. Feed preparation

Chicken starter diet (Red Hen, Laucke Mills, Australia) with no antimicrobials or coccidiostats was used throughout the trial. The feed was formulated to meet or exceed the National Research Council guidelines for broiler chickens [5]. The oregano was mixed into the feed to make 3 treatment groups 2% (0.02 kg/kg w/w), 1% (0.01 kg/kg w/w) and 0.5% (0.005 kg/kg w/w) in an electric mixer (125L cement mixer CMX-125, Ozito, China).

2.3. Birds and management

The study was approved by the Animal Ethics Committee of Central Queensland University under the approval number 0000020312. Forty eight one-day-old chicks (Ross Broiler 308, Bond Enterprises, Toowoomba) were randomly distributed into four pens, with 12 birds per pen. Each pen received feed supplemented with a different amount of oregano: the control 0%, 0.5%, 1% and 2%. The purpose of this experiment was not to evaluate bird performance but rather to evaluate the effects of oregano on gastrointestinal sections and on pathogen load reduction. All birds were fed *ad libitum* and had unrestricted access to drinking water. Birds were individually tagged using leg bands and weighed every week. The trial ran for a total of 42 days. Faecal material was collected for each bird by placing a transparent loose chicken wire enclosure around individual birds and waiting until fresh faeces were deposited and collected. Birds were euthanised at day 42 post hatch (CO_2, BOC, Australia) and dissected. Jejunum, ileum and caecum contents were taken for microbiota analysis, and liver and ileum sections were collected for histology.

2.4. Short chain fatty acids measurement

The standards and samples were analysed on a GC-MS (GC-MS-QP2010 Ultra) fitted with a AOC-20s Shimadzu autosampler and a Shimadzu AOC-20i auto-injector with a polar column (Agilent J&W GC, 30m, 0.250 diameters (mm), film 0.25 (μm) temperature limits form 40 °C–260 °C).

SCFAs were determined by injecting a 1 μl sample at 250 °C with helium (1.97 ml/min, 5.0, Coregas, Australia) as the carrier gas. The injection mode had a 5.0 split. The pressure was maintained at 143.3 kPa and a helium flow of 103.4 ml/min. The mass spectrometer operated in the electron ionization mode at 0.2kV, source temperature was 220 °C, and the scan mode was between 33 to 150m/z.

2.5. Histology

The tissue samples of liver and the midpoint of the ileum were collected and fixed in 10% buffered formalin solution. Fixation, paraffin embedding, deparaffinization, rehydration and staining with hematoxylin and eosin (H&E), were done by routine laboratory procedures. Glass slides were scanned at the TRI Microscopy Core Facility (Brisbane, Australia) using a Nikon Brightfield, Olympus VS120 slide scanner and analysed using Olympus microscopy software Olyvia. Morphometric analysis of the ileum was performed using Olympus software SensEntry 1.13. For each tissue sample, 20 well-oriented villi and crypts were examined. The measured parameters were: villus height (distance from the tip to the bottom of the villi), villus width (mean value between basal and apical villi width) and the crypt depth (distance between the crypt neck and its base). These morphometric measures were also utilised for the calculation of the villus surface area and the villus height to crypt depth ratio. The villus surface area was calculated using the equation [6]: Villus surface area [μm2] $= \pi \times$ Villus height [μm] $\times$ Villus width [μm].

As datasets were homogenous (cv<30%), the groups were compared using one-way ANOVA followed by Tukey's multiple comparison test. A significant difference was estimated at $P < 0.05$ and $P < 0.01$ significance levels. Statistical analysis of the results obtained in the experiment was carried out using statistical software GraphPad Prism version 6 (GraphPad, San Diego, CA, USA).

2.6. DNA extraction

DNA was extracted from jejunal, ileal, caecal and faecal samples. Approximately 0.2 g of sample was transferred into tubes containing 0.2 g of glass beads (0.1 mm diameter) and 0.7 ml of lysis buffer (500 mM NaCl, 50 mM EDTA, 50 mM TrisHCl (pH 8), 4% SDS). Samples were homogenised at maximum speed for 5 min (Mini-Beadbeater, Biospec products) and incubated at 75 °C for 15 min with vortexing at 5 min intervals. Samples were then centrifuged (16,000 rcf, 5 min) and 0.4 ml of the supernatant was combined with 500 μl of binding buffer (5 M Gu-HCl, 30% isopropanol) and transferred into a DNA spin column with a collection tube (Enzymax LLC, Cat# EZC101, Kentucky, US). The spin column was centrifuged (8,000 rcf, 1 min) and the contents of the collection tube discarded. The spin column was then washed twice with 800 μl of wash buffer (10 mM Tris-HCl, 80% ethanol (pH7.5) centrifuging at 8,000 rcf for 1 min. The spin columns were dried by centrifugation (8,000 rcf, 1 min) and placed in new collection tubes and eluted with 50 μl of elution buffer (10 mM Tris-HCl). The DNA quality and quantity was estimated using a NanoDrop spectrophotometer.

2.7. 16S rRNA gene sequencing

Primers used for amplification of the V3–V4 region of 16S rRNA genes were: forward ACTCCTACGGGAGGCAGCAG, reverse

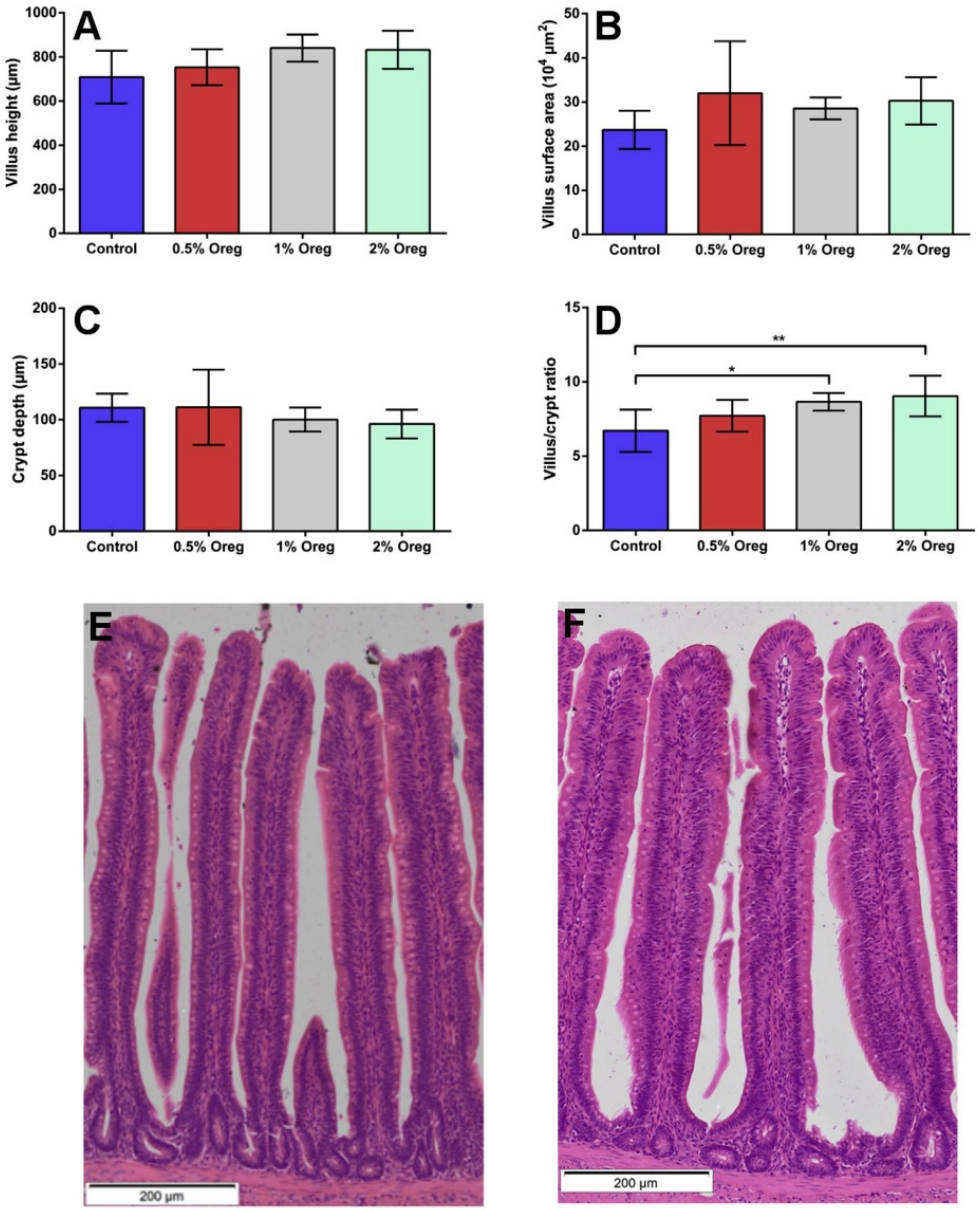

Fig. 1. Effect of oregano supplementation on the ileal morphology. Statistical analysis of villus height (A); villus surface area (B); crypt depth (C); villus/crypt ratio (D). Results shown as mean ± SD and $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$ and $^{**}P < 0.01$). Microphotography of ileum of the control (E) and 2% oregano supplemented broiler (F); H&E, bar = 200µm.

GGACTACHVGGGTWTCTAAT. The primers contained barcodes, spacers and Illumina sequencing linkers that have been previously described [7]. The 16S rRNA gene sequencing library preparation and amplification followed the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Sequencing was conducted on the Illumina MiSeq platform using 2 × 300 bp paired-end sequencing.

The microbial communities of each sample were initially analysed using Quantitative Insights Into Microbial Ecology (QIIME v.1.9.1) [8]. Paired-end sequences were combined using the Fastq-Join algorithm, allowing no mismatches within the region of overlap. Phred quality threshold had a minimum of 20. OTUs were picked at 97% similarity using Uclust [9] and inspected for chimeric sequences using Pintail [10]. All taxonomic assignments were performed in QIIME against the GreenGenes database and QIIME default parameters [11]. A UniFrac matrix was calculated in QIIME using a rarefied table of OTU abundance. Calypso was used to further explore and present the data [12]. After quality filtering, 16S rRNA gene amplicon data for 36 faecal, 39 caecum, 41 ileum and 41 jejunum, samples were included in the analysis. The sequence data is publicly available at the MG-RAST database under library accession number mgl745316 and a project ID mgp89580.

All OTUs with less than 0.01% abundance were removed. Statistical analysis including Spearman correlations, alpha and beta diversity were done on Hellinger transformed [13] OTU table. Significantly differentially abundant taxa were analysed using ANOVA. Bodyweight data were analysed using a one-way ANOVA in IBM SPSS Statistics. Significance was considered at $P < 0.05$.

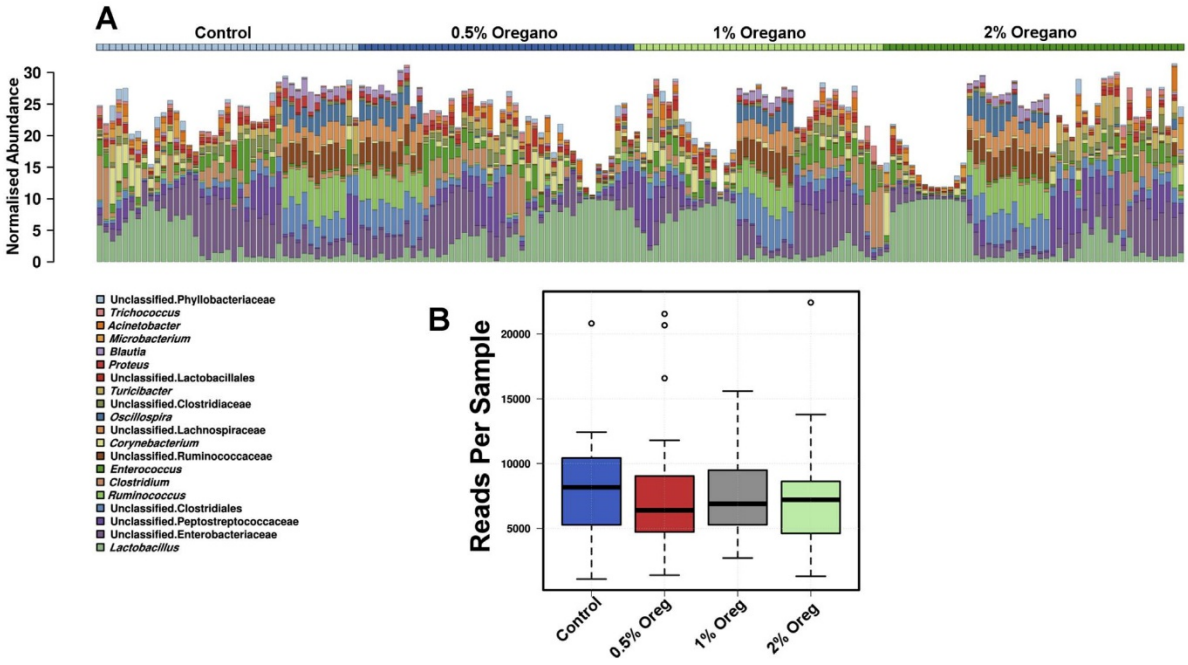

Fig. 2. Hierarchical sample clustering bar-chart showing 20 most abundant genera (A) and the sequencing reads per sample barchart (B).

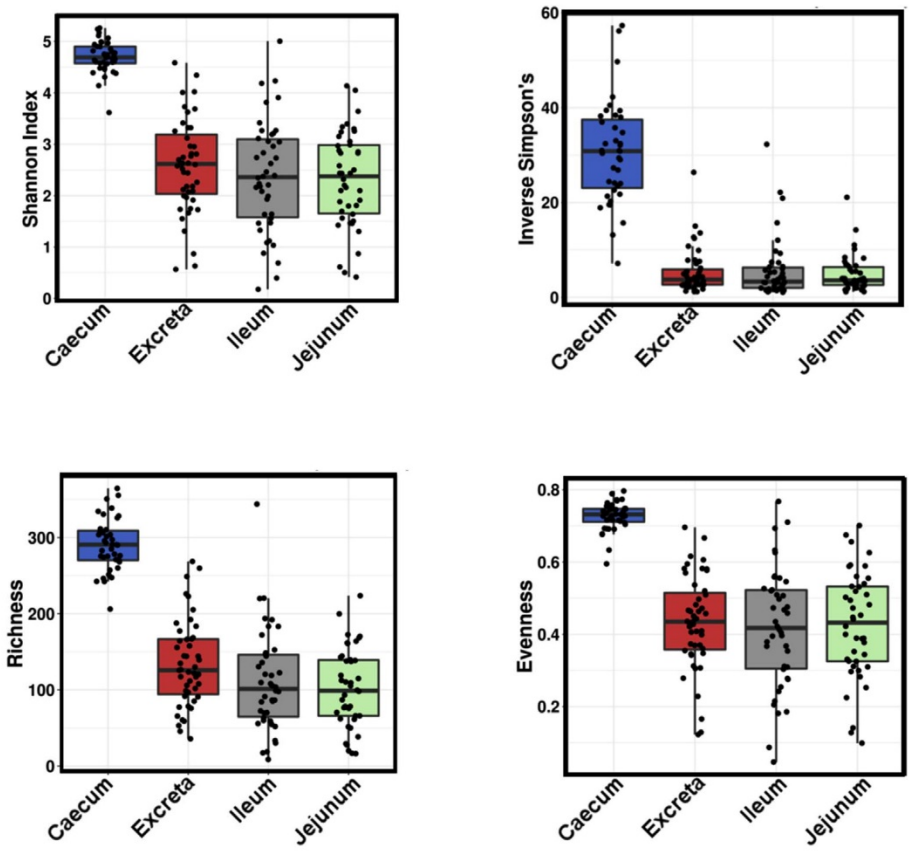

Fig. 3. Influence of oregano on alpha diversity.

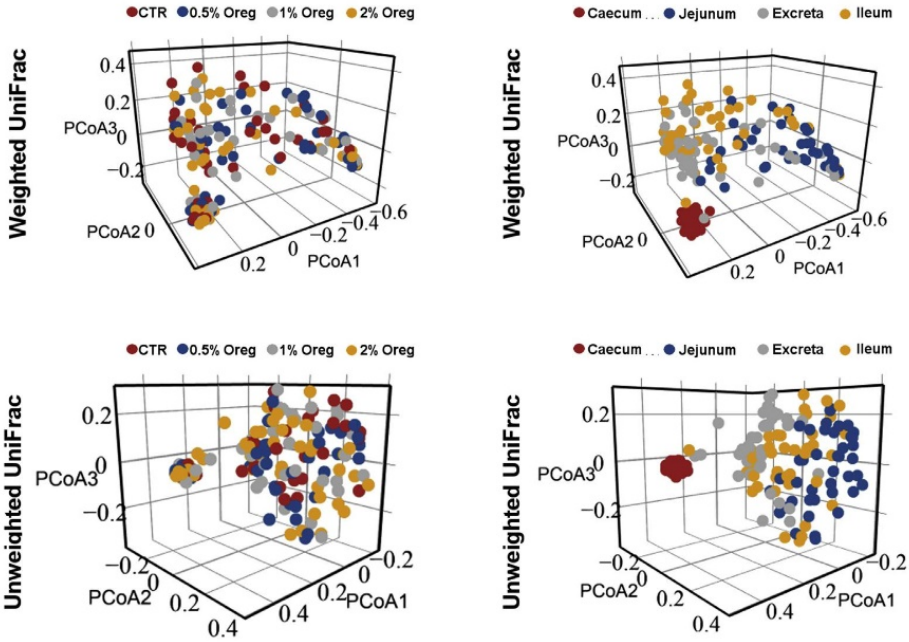

Fig. 4. Oregano and beta diversity expressed as weighted and unweighted UniFrac. Samples are coloured by concentrations of oregano (left) and sampling origin (right).

3. Results

3.1. Bird performance

The experiment had a relatively low mortality rate (4%) and there were no significant differences in bird weights ($P = 0.514$) or feed intake between treatment groups over the 42 day grow-out period.

3.2. Gut histology and SCFA

Light microscopic observation revealed there were no significant differences in height, width and the villus surface area in the ileum in oregano supplemented groups (Fig. 1). The morphology of mucosal epithelium was well preserved in all groups. Depth of the crypts was not significantly different; however, the villus height to crypt depth ratio was significantly increased in groups treated with 1% $P < 0{,}05$) and 2% oregano ($P < 0{,}01$) (Fig. 1). Histological analysis of liver tissue showed no histological appearance of pathological changes and no visible difference between the groups. There were no significant differences in either acetic, butyric, isobutyric nor in valeric acid concentrations in caecal content.

3.3. Microbiota data summary

A total of 157 samples from different oregano concentrations (0, 0.5, 1 and 2%) and the gut origins (cecum, ileum, jejunum and faeces) were sequenced (Fig. 2).

3.4. Alpha diversity

There were no statistically significant differences in alpha diversity indices due to the oregano supplementation, as assessed by Shannon ($P = 0.66$), Inverse Simpson ($P = 0.70$), Richness ($P = 0.61$) or Evenness indices ($P = 0.64$). However, as expected, the gut sections had very different diversity structure, with caecum showing higher richness and taxa evenness using all indexes analysed: Shannon ($P = 3.1e^{-29}$), Inverse Simpson ($P = 9.1e^{-45}$), Richness ($P = 7.2e^{-39}$) and Evenness index ($P = 2.7e^{-24}$) as shown in Fig. 3.

Table 1
Phylum level: ANOVA significant and Pearson significantly correlated with oregano concentration phyla.

Taxa (ANOVA)	P- value	FDR corrected
Tenericutes	0.00019	0.0011
Chloroflexi	0.026	0.062
Proteobacteria	0.031	0.062
Firmicutes	0.068	0.1
Bacteroidetes	0.58	0.7
Actinobacteria	0.77	0.77
Taxa (Pearson correlated)	**P-value**	**R**
Proteobacteria	0.0096	-0.1981
Firmicutes	0.045	0.1541

Weighted and unweighted UniFrac at an OTU level and a Bray Curtis matrix at genus and phylum levels were used inspect the differences in beta diversity. Based on weighted UniFrac there was no significant difference between the microbiotas from the treatment groups fed different oregano concentrations (Adonis $P = 0.359$) nor based on bird sex ($P = 0.396$) or weight ($P = 0.554$). However, the gut origin had a very distinct microbiota structure (Adonis $P = 3.3e^{-4}$). Similarly, based on unweighted UniFrac oregano concentration was not a major influencer ($P = 0.456$) and neither were the bird sex ($P = 0.234$) or their weight ($P = 0.178$), but the gut origin had a significant role in microbiota structure (Adonis $P = 3.3e^{-4}$) (Fig. 4). Additionally, using Primer-e based PERMANOVA and Bray Curtis distance, at the genus level oregano has no significant influence ($P = 0.064$), the gut origin was very distinct ($P = 1e^{-4}$) and there was no significant interaction between the gut origins and oregano concentration ($P = 0.194$). Based on the above, oregano had a marginal influence on overall gut microbiota independent from the gut sections inspected, thus targeting specific taxa rather than a total community.

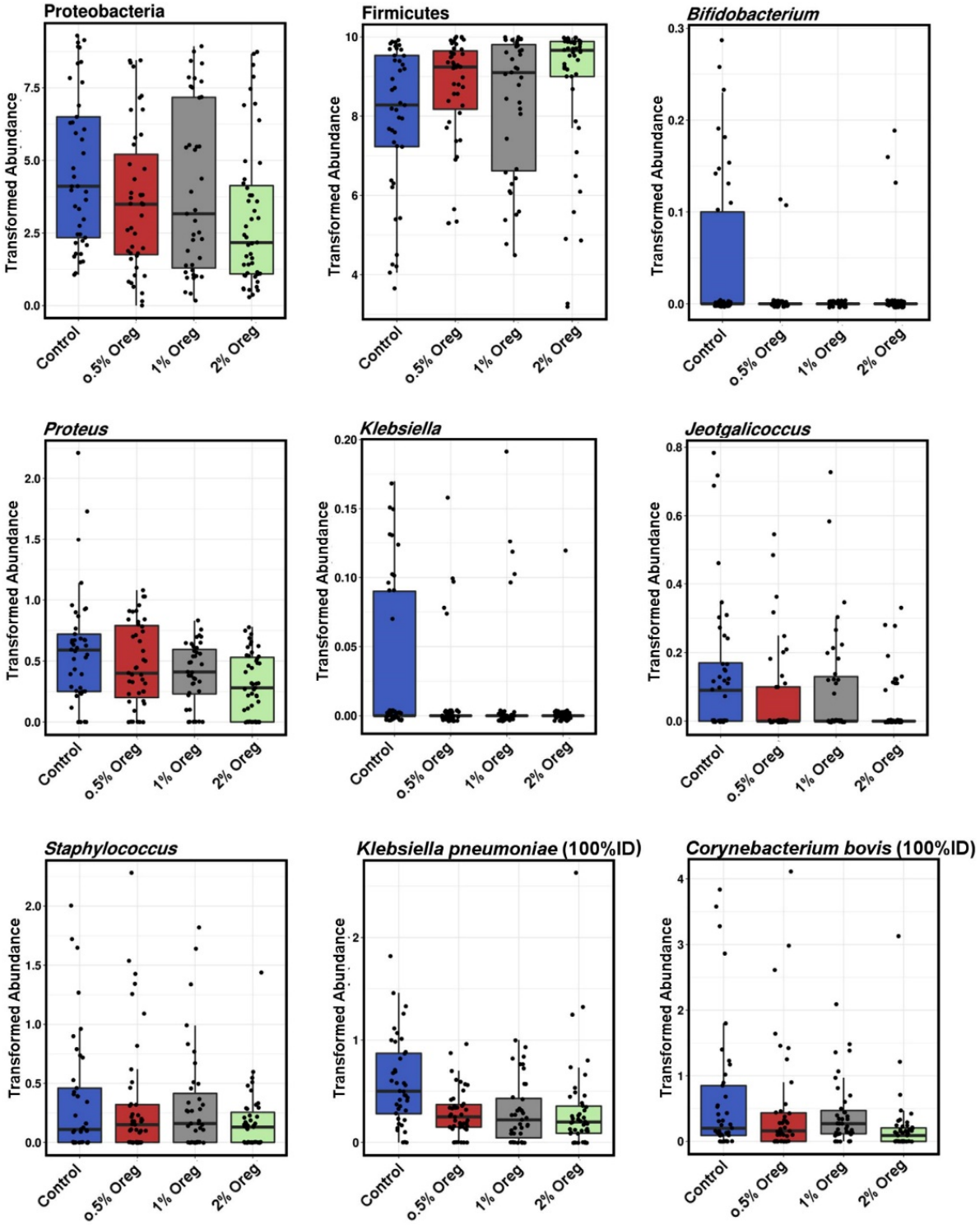

Fig. 5. Taxa at different phylogenetic levels significantly ($P < 0.05$) responding to different oregano concentrations.

3.5. Oregano and microbiota

Several taxa at all taxonomic levels were differentially abundant between the groups treated with different oregano concentrations. The phyla that significantly ($P < 0.05$) differed between the treatments included Tenericutes (higher in oregano treatments), Chloroflexi (lower in oregano groups) and Proteobacteria, reduced in oregano treatments (Table 1). Both Firmicutes and Proteobacteria were significantly correlated (Pearson) with oregano concentration, with Proteobacteria significantly ($P = 0.009$, R = -0.19) reduced in the higher concentrations of

Table 2
Genus level: ANOVA significant and Pearson significantly correlated with oregano concentration genera.

Taxa (ANOVA)	P- value	FDR corrected
Oligella	0.0000059	0.00067
Bifidobacterium	0.000044	0.0025
Unclassified.RF39	0.00019	0.0072
Proteus	0.00067	0.019
Klebsiella	0.0035	0.068
Solibacillus	0.0036	0.068
Jeotgalicoccus	0.015	0.24
Unclassified.Alcaligenaceae	0.017	0.24
Unclassified.Bacillales	0.021	0.27
Unclassified.JG30KFCM45	0.026	0.27
Salinicoccus	0.026	0.27
Granulicatella	0.041	0.36
Unclassified.Carnobacteriaceae	0.043	0.36
Rhodococcus	0.049	0.36
Taxa (Pearson correlated)	**P- value**	**R**
Proteus	0.000063	-0.3018
Unclassified Planococcaceae	0.018	-0.1811
Unclassified Enterobacteriaceae	0.025	-0.1713
Staphylococcus	0.027	-0.1693
Unclassified Gemellales	0.027	-0.1701
Unclassified Lactobacillales	0.044	-0.155

oregano and Firmicutes significantly ($P = 0.045$, R = 0.15) increased in higher oregano concentrations (Fig. 5). At the genus level (detailed in Table 2), the genera that were differentially abundant between the treatments (Fig. 5) included *Bifidobacterium* ($P = 4.4e^{-5}$), *Proteus* ($P = 6.7e^{-4}$), *Klebsiella* ($P = 0.003$) and *Jeotgalicoccus* ($P = 0.015$); all reduced in oregano groups. *Proteus* ($P = 6.3e^{-5}$ R = -0.3) and *Staphylococcus* ($P = 0.027$, R = -0.17; Fig. 5) were significantly negatively correlated with oregano concentrations.

At an OTU level, there were several OTUs that varied in relative abundance between treatments (Fig. 5), with Enterobacteriaceae OTU 291838 (100% identical to *Klebsiella pneumoniae* subsp. ozaenae strain ATCC 11296 using Blastn across the amplified region), and *Corynebacterium* OTU 173995 (100% identical to *Corynebacterium bovis* DSM 20582), negatively correlated with oregano concentration ($P = 8.7e^{-3}$, R = -0.2 and $P = 3.9e^{-3}$, R = -0.22, respectively, Fig. 5) and a Clostridiales OTU 284045 (most similar to *Ruminococcus lactaris* ATCC 29176 (99%)) that was slightly positively correlated ($P = 0.012$, R = 0.19) with oregano concentration.

3.6. High concentration of oregano

The comparison between the microbiota of the birds at 0% and 2% oregano shows the effects of relatively high concentrations of oregano. Proteobacteria and Firmicutes phyla significantly changed in relative abundance, Enterobacteriaceae and Planococcaceae families were reduced while Bifidobacteriaceae were dramatically inhibited by 2% oregano. At the genus level *Proteus*, *Klebsiella*, *Bifidobacterium* and *Staphylococcus* were all significantly reduced by 2% oregano in the feed (Fig. 6).

LefSe analysis (Fig. 7) confirmed that poultry pathogen-rich genera *Clostridium*, *Proteus*, *Serratia*, *Klebsiella* and *Sporosarcina* as well as beneficial *Bifidobacterium*, were the genera that most defined the microbiota differences between the control group and the 2% oregano treated group. Proteobacteria was the most defining phylum carried by the control group and higher levels of Firmicutes and Tenericutes were the most defining phyla of the microbiota within the 2% oregano group.

3.7. Influence of oregano in different gut sections

PERMANOVA analysis demonstrated no difference in response to oregano in different gut sections. Due to the high data volume presented in the manuscript, the detailed account of oregano influence in each intestinal compartment; caecum, ileum, jejunum and faeces, is given in Supplementary File 1. Each sheet within the file presents a data summary for a different sample origin, each containing genus level clustered bar chart, RDA multivariate plot and significance, DAPC group to group association plot, richness and evenness bar charts and a table with ANOVA significant genera with a bar chart figure for all ANOVA significant taxa. Based on RDA analysis, differences between oregano concentrations significantly affected microbial communities in caecum ($P < 0.001$) and in faeces ($P = 0.037$) while there were no significant differences in the ileum and jejunum microbiota compositions. There were significant differences in richness only in the jejunum and faecal microbiota, but not in caecum or ileum microbiota (Supplementary File 1).

4. Discussion

A desirable property of potential AGP replacements for pathogen control is that they should not induce major shifts in the gut microbiota, thus avoiding unforeseen consequences of microbial disruption [14].

Proteobacteria are a microbial signature of dysbiosis, and an increase in Proteobacteria has been associated with several GIT diseases including metabolic syndromes, diabetes, cancer, obesity, Chron's disease, inflammatory bowel disease and ulcerative colitis [15]. Additionally, their reduction has been shown to increase bird performance [16]. *Enterobacteriaceae* was the only family significantly reduced by oregano treatments. Four out of five *Enterobacteriaceae* genera detected in the study were significantly decreased in various oregano concentrations: *Klebsiella, Proteus*, Unclassified Enterobacteriaceae genus and *Serratia*. *Klebsiella* and *Proteus* members are associated with a wide range of diseases within human and animal health, as well as antimicrobial resistance. They are known to be related to diseases that result from biofilm production [17, 18], host cell invasion [19, 20], translocation-related [21, 22, 23] and foodborne illness [24, 25].

Enterobacteriaceae family members have been shown to rapidly produce biofilms in heterogeneous bacterial populations within the mucus layer of the intestinal epithelium [26]. Biofilm communities are capable of exclusion of antimicrobial agents and are associated with many persistent bacterial infections [27] and adherence to epithelial cells, which is essential for bacterial host invasion. However, the mechanisms are dynamic and still not well understood. For example, *Klebsiella* has been shown to use a transcellular pathway to translocate without the requirement for degradation of tight junction proteins, likely by hijacking eukaryotic signalling machinery to control downstream cytoskeleton dynamics [28]. In a study conducted in mice, *Klebsiella* increased inflammation by producing β-glucuronidase and endotoxin lipopolysaccharide, which resulted in reduced tight junction proteins [29]. Additionally, *Klebsiella* strains are becoming of increasing concern due to antimicrobial resistance particularly within the broiler industry where a study conducted in China has shown that 96.7% of *Klebsiella* were multidrug resistant [30, 31, 32]. *Proteus* has been correlated with 5.8% of deaths associated with bacterial related foodborne disease in China between 1994-2005 [33].

Carvacrol is the main antimicrobial compound found in oregano plants and is known to interact with cell membranes [34]. Carvacrol hydroxyl group has shown to facilitate the transport of cations across the cell membrane, which reduces membrane potential, eventually leading to cell death [35], subsequently, carvacrol's ability to interact with cell membranes means that there is potential for cytotoxicity [2, 36]. Our study revealed no significant changes in villi height, area or crypt depth or difference in weight gain, suggesting that birds fed 2% oregano did not compromise intestinal integrity or performance. While carvacrol has been shown to be a potent antimicrobial, it has also displayed the ability to disrupt the invasive ability of motile pathogens at sub-inhibitory levels [37]. *Escherichia coli* has been shown to produce increased amounts of heat shock protein 60 (HSP60) in the presence of 1mM of carvacrol and become aflagellate and therefore non-motile [38]. In a recent study,

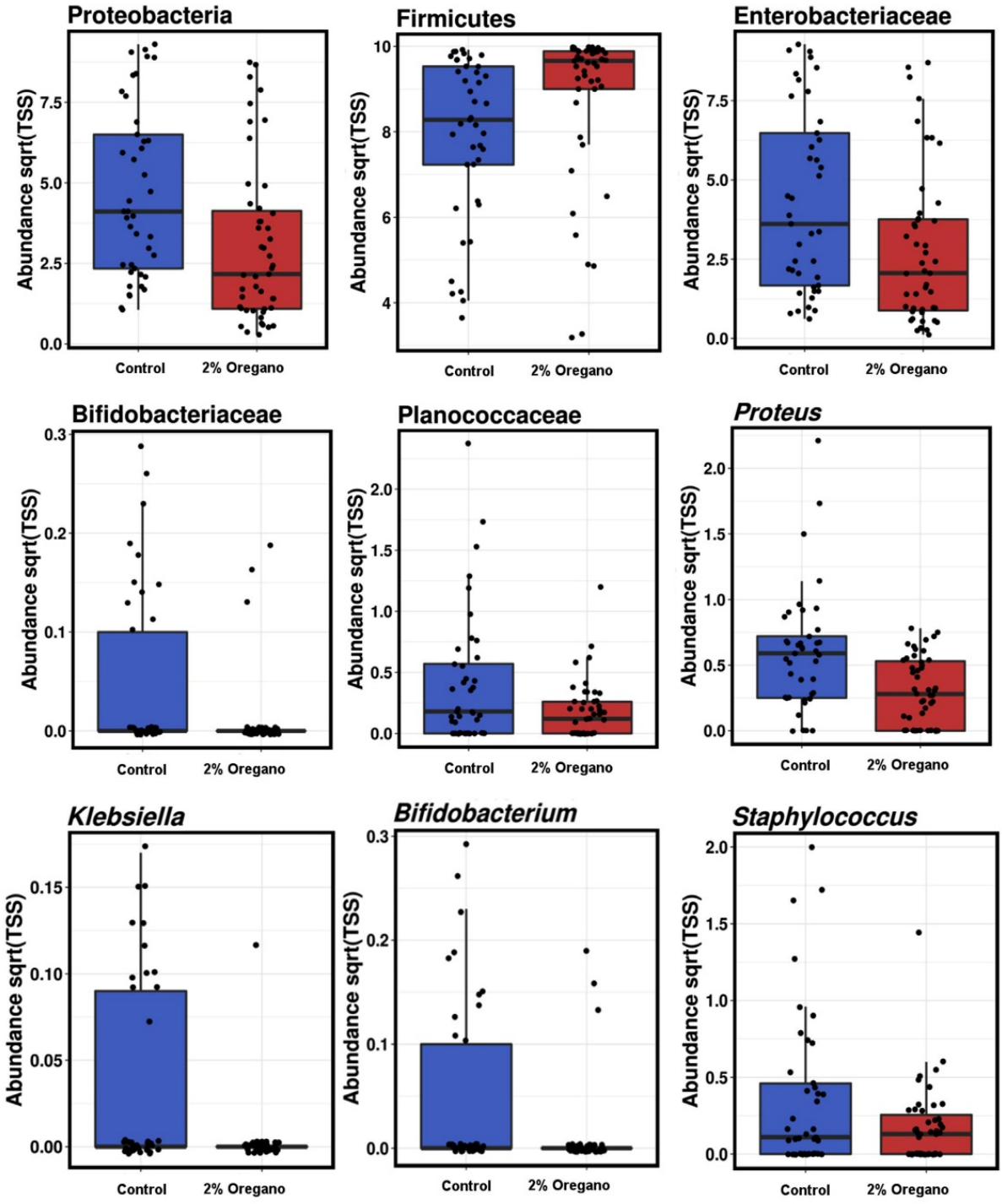

Fig. 6. Taxonomic levels significantly affected by high concentrations of oregano.

Inamuco [39] revealed that *Salmonella typhimurium* lost motility and invasive potential not as a result of malformed flagella, but potentially a loss of flagellum functionality. Carvacrol has been demonstrated to inhibit various strains of *Klebsiella* biofilm formation [40], although the precise mechanisms are not identified. Many other studies also show that carvacrol is able to disrupt biofilm formation at concentrations far below the minimum inhibitory levels [41, 42, 43]. A more comprehensive study conducted by Burt [44], demonstrated that carvacrol at sublethal levels (<0.5mM) could disrupt quorum sensing of *Chromobacterium violaceum*, inhibiting the formation of biofilms. The reduced load of *Klebsiella* and *Proteus* in faeces is likely to decrease the transfer of pathogens by coprophagous activities and other litter interactions.

Staphylococcus is another pathogen that is significantly affected by higher concentrations of oregano. Some *Staphylococcus* species are

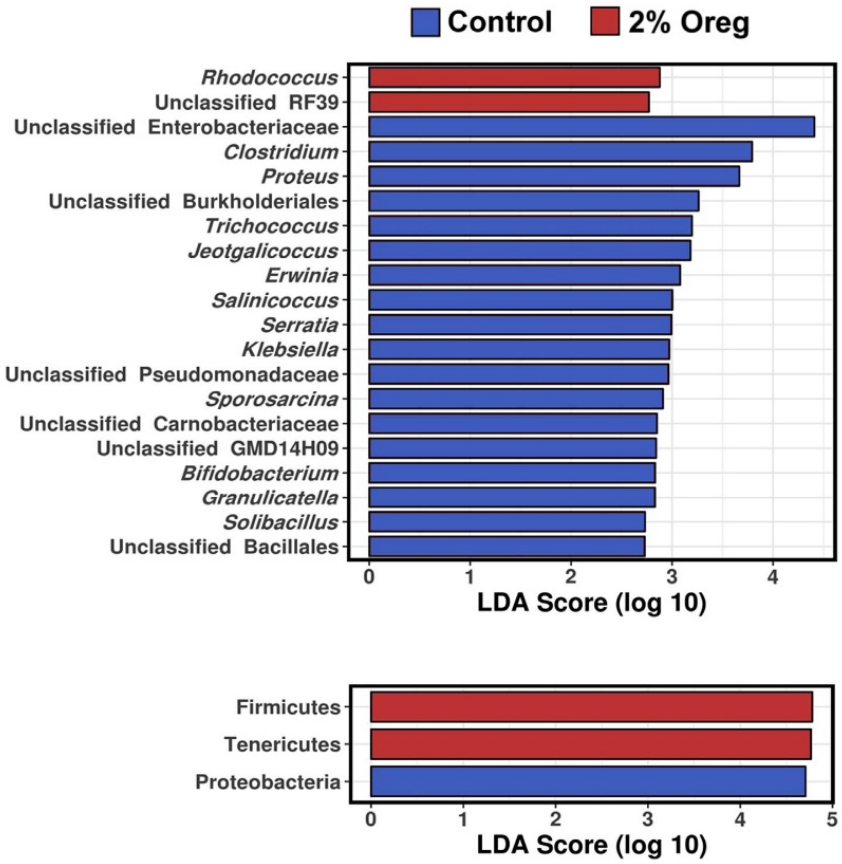

Fig. 7. LefSe analysis representing taxa that define control and oregano microbial structures.

potential poultry pathogens with zoonotic significance. *Staphylococcus aureus* is a widely prevalent enterotoxin-producing pathogen in poultry, which is recognised to acquire resistance against methicillin and other common antibiotics such as ciprofloxacin, erythromycin, tetracyclin etc. [45, 46, 47, 48] and can transfer from poultry to humans and vice versa [49]. *Staphylococcus* can cause a wide variety of infections both in humans and in animals [50], with severe food poisoning with staphylococcal enterotoxins [51], often associated with poultry meat [52]. Moreover, translocation of *Staphylococcus* from intestine can infect the proximal epiphyseal plate of the femur, tibiotarsus and flexible thoracic vertebrae causing bacterial chondronecrosis with osteomyelitis (commonly called femoral head necrosis), which is one of the major causes of lameness in chickens [53]. Therefore, staphylococcal inhibitory effects of oregano are potentially of significant benefit to the poultry industry.

Microbiota analysis indicated that the relative abundance of *Corynebacterium bovis* in the intestine was negatively correlated to the oregano treatment. Although this bacterium is a normal inhabitant of mammary gland of bovine and opportunistically causes mastitis [54], the infection of chicken with this bacterium is not commonly reported.

Although oregano did not have any major influence on the total intestinal *Lactobacillus*, all oregano concentrations reduced *Bifidobacterium* carriage in the community. Intestinal *Bifidobacterium* are generally regarded as beneficial to the host due to digestion of oligo- and polysaccharides, producing beneficial short chain fatty acids and lactic acid [55, 56], inhibition of potential pathogens like *Compylobacter jejuni* [57] and reduction in carcass condemnation due to cellulitis [58]. However, different bifidobacterial strains have different abilities to utilise carbohydrates [56]. Therefore further studies are necessary to elucidate the specific impacts of bifidobacterial depletion on the host. Enrichment of the product with pre-biotics could be an option to nullify this bifidotoxic effect of oregano which needs further study to substantiate.

5. Conclusions

Oregano powder as a feed supplement suppressed the relative abundance of various classes of pathogens and did not cause any negative effects on liver and ileum histology. Oregano could have application in the poultry industry as one of a series of compounds that can be used to ameliorate some of the difficulties introduced with the removal of in-feed AGPs. The strong reduction of *Bifidobacterium* is concerning and requires further study to understand the breadth of *Bifidobacterium* strains affected. Finally, we wish to acknowledge that our conclusions are drawn from a single animal trial and that more work needs to be done to understand the role oregano supplementation has in the poultry intestine. We are currently running oregano trials in industry with multiple sheds and high bird number.

Declarations

Author contribution statement

Benjamin W Bauer: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nicky-Lee Willson, Thi Thu Hao Van: Performed the experiments.

Anita Radovanovic, Yadav Sharma Bajagai: Analyzed and interpreted the data.

Robert J Moore: Contributed reagents, materials, analysis tools or

data.

Dragana Stanley: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

The sequence data associated with this study has been deposited at the MG-RAST database under the accession number mgl745316 and a project ID mgp89580.

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Nature of Candidate's Contribution, including percentage of total

BB was involved in the data collection, statistical analysis, and writing of this scientific article. BB was responsible for an estimated 70% of results presented here.

Nature of all Co-Authors' Contributions, including percentage of total

All co-authors assisted with the conception attended lab meetings where the data was discussed and contributed to the revision of the article.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No.

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure

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Chapter 4

Effects of supplemented oregano on the maturation of broiler microbiota

This chapter presents the peer-reviewed conference paper for the poster presented at the Australian Poultry Science Symposium, 2020. The influence that oregano had when given from the first day post-hatch, on development and maturation of intestinal microbiota was investigated. Surprisingly even high concentration of oregano did not result in very different mature microbiota; the differences were small and variable from week to week.

EFFECTS OF SUPPLEMENTED OREGANO ON THE MATURATION OF BROILER FAECAL MICROBIOTA

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Summary

Oregano-based products are being increasingly used in livestock feed as a replacement for antibiotics. Despite the increasing use of oregano on poultry farms, little is known about the influence it may have on the development of the intestinal microbiota of chickens, especially when used from first days of life. We investigated the effect of oregano supplementation in the development of intestinal microbiota in Ross 308 broilers grown with and without 2% oregano in the feed. Faecal samples were collected weekly from week 1 to week 6, and microbial communities were investigated via sequencing of 16S rRNA gene amplicons. Linear regression based on Pearson's correlation showed no difference in taxa positively or negatively correlated with the timeline, in either known pathogenic or beneficial genera, despite some taxa identified as differentially abundant ($P < 0.05$) between control and oregano treatments. The results we present question the validity of using faecal samples to pinpoint slight differences in microbiota.

I. INTRODUCTION

Oregano essential oil contains many compounds, of which carvacrol, thymol and their precursors are the major components, accounting for approximately 80% of the contents. Carvacrol and thymol have been shown to actively disrupt the cell membranes of bacteria leading to cell death, promoting the use of oregano as a phytobiotic (Rao et al., 2010). Phytoadditives such as oregano are becoming more popular as organic and natural alternatives to antibiotics. Many commercial poultry feed supplements are based on oregano antimicrobial products.

Although there is evidence of the presence of bacteria in ovo, the bulk of the microbiota colonisation of the chicken gastrointestinal tract starts from hatch, resulting in a highly populated gut within three days (Lu et al., 2003; Apajalahti et al., 2016). Commercial chickens have a highly variable microbiota (Stanley et al., 2012) that could be easily influenced by various feed additives and feed ingredients during first days of colonisation.

Numerous studies have investigated the effects of oregano on human and animal pathogens. Akdemir Evrendilek (2015) reported that oregano could inhibit growth of *Listeria innocua*, coagulase-negative staphylococci, *Staphylococcus aureus*, *Bacillus subtilis*, *Yersinia enterocolitica*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Proteus mirabilis*, *Escherichia coli* O157:H7, and *Klebsiella oxytoca*. Silva et al., (2013) tested essential oils of known antibacterial herbs including thyme, oregano, rosemary, verbena, basil, peppermint, pennyroyal and mint for their activity against the food spoilage bacteria. Oregano showed the highest antimicrobial activity against the food spoilage bacteria *Listeria monocytogenes, Clostridium perfringens, Bacillus cereus, S. aureus, Enterococcus faecium, Enterococcus faecalis, Staphylococcus epidermidis, Salmonella enterica, E. coli* and *Pseudomonas aeruginosa*. Others suggested that oregano's main active ingredient, carvacrol, can control biofilm formation by disrupting bacterial quorum sensing (Burt et al., 2014).

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With the established ability of oregano to control some of the major poultry pathogens, we hypothesised that a high concentration of oregano in the feed given from day 1 would remodel gut development and prevent, or reduce, the colonisation of some major poultry pathogens such as *Salmonella* and *Campylobacter* species.

II. METHOD

Oregano and feed preparation: Dried oregano leaves (Saucy Spice Company, North Boambee Valley, NSW, Australia) were blended (100 g, 1.5 min/max, 1500W, Nutri Ninja Auto iQ Duo, SharkNinja, USA) and processed in a Planetary Ball Mill Machine (speed no. 5, 2 hrs, 40 g per each run, Changsha Yonglekang Equipment, China). The oregano was then sized using an electric sieve machine (Changsha Yonglekang Equipment, China) and the particle size of the powder was determined by laser diffraction (Mastersizer 2000, Malvern, ATA scientific, Australia); the average size was 10 µm. Antimicrobial and coccidiostat free chicken starter diet crumble (Red Hen, Laucke Mills, Australia) was formulated to meet or exceed the National Research Council guidelines for broiler chickens (NRC, 1994). The 2% of oregano (0.02 kg/kg w/w), was evenly distributed through the feed using an electric mixer (125 L mixer, Ozito, China).

Birds and management: The study was approved by the Animal Ethics Committee at Central Queensland University under the approval number 0000020312 and the data on microbiota structure of different intestinal sections (ileum, jejunum and caecum) at slaughter day at 6 week old with pathogen reduction profiles in these sections, histology and SCFA profiles have been published separately (Bauer et al, 2019). Day-old broiler chicks (Ross Broiler 308, Bond Enterprises, Toowoomba) were delivered to the experimental facility and randomly distributed into two treatments, control (n = 12) and 2% w/w oregano (n=12). All birds were fed ad libitum and had unrestricted access to drinking water. The purpose of this experiment was not to evaluate bird performance, but the development of the microbial communities. Birds were individually tagged using coloured leg rings. Feed consumption and individual bird weights were measured each week, for six weeks. Freshly voided faecal samples (not swabs) were taken weekly from each birds separately.

DNA extraction and 16S rRNA sequencing: DNA was extracted from faecal samples for 16S rRNA gene sequencing. The detailed protocol has been previously described (Gangadoo et al., 2019). The primers contained barcodes, spacers, and Illumina sequencing linkers that have been previously described (Fadrosh et al., 2014). The 16S rRNA gene sequencing library preparation and amplification followed the Illumina recommended protocol (Illumina Inc., San Diego, CA, USA). Sequencing was conducted on the Illumina MiSeq platform using 2x300 bp paired-end sequencing. The sequence data generated from each sample were initially analysed using Quantitative Insights Into Microbial Ecology (QIIME v.1.9.1) as previously described (Gangadoo et al., 2019). All OTUs with less than 0.01% abundance were removed. A Hellinger transformed OTU table was used in statistical investigation. Significantly differentially abundant taxa were identified using ANOVA, and linear regression using Pearson's correlation was also investigated. Calypso was used for further data exploration and visualization (Zakrzewski et al., 2017). The sequence data is publicly available on the MG-RAST database under project mgp89580 and library accession number mgl745316.

III. RESULTS

Oregano and the timeline of microbiota development: The overall structure of the faecal microbiota did not differ significantly between control and oregano supplemented communities (Adonis on weighted UniFrac matrix) at week 1 (P = 0.234), week 2 (P = 0.689), week 3 (P =

0.648), week 4 (P = 0.405), or week 5 (P = 0.176); however, they differentiated at week 6 (P = 0.036). The number of differentially abundant taxa at the genus level was not very high at any of the weekly data, and different genera were affected every week, for example, *Lactobacillus* was reduced in the oregano supplemented group 4.9 fold during week 3 only, and not significantly affected after that. There were also a range of pathogen-rich genera equally inconsistently reduced in oregano at multiple different sampling points, for example, *Streptococcus* was reduced only in week 1, *Clostridium* only in week 3 or *Staphylococcus* only in week 4. However, the genus correlation with the age of birds, across all 6 weeks, was not different in oregano supplemented birds when compared to the un-supplemented control. All significantly correlated genera (Pearson) changed with age in the same direction in both control and oregano supplemented birds (Table 1), significantly increasing with the birds' age in both control and oregano, or reducing in both groups. Oregano supplementation was not able to change the correlation direction or significance even in those taxa that were significantly reduced in abundance in the oregano supplemented birds at particular sampling times.

Table 1 - Pearson-based correlations of genera with the birds' age, performed separately for control and oregano birds. Correlations were not reversed or removed by supplemented oregano but instead remained in the same direction. Every genus that increased over time in the control group was also significantly increased over time in the oregano group and vice versa. Significant positive correlations are shown in italic font and negative in bold.

	CONTROL		2% Oregano	
Taxa	P-value	R	P-value	R
Arthrobacter	0.000007	-0.529	1.2E-06	-0.553
Lactococcus	8.7E-06	-0.524	1E-08	-0.631
Allobaculum	0.000026	-0.501	1.9E-06	-0.544
Bacillus	0.000027	0.499	0.000003	0.535
Turicibacter	0.000031	0.496	2.1E-06	0.542
Leuconostoc	0.000035	-0.493	2.8E-08	-0.616
Microbacterium	0.000046	-0.487	2.7E-08	-0.617
Blautia	0.000066	-0.477	0.000075	-0.467
SMB53	0.00013	0.461	1.8E-09	0.655
Lactobacillus	0.00035	0.433	0.00016	0.445
Serratia	0.0004	-0.429	6.7E-07	-0.564
Facklamia	0.0004	0.429	4E-07	0.573
Paracoccus	0.0011	0.400	0.000034	0.483
Epulopiscium	0.0016	0.387	0.0013	0.385
Leucobacter	0.0021	-0.378	0.00016	-0.445
Trichococcus	0.0022	0.377	3.2E-07	0.577
Proteus	0.0024	-0.373	0.000078	-0.463
Sarcina	0.0045	0.351	1.1E-06	0.554
Corynebacterium	0.006	0.340	0.00048	0.415
Acinetobacter	0.011	0.314	0.00033	0.425
Sphingobacterium	0.031	0.270	0.000019	0.496

IV. DISCUSSION

Oregano based products are among the most frequently used antibiotic alternatives in poultry farming with positive anecdotal feedback in terms of farmer perceived pathogen control. There is an abundance of *in vitro* studies showing that oregano can inhibit a range of common

livestock and poultry pathogens, indicative that oregano would be expected to influence the development of gut microbiota. Although the microbial communities in the excreta became significantly different by week 6, we found that oregano did not prevent the gradual increase/decline of any microbial taxa or reverse its temporal development. At best, oregano in the formulation, at the level tested here, influenced the extent of such gradual changes in microbiota which in turn resulted in slightly differential microbial communities by the end of 6 weeks of continual oregano supplementation. An interesting outcome in this study was the variability of the differential microbiota from week to week. This raises several questions regarding the reliability of significantly differential taxa when there are no significant overall community differences, as well as the importance of timing of the sampling and diet influence. A critical criterion for a phytochemical to be considered as a viable replacement for antibiotics is a minimal disturbance of the intestinal community and, ideally, high inhibitory preference towards poultry pathogens. In the current study, oregano did not disturb the microbial communities over time, even at the high dose of 2% supplementation. In the separate publication (Bauer et al., 2019) we published consistent reduction of some pathogens across gut sections (ileum, jejunum and caecum). Weekly faecal data indicate that oregano supplementation inhibited some pathogens, however not reproducibly over time, and did not exhibit any major influence on the temporal microbiota development trends. The finding that using faecal samples, different genera were affected every week may be result of well-established faecal microbiota sample variability (Stanley et al., 2015) that is a result of periodic emptying of different gut sections in chicken, temporal microbiota fluctuation or simply methodology noise. Either way, our results indicate that intestinal samples, such as caecum, are more appropriate to detect subtle differences in microbiota in chicken than are faecal samples.

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Nature of Candidate's Contribution, including percentage of total

BB was involved in the data collection, statistical analysis, and writing of this scientific article. BB contribution to this manuscript is estimated to 50%. YSB performed complex bioinformatical functional analysis, **Shared first authorship**

Nature of all Co-Authors' Contributions, including percentage of total

All co-authors assisted with the conception, attended lab meetings where the data was discussed, and contributed to revision of the article.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No.

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Chapter 5

Functional analysis of changes in gut microbiota genetic potential in broilers supplemented with 2% oregano

Here we present the investigation into the change of functional abilities of microbiota from the gut of broilers supplemented with 2% oregano. The 16S amplicon sequencing was used in combination with PiCrust algorithm to predict the genes and functions present in microbiota of control and oregano. The data suggested significant changes in function rather than in microbiota per se. Oregano reduced the abundance of genes involved in the ability of bacteria to invade epithelia, make toxins and move. It also reduced a range of infection and disease-related genes and reduced abundance of genes involved in carbohydrate and lipid metabolism and steroid synthesis.

FUNCTIONAL ANALYSIS OF CHANGES IN GUT MICROBIOTA GENETIC POTENTIAL IN BROILERS SUPPLEMENTED WITH 2% OREGANO

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Summary

Oregano-based products, especially oregano's most potent antimicrobial volatile compounds, carvacrol and thymol, are common ingredients of many phytobiotic products on the market. Here we investigated the changes oregano inclusion in broiler feed induces in the genetic function capabilities of the gut microbiota. Oregano changed the phylogenetic composition of the gut microbiota, resulting in a significantly reduced abundance of bacterial genes involved in bacterial motility, flagella, bacterial secretion, and bacterial ability to invade epithelial cells. Oregano may therefore reduce the genetic ability of intestinal bacteria to contribute to a range of infectious diseases. There were also reductions in the abundance of genes involved in intestinal metabolic and digestive functions.

I. INTRODUCTION

Throughout the world, poultry are a major and growing source of high-quality protein, as they outperform all other terrestrial meat production systems in water, feed, carbon, and land use efficiency. A challenge for intensive production systems has been the potential to introduce high pathogen loads and stresses on the animals. Such challenges have traditionally been managed with the assistance of antibacterial growth promoters. However, the poultry industry in Australia, and in many other regions, is working to reduce the use of in-feed antibiotics and alternative products are required to maintain health and productivity. We can look to nature for alternative antimicrobial compounds. Bacteria can infect almost all organisms, and, in response, many hosts have evolved the ability to produce antibacterial compounds to help them fight off pathogenic bacteria. Plants, especially medicinal herbs and spices, produce a range of antibacterial phytochemicals. Phytobionts such as oregano, clove and cinnamon's antimicrobial ingredients, are showing comparable, if not better, results than subclinical antibiotics in pathogen control. There are multiple reports on their effect on individual poultry pathogens such as *Salmonella*, and on performance. Lately, more reports are becoming available on their effect on whole bacterial communities. However, the changes they introduce to the functional capabilities of microbiota, i.e., what microbiota can do for the host, are equally important and give a very different picture to that produced by simple taxonomic profiling of microbiota. Here we present the first insights into the effects of oregano on the functional capabilities of intestinal microbiota in broilers.

II. METHOD

Dried oregano (Turkish, Saucy Spice Company, NSW, containing 2-3% carvacrol) was used to make a powder with an average particle diameter of 10μm. Chicken starter diet (Red Hen, Laucke Mills, Australia) with no antimicrobials or coccidiostats was used for the duration of the trial. The oregano was mixed into the feed at a 2% inclusion rate (0.02 kg/kg w/w). The study was approved by the Animal Ethics Committee of Central Queensland University under

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approval number 0000020312. One day old Ross Broiler 308 birds (Bond Enterprises, Toowoomba) were randomly distributed into two groups with n=12 per treatment. All birds were fed *ad libitum* and had unrestricted access to drinking water. Birds were individually tagged using leg bands and weighed every week for 42 days. Fresh faecal material was collected for each bird. The contents from the jejunum, ileum and caecum intestinal contents, together with faecal samples were taken for sequencing analysis.

DNA was extracted from samples using a previously described protocol (Bauer et al., 2019). The 16S rRNA gene sequencing library preparation and amplification followed the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Sequencing was conducted on the Illumina MiSeq platform using 2x300 bp paired-end sequencing according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). The microbial communities of each sample were initially analysed using QIIME. Phred quality threshold had a minimum of 20. OTUs were picked at 97% similarity using UCLUST (Edgar, 2010). The PICRUSt algorithm (Langille et al., 2013) was used to predict and enumerate genetic functional categories. The sequence data are publicly available at the MG-RAST database under library accession number mgl745316 and project ID mgp89580.

III. RESULTS

We predicted the functional abilities of microbiota using the KEGG database to compare microbiota of control and 2% oregano treated birds. Adonis multivariate analysis using Jaccard distance showed significant functional differences (Adonis $P<0.05$) (Figure 1). The data show that oregano supplementation reduced the abundance of genes in functional categories involved in a number of diseases such as bladder cancer (P=5e-4), prostate cancer (P=0.045), prion diseases (P=0.007) and Shigellosis (P=2.3e-4). The microbiota from oregano supplemented birds had a lower abundance of genes involved in bile secretion (P=5e-5), bacterial motility proteins (P=0.018), flagellar assembly proteins (P=0.023), bacterial secretion (P=0.012) and bacterial invasion of epithelial cells (P=2e-4). Although there were no differences in weight throughout the lifetime of the birds, a range of metabolic functional related genes, such as carbohydrate digestion and absorption (P=1.9e-3), vitamin B6 metabolism (P=0.028), fatty acid metabolism (P=0.001), and steroid hormone biosynthesis (P=0.025) were reduced in oregano supplemented microbiota. Therefore, the data suggest that oregano changes the microbiota structure to reduce bacterial species with the genetic potential for motility, flagella and secretion and their ability to invade epithelial cells, as well as reducing functional capability to contribute to a range of diseases. However, these health beneficial functions come with the cost of reducing microbiota metabolic and digestive ability.

IV. DISCUSSION

Oregano supplementation in the current study significantly reduced the presence of genes involved in flagellar assembly, bacterial motility and bacterial invasion of epithelial cells. It was previously reported (van Alphen et al., 2012) that oregano's main antimicrobial, carvacrol, inhibited the motility of *Campylobacter jejuni* without affecting bacterial growth. This is of significance because it indicates that oregano can reduce pathogenic potential in a way that is not detectable using current culturing or microbiota sequencing methods since the bacterial numbers do not have to be affected. Sub-inhibitory concentrations of carvacrol were shown to block motility and invasion of epithelia by *C. jejuni* via interfering with flagella function (van Alphen et al., 2012); the reduction of flagellar genes in the oregano supplemented microbiota was also significant in the current study. Additionally, carvacrol has been shown to induce heat shock protein 60 and inhibit the synthesis of flagellin in *Escherichia coli* (Burt et al., 2007). Oregano essential oil also shows anti-*Giardia* activity via disruption of its adherence (Machado

et al., 2010). Moreover, oregano essential oil abolishes *Salmonella enterica* serovar Enteritidis in pre-formed biofilms on stainless steel via multi-target action mode on bacterial cell membrane (Lira et al., 2019) and is efficient against antibiotic-resistant *Salmonella enterica* (Moore-Neibel et al., 2013). The anti-*Salmonella* activity is in agreement with our functional analysis findings that oregano reduces motility, bacterial secretion, and invasion of epithelia, all major *Salmonella* weaponries.

The anticancer activity of oregano reported in our functional analysis is aligned with previously reported anticancer properties of carvacrol in breast, liver, and lung carcinomas (reviewed in Sharifi-Rad et al., 2018), coupled with strong antioxidant and anti-inflammatory capabilities (Sharifi-Rad et al., 2018).

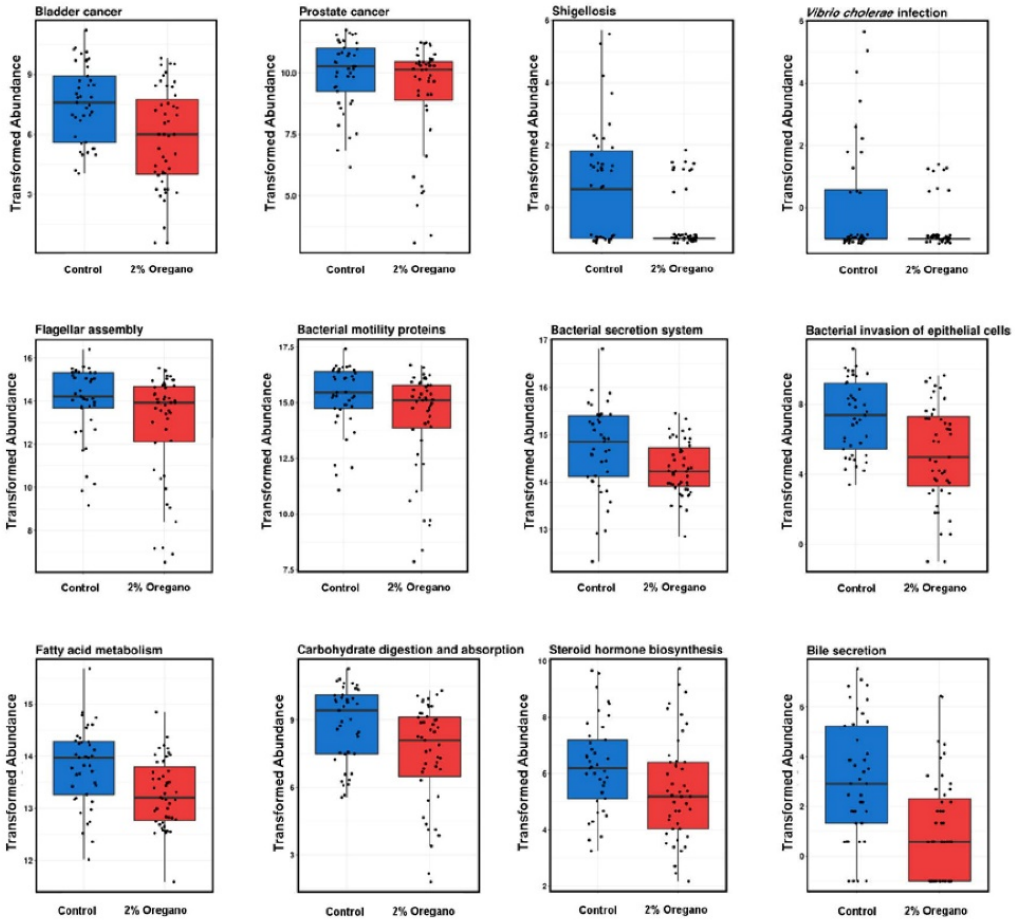

Figure 1 - Functional analysis of changed microbiota genetic potential due to influence of oregano performed in PICRUSt.

Our results indicate that oregano reduced the abundance of genes involved in digestion of carbohydrates and fats as well as steroid hormones and bile. The reports on oregano helping digestion (Reyer et al., 2017; Sharifi-Rad et al., 2018) are opposite to our findings; however, even if significantly altered, the reduction of the digestion-related genes in intestinal bacteria will not necessarily result in measurable reduction in the host digestion, but could also reduce growth of bacteria. Host digestive efficiency depends on a range of host factors, feed composition, and bacterial contribution, to name a few (Swallow, 2003). On the other hand, oregano also reduced the abundance of bacterial genes involved in host bile secretion which strengthens the possibility that oregano can indeed slow down the host digestion process. Our

Chapter 6

Discussion, implications, future work and directions

The data presented in this thesis brings new knowledge to understanding the effects of prolonged oregano supplementation to the broiler chicken. All of the research presented in the literature review is based on the classic culturing methodology where only single species were cultured and investigated separately from the community. With the emergence of sequencing technology and the rising popularity of 16S microbiota sequencing methodology, an understanding has been developed that those same species behave very different when grown in a single culture as opposed to the growth in the complex microbial community. Classic bacterial culturing does not take into account complex microbiota interactions with each other and with the metabolites present in the gut as well as with the host produced factors. This is emphasised with an inability to culture most of the species from the gut, despite all of the modern technology at one's disposal as it is seemingly impossible to reproduce the complexity of the intestinal environment in vitro.

The data showed that oregano did not change microbiota as much as could be expected based on the literature review grounded on single species inhibition data. The same taxa were much more resilient when grown in the community than when grown on their own, presumably due to a very different intestinal chemistry compared to media optimised for perfect growth. The same taxa had ample more metabolites produced by all of the collective community members at their disposal, some of which could provide beneficial effects in mounting a response to the phytogens from oregano. This emphasises the importance of in vivo data.

Interestingly, this translated to a minor influence of oregano on the development of intestinal microbiota, to the level that could be called almost negligible. This is surprising as such potent antimicrobial phytogenic product should remodel colonisation of naïve chicks intestinal tract, however, even at doses as high as 2%, there were no significant differences in the final developed community. On the one hand, this could be disappointing as it was expected that oregano would fully prevent the pathogens from colonising. Even the species with an abundance of literature showing that they are strongly inhibited by oregano did manage to colonise. On the other hand, this emphasises the level of the irrelevance of single strain data to the behaviour in vivo and gives confidence that oregano will not cause significant disruption of normal chicken microbiota.

Instead of bringing chaos to the microbial community, oregano selectively reduced certain species and increased others. Some *Lactobacillus* sp. were strongly induced, and others strongly inhibited. This opens another major question: is there antimicrobial resistance (AMR)

associated with phytogenic products and will they also contribute to the rise of AMR in poultry sheds?

Antimicrobial resistance refers to bacteria becoming resistant to antibiotics that are designed to kill that particular species. AMR is recognised by the World Health Organization (WHO) as a priority research area with growing public awareness of the danger it represents to human health. The Review on Antimicrobial Resistance, a project commissioned in July 2014 by the UK Prime Minister, predicted that "the death toll of AMR could be one person every three seconds", amounting to 10 million per year and cost over 100 trillion USD by 2050. Although hospitals and human overuse of antibiotics are the major sources of AMR pathogens, the use of antibiotics in livestock, mainly pig and poultry, came under the magnifying glass with growing AMR awareness. Leaking AMR genes from farm manure into the soil and waterways are considered a more significant issue then AMR in poultry products.

Recently our understanding of AMR changed significantly. The scientific community now knows that AMR is a natural progression which occurs in response to microbial stressors. Metagenomic analysis of archeological samples showed that thousand years old mummies had AMR genes to all of today's antibiotic classes, even those discovered most recently. This means that AMR is a natural phenomenon that predates the modern era of antibiotics and that some members of the collective of global bacteria are adapted to just about anything antimicrobial that nature has to offer. The modern environment is enriched in mutagenic chemicals, which resulted in some species that have a very high mutation, and hence adaptation rate. Any type of selective antibacterial pressure would, therefore, select for the most mutagenic and most adaptive species, thus increasing total AMR genes. It is quite possible that phytogens will also gradually increase AMR on farms returning the industry few steps back to revisit other ways of pathogen control.

To conclude, the investigation provided some answers and opened many questions warranting further research into the effects of phytogens and their long term AMR interactions. The data also opened several exciting topics that should be investigated further. For example, the functional data analysis pointed out to the ability of oregano to reduce the presence of genes involved in prostate and urinary cancer, as well as indicated possible reduction of sex hormones which can be the reason of reduction of these specific types of cancer. Based on functional analysis, oregano interferes with the digestion of lipids and carbohydrates, the functional

analysis, however, cannot be more specific. Is oregano reducing the ability to absorb or to store the fat and carbohydrates?

Finally, this study focused on microbiota and microbiota function as well as behaviour and maturation of the total microbial community where the number of animals that were used is acceptable. This study did not focus on evaluating performance on an industrial scale, but instead, it focused on microbiota, metabolites and histology. However, the next stage of the project are the trials in layers in an industry setting using n=20,000 investigating resistome and gene expression changes as well as performance. Since these trials follow the layers from hatch to the end of production, they exceed the scope of this thesis and are timing restrictive; however, it was the work presented here that set up the scene and provided information need for optimising the industrial-scale trials.

Appendix A

Chapter 2: Oregano powder reduces *Streptococcus* and increases SCFA concentration in a mixed bacterial culture assay

Supplementary material

Oregano powder reduces *Streptococcus* and increases SCFA concentration in a mixed bacterial culture assay

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Supplementary File 1

Contents:

Table 1: Concentrations of vitamins and trace elements in enhanced LYHBHI media.

Ingredient	Origin	Conc μg/L
Calcium (carbonate)	Cenovis Multivitamin	2.10E+01
Riboflavine (Vit B2)	Cenovis Multivitamin	4.00E-01
Thiamine Nitrate (B1)	Cenovis Multivitamin	4.00E-01
Cyanocobalamin (B12)	Cenovis Multivitamin	5.00E-03
Pyridoxine Hydrochloride (B6)	Cenovis Multivitamin	8.00E-01
Nicotinamide (B3)	Cenovis Multivitamin	4.00E+00
Calcium Panthonenate (B5)	Cenovis Multivitamin	2.20E+00
Zinc (Oxide)	Cenovis Multivitamin	1.50E+00
Ascorbic acid	Cenovis Multivitamin	4.50E+00
Cod-liver oil	Cenovis Multivitamin	1.75E+01
Magnesium (oxide heavy)	Cenovis Multivitamin	1.50E+00
Total Vit A	Cenovis Multivitamin	5.93E-02
dl-alpha-Tocopherol (Vit E 20IU)	Cenovis Multivitamin	1.82E+00
Iron (Ferrous Fumarate)	Cenovis Multivitamin	5.00E-01
Folic acid	Cenovis Multivitamin	3.00E-02
Betacarotene	Cenovis Multivitamin	1.20E-01
Cholecalciferol (Vit D3 154IU)	Cenovis Multivitamin	3.85E-04
Citrus Bioflavonoids Extract	Cenovis Multivitamin	2.00E-01
Biotin (Vit H)	Cenovis Multivitamin	1.50E-02
Phytomenadine (Vit K1)	Cenovis Multivitamin	1.50E-03
Iodine (Potassium Iodide)	Cenovis Multivitamin	1.50E-02

Copper (Cupric Sulfate Anhydrous)	Cenovis Multivitamin	1.00E-01
Chromium (Picolinate)	Cenovis Multivitamin	2.50E-03
Manganese (Sulfate Monohydrate)	Cenovis Multivitamin	1.00E-01
Selenium (Selenomethionine)	Cenovis Multivitamin	2.50E-03
Boron (Boric acid)	Cenovis Multivitamin	3.00E-01
Menaquinone 7-MK7	Caruso's	9.00E+00

Table 2: Differentially abundant OTUs.

Positive Fold Change indicates fold higher in oregano, negative fold change represents fold reduced in oregano.

Multiple genera hits text in the table indicate blast hits with species from different genera with the same %ID, impossible to resolve on the current amplicon size.

Multiple sp. hits text in the table indicate blast hits with different species within the same genera that have the same %ID, impossible to resolve on the current amplicon size.

Uncult. bact. clone text in the table indicates number of hits to uncultured 16S bacterial gene sequences.

OTUs (GreenGenes taxonomy)	***p*-val (DESeq2)**	**Blastn hit**	**Blast % ID**	**Fold Change**
***Lactobacillus* OTU 152229**	2.70E-05	*L. ingluviei*	100	34.14
***Lactobacillus* OTU 272449**	3.20E-05	*L. acidophilus*	100	12.3
***Lactobacillus* OTU 104484**	6.10E-05	*L. ingluviei*	100	113100
***Lactobacillus* OTU 119821**	1.10E-04	*L. johnsonii*	100	8.123
Lactobacillales OTU 33472	3.40E-04	Multiple genera hits	100	6.034
***Lactobacillus* OTU 155425**	4.90E-04	*L. crispatus*	100	5.313
***Lactobacillus* OTU 27332**	9.70E-04	Uncult. bact. clone	98	73.79
***Streptococcus* OTU 207140**	1.00E-03	Uncult. bact. clone	99	-6.339
***Pediococcus* OTU 284103**	1.10E-03	Uncult. bact. clone	98	310100
***Lactobacillus* OTU 251983**	1.20E-03	Uncult. bact. clone	98	-6.113
***Lactobacillus* OTU 266421**	1.30E-03	Uncult. bact. clone	97	7.378

OTUs (GreenGenes taxonomy)	***p*-val (DESeq2)**	**Blastn hit**	**Blast % ID**	**Fold Change**
***Lactobacillus* OTU 170328**	2.00E-03	*L. ingluviei*	96	6.261
Lactobacillaceae OTU 101791	2.40E-03	Multiple genera hits	99	159300
***Lactobacillus* OTU 17603**	2.70E-03	*L. ingluviei*	100	5.941
***Lactobacillus* OTU 80381**	3.50E-03	Uncult. bact. clone	97	17.24
***Lactobacillus* OTU 85290**	4.00E-03	*L. reuteri*	97	-9.651
Enterobacteriaceae OTU 263876	4.30E-03	Uncult. bact. clone	95	7.036
***Lactobacillus* OTU 169080**	5.20E-03	Uncult. bact. clone	95	29700
***Lactobacillus* OTU 76087**	5.20E-03	Multiple genera hits	99	6.504
***Lactobacillus* OTU 116794**	6.10E-03	Uncult. bact. clone	97	26820
***Streptococcus* OTU 136500**	6.20E-03	Uncult. bact. clone	97	-16.13
Carnobacteriaceae OTU 9724	6.70E-03	Multiple genera hits	98	4.884
***Streptococcus* OTU 167252**	6.80E-03	Uncult. bact. clone	98	-8.999
***Lactobacillus* OTU 213664**	7.40E-03	Uncult. bact. clone	98	9.258
Streptococcaceae OTU 180394	7.60E-03	Uncult. bact. clone	96	30660
***Lactobacillus* OTU 98212**	8.50E-03	Uncult. bact. clone	96	36270
***Enterococcus* OTU 123391**	9.20E-03	Multiple genera hits	95	21240
***Streptococcus* OTU 214315**	9.20E-03	Uncult. bact. clone	98	-5.219
Streptococcaceae OTU 19535	9.80E-03	*S. anginosus*	97	5.146
Lactobacillaceae OTU 21754	0.010	Uncult. bact. clone	97	55700
***Lactobacillus* OTU 211163**	0.010	Uncult. bact. clone	97	3.497
***Lactobacillus* OTU 238976**	0.010	Uncult. bact. clone	97	-16.66
Lactobacillaceae OTU 186446	0.011	Multiple genera hits	98	52220
***L. manihotivorans* OTU 159388**	0.011	Uncult. bact. clone	97	4.376
***Lactobacillus* OTU 300049**	0.011	Uncult. bact. clone	98	113.9
Streptococcaceae OTU 182884	0.012	Uncult. bact. clone	96	9.836
***L. coleohominis* OTU 224518**	0.013	*L. coleohominis*	100	2.939

OTUs (GreenGenes taxonomy)	***p*-val (DESeq2)**	**Blastn hit**	**Blast % ID**	**Fold Change**
***Lactobacillus* OTU 228314**	0.013	Uncult. bact. clone	96	10.44
***Lactobacillus* OTU 25404**	0.013	Multiple genera hits	96	4.216
***Lactobacillus* OTU 225126**	0.014	*L.* KC45b	97	44720
***Lactobacillus* OTU 88490**	0.014	*L. ingluviei*	97	12.64
***Lactobacillus* OTU 71614**	0.015	Uncult. bact. clone	96	8.038
***Streptococcus* OTU 97924**	0.015	Uncult. bact. clone	98	-6.752
***Lactobacillus* OTU 286390**	0.016	Uncult. bact. clone	97	20270
***Lactobacillus* OTU 708**	0.016	*L. ingluviei*	97	8.157
***Lactobacillus* OTU 123426**	0.017	Uncult. bact. clone	97	-20.05
***Enterococcus* OTU 38024**	0.019	Uncult. bact. clone	97	2.222
***Lactobacillus* OTU 61159**	0.019	*L. ingluviei*	96	21750
***Lactobacillus* OTU 145258**	0.021	Uncult. bact. clone	97	6.821
***Lactobacillus* OTU 250410**	0.021	Uncult. bact. clone	99	11.34
***Streptococcus* OTU 159558**	0.021	Uncult. bact. clone	97	-5.26
***Lactobacillus* OTU 192829**	0.023	Uncult. bact. clone	99	-4.384
***Lactobacillus* OTU 86477**	0.024	Uncult. bact. clone	98	-8.162
***Lactobacillus* OTU 151770**	0.025	*L. ingluviei*	97	2.977
***Lactobacillus* OTU 158017**	0.025	Uncult. bact. clone	95	31060
***Lactobacillus* OTU 269761**	0.025	Uncult. bact. clone	96	30260
***Streptococcus* OTU 106153**	0.025	*S. macedonicus*	100	-3.892
Lactobacillaceae OTU 147775	0.026	Uncult. bact. clone	96	29460
Lactobacillales OTU 278065	0.026	Uncult. bact. clone	98	44.97
***Lactobacillus* OTU 174211**	0.027	*L. ingluviei*	97	16530
***Streptococcus* OTU 82525**	0.027	Uncult. bact. clone	97	-5.678
***Lactobacillus agilis* OTU 261200**	0.028	*L. agilis*	99	-5.439
***Lactobacillus* OTU 56616**	0.028	Uncult. bact. clone	99	3.656
***Streptococcus* OTU 237114**	0.029	Uncult. bact. clone	97	-8.107
***Lactobacillus* OTU 109663**	0.030	Uncult. bact. clone	98	6.144
***Lactobacillus* OTU 258257**	0.030	Uncult. bact. clone	99	-20030

OTUs (GreenGenes taxonomy)	***p*-val (DESeq2)**	**Blastn hit**	**Blast % ID**	**Fold Change**
***Lactobacillus* OTU 275395**	0.030	Uncult. bact. clone	97	6.727
***Streptococcus* OTU 144009**	0.030	Uncult. bact. clone	98	-12.45
***Streptococcus* OTU 61448**	0.030	Uncult. bact. clone	98	-26380
***Enterococcus* OTU 187731**	0.031	Uncult. bact. clone	98	2.284
***Lactobacillus* OTU 174201**	0.032	Uncult. bact. clone	98	2.526
***Lactobacillus* OTU 207893**	0.033	Uncult. bact. clone	98	-24070
***Lactobacillus* OTU 52161**	0.033	Uncult. bact. clone	98	2.42
***Lactobacillus* OTU 60926**	0.033	Uncult. bact. clone	98	12780
Enterobacteriaceae OTU 92136	0.033	Uncult. bact. clone	96	4.642
***Streptococcus* OTU 264005**	0.037	Uncult. bact. clone	99	-6.379
***Lactobacillus* OTU 192558**	0.038	Uncult. bact. clone	96	6.253
***Streptococcus* OTU 4154**	0.038	Uncult. bact. clone	97	-4.859
***Lactobacillus* OTU 184772**	0.039	Uncult. bact. clone	92	11220
***Enterococcus* OTU 175058**	0.040	Multiple genera hits	99	11420
***Lactobacillus* OTU 34587**	0.040	Uncult. bact. clone	99	4.445
***Lactobacillus* OTU 136182**	0.041	Multiple sp. hits (*Lactobacillus*)	97	4.301
***Lactobacillus* OTU 263116**	0.041	*L. reuteri*	97	-4.942
***Enterococcus* OTU 170058**	0.042	Multiple genera hits	96	5.834
***Lactobacillus* OTU 154837**	0.042	Uncult. bact. clone	96	11030
***Streptococcus* OTU 293956**	0.042	Uncult. bact. clone	98	-5.861
Enterobacteriaceae OTU 253114	0.042	Multiple sp. hits (*Enterobacteriaceae*)	97	13740
***Lactobacillus* OTU 226170**	0.043	Uncult. bact. clone	99	2.021
***Lactobacillus* OTU 207731**	0.044	Uncult. bact. clone	99	10390
***Streptococcus* OTU 122385**	0.044	Uncult. bact. clone	96	-4.077
***Lactobacillus* OTU 284267**	0.045	Uncult. bact. clone	95	7.555
***Lactobacillus* OTU 126939**	0.046	Uncult. bact. clone	97	5.421
***Lactobacillus* OTU 3668**	0.046	*L. ingluviei*	97	13770

OTUs (GreenGenes taxonomy)	***p*-val (DESeq2)**	**Blastn hit**	**Blast % ID**	**Fold Change**
***L. agilis* OTU 116383**	0.048	*L. agilis*	96	11300
***Lactobacillus* OTU 9908**	0.048	Uncult. bact. clone	95	20080
***Lactobacillus* OTU 264260**	0.049	Uncult. bact. clone	96	-15460

Table 3: Genera and OTUs Spearman correlated to acetic acid.

Significant correlations for top 100 most abundant OTUs are shown.

Multiple genera hits text in the table indicate blast hits with species from different genera with the same %ID, impossible to resolve on the current amplicon size.

Uncult. bact. clone text in the table indicates number of hits to uncultured 16S bacterial gene sequences.

OTUs (GreenGenes taxonomy)	***p*-val**	**R**	**Blastn hit**	**Blast % ID**
Enterobacteriaceae OTU 271191	1.70E-04	0.88	Uncult. bact. clone	96
Enterobacteriaceae OTU 261461	5.00E-04	0.85	Uncult. bact. clone	97
***Lactobacillus* OTU 200355**	6.30E-04	0.84	Uncult. bact. clone	98
***Enterococcus* OTU 15749**	1.00E-03	0.82	*E. faecium*	100
***Enterococcus* OTU 55837**	1.70E-03	0.80	Uncult. bact. clone	98
***Enterococcus* OTU 11689**	1.80E-03	0.80	Uncult. bact. clone	97
Enterobacteriaceae OTU 267130	3.40E-03	0.77	Uncult. bact. clone	99
***Enterococcus* OTU 187731**	4.70E-03	0.75	Uncult. bact. clone	98
***Lactobacillus* OTU 17603**	5.30E-03	-0.75	*L. ingluviei*	100
Enterobacteriaceae OTU 146685	5.30E-03	0.75	Uncult. bact. clone	98
Enterobacteriaceae OTU 224864	6.60E-03	0.73	Uncult. bact. clone	99
***Lactobacillus* OTU 35668**	0.010	-0.71	*L. marseille*	98
Enterobacteriaceae OTU 135153	0.011	0.70	*E. coli*	100
***Lactobacillus* OTU 203797**	0.014	-0.69	Uncult. bact. clone	96
***Streptococcus* OTU 154406**	0.014	-0.68	Uncult. bact. clone	98
***Lactobacillus* OTU 708**	0.015	-0.68	*L. ingluviei*	97
***Lactobacillus* OTU 109663**	0.020	-0.66	Uncult. bact. clone	98
***Enterococcus* OTU 264003**	0.021	0.66	Uncult. bact. clone	98
***L. manihotivorans* OTU 159388**	0.021	-0.66	Uncult. bact. clone	97
Enterobacteriaceae OTU 183643	0.023	0.65	Uncult. bact. clone	100
***Lactobacillus* OTU 76947**	0.024	0.64	Uncult. bact. clone	97
***S. alactolyticus* OTU 147828**	0.024	0.64	Uncult. bact. clone	99

OTUs (GreenGenes taxonomy)	***p*-val**	**R**	**Blastn hit**	**Blast % ID**
***Lactobacillus* OTU 148643**	0.025	-0.64	*L. johnsonii*	100
***L. manihotivorans* OTU 198669**	0.026	0.64	Uncult. bact. clone	98
***Lactobacillus* OTU 151770**	0.027	-0.63	*L. ingluviei*	97
***Lactobacillus* OTU 52161**	0.035	0.61	Uncult. bact. clone	98
***Lactobacillus* OTU 92052**	0.035	-0.61	Uncult. bact. clone	99
***Lactobacillus* OTU 1550**	0.038	-0.60	*L. KC45b*	97
Streptococcaceae OTU 233731	0.040	-0.60	Uncult. bact. clone	99
***L. coleohominis* OTU 224518**	0.040	-0.60	Uncult. bact. clone	100
***Lactobacillus* OTU 287135**	0.041	-0.60	Uncult. bact. clone	100
***Lactobacillus* OTU 44522**	0.042	-0.59	Uncult. bact. clone	99
***Lactobacillus* OTU 1270**	0.050	-0.58	Uncult. bact. clone	98
GENERA				
Enterococcus	2.20E-04	0.87	-	-
Ruminococcus	4.00E-04	0.85	-	-
Unclassified Coriobacteriaceae	8.20E-04	0.83	-	-
Unclassified Burkholderiales	4.50E-03	0.75	-	-
Proteus	5.50E-03	0.74	-	-
Unclassified Betaproteobacteria	5.70E-03	0.74	-	-
Coprobacillus	8.50E-03	0.72	-	-
Bacteroides	9.60E-03	0.71	-	-
Unclassified Enterobacteriaceae	0.011	0.70	-	-
Sutterella	0.012	0.69	-	-
Clostridium	0.015	-0.68	-	-
Unclassified Enterococcaceae	0.017	0.67	-	-
Unclassified Erysipelotrichaceae	0.019	0.66	-	-
Bifidobacterium	0.025	0.64	-	-
Collinsella	0.028	0.63	-	-
Unclassified Ruminococcaceae	0.032	0.62	-	-
Unclassified Pseudomonadaceae	0.040	0.60	-	-

Table 4: Genera and OTUs Spearman correlated to butyric acid.

Significant correlations for top 100 most abundant OTUs are shown.

Multiple genera hits text in the table indicate blast hits with species from different genera with the same %ID, impossible to resolve on the current amplicon size.

Uncult. bact. clone text in the table indicates number of hits to uncultured 16S bacterial gene sequences.

OTUs (GreenGenes taxonomy)	***p*-val**	**R**	**Blastn hit**	**Blast % ID**
Enterobacteriaceae OTU 261461	3.20E-04	0.86	Uncult. bact. clone	99
Enterobacteriaceae OTU 271191	5.80E-04	0.84	Uncult. bact. clone	96
***Lactobacillus* OTU 200355**	1.10E-03	0.82	Uncult. bact. clone	98
***Enterococcus* OTU 55837**	1.30E-03	0.81	Uncult. bact. clone	98
***Enterococcus* OTU 187731**	1.60E-03	0.80	Uncult. bact. clone	98
***Enterococcus* OTU 15749**	1.80E-03	0.80	*E. Faecium*	100
***Enterococcus* OTU 11689**	4.30E-03	0.76	Uncult. bact. clone	98
Enterobacteriaceae OTU 267130	6.30E-03	0.74	Uncult. bact. clone	99
***Lactobacillus* OTU 76947**	6.90E-03	0.73	Uncult. bact. clone	95
Enterobacteriaceae OTU 146685	7.00E-03	0.73	Uncult. bact. clone	98
***Lactobacillus* OTU 17603**	9.00E-03	-0.71	*L. ingluviei*	100
Enterobacteriaceae OTU 224864	0.011	0.70	Uncult. bact. clone	99
***Enterococcus* OTU 264003**	0.012	0.70	Uncult. bact. clone	98
Enterobacteriaceae OTU 135153	0.014	0.69	*E. coli*	99
***Lactobacillus* OTU 35668**	0.015	-0.68	Multiple sp. hits (*Lactobacillus*)	98
***S. alactolyticus* OTU 147828**	0.018	0.67	Uncult. bact. clone	99
***Lactobacillus* OTU 109663**	0.021	-0.65	Uncult. bact. clone	99
Enterobacteriaceae OTU 183643	0.021	0.65	Uncult. bact. clone	98
***L. manihotivorans* OTU 198669**	0.028	0.63	Uncult. bact. clone	98
***Lactobacillus* OTU 52161**	0.028	0.63	Uncult. bact. clone	98
***Lactobacillus* OTU 203797**	0.034	-0.61	Uncult. bact. clone	96
***Lactobacillus* OTU 151770**	0.034	-0.61	*L. ingluviei* JCM 12531	97

OTUs (GreenGenes taxonomy)	***p*-val**	**R**	**Blastn hit**	**Blast % ID**
***Enterococcus* OTU 93226**	0.035	0.61	*L. ingluviei*	97
***Lactobacillus* OTU 708**	0.036	-0.61	Uncult. bact. clone	97
***L. manihotivorans* OTU 159388**	0.038	-0.60	Multiple genera hits	100
***Lactobacillus* OTU 148643**	0.040	-0.60	Uncult. bact. clone	97
Streptococcaceae OTU 233731	0.041	-0.60	Uncult. bact. clone	99
***Lactobacillus* OTU 92052**	0.043	-0.59	Uncult. bact. clone	99
***Lactobacillus* OTU 66484**	0.044	0.59	Uncult. bact. clone	97
***Lactobacillus* OTU 1550**	0.046	-0.58	*L. KC45b*	97
***Lactobacillus* OTU 44522**	0.046	-0.59	Uncult. bact. clone	99
GENERA				
Enterococcus	5.00E-04	0.85	-	-
Ruminococcus	1.50E-03	0.81	-	-
Unclassified Coriobacteriaceae	2.70E-03	0.78	-	-
Unclassified Burkholderiales	5.20E-03	0.75	-	-
Proteus	5.90E-03	0.74	-	-
Coprobacillus	6.50E-03	0.73	-	-
Sutterella	6.80E-03	0.73	-	-
Bacteroides	0.012	0.69	-	-
Unclassified Betaproteobacteria	0.013	0.69	-	-
Unclassified Enterobacteriaceae	0.014	0.69	-	-
Clostridium	0.017	-0.67	-	-
Unclassified Enterococcaceae	0.023	0.65	-	-
Unclassified Ruminococcaceae	0.032	0.62	-	-

Table 5: OTUs Spearman correlated to carvacrol.

Significant correlations for top 100 most abundant OTUs are shown. There were no genera significantly correlated with carvacrol.

Multiple genera hits text in the table indicate blast hits with species from different genera with the same %ID, impossible to resolve on the current amplicon size.

Uncult. bact. clone text in the table indicates number of hits to uncultured 16S bacterial gene sequences.

OTUs (GreenGenes taxonomy)	***p*-val**	**R**	**Blastn hit**	**Blast % ID**
***Lactobacillus* OTU 192829**	6.30E-04	-0.84	Uncult. bact. clone	99
***Lactobacillus* OTU 152229**	1.20E-03	0.82	Uncult. bact. clone	97
***Streptococcus* OTU 214315**	1.60E-03	-0.80	Uncult. bact. clone	98
***Lactobacillus* OTU 251983**	5.20E-03	-0.75	Uncult. bact. clone	98
***Streptococcus* OTU 207140**	6.40E-03	-0.74	Uncult. bact. clone	99
***Streptococcus* OTU 230511**	0.011	-0.70	Uncult. bact. clone	99
***L. manihotivorans* OTU 159388**	0.012	0.69	Uncult. bact. clone	97
***Lactobacillus* OTU 119821**	0.014	0.68	*L. KC38*	99
***Lactobacillus* OTU 85290**	0.016	-0.67	Uncult. bact. clone	97
***Streptococcus* OTU 52564**	0.019	-0.66	Uncult. bact. clone	98
Lactobacillales OTU 33472	0.019	0.66	Uncult. bact. clone	99
***Streptococcus* OTU 122385**	0.023	-0.65	Uncult. bact. clone	96
***Lactobacillus* OTU 272449**	0.026	0.64	Uncult. bact. clone	99
***Streptococcus* OTU 159558**	0.035	-0.61	Uncult. bact. clone	99
***Lactobacillus* OTU 92052**	0.038	0.60	Uncult. bact. clone	99
***Lactobacillus* OTU 1550**	0.039	0.60	*L. KC45b*	97