Phytogenic products, used as alternatives to antibiotic growth promoters, modify the intestinal microbiota derived from a range of production systems: an in vitro model

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Abstract
The removal of antibiotics from the feeds used in the livestock industry has resulted in the use of a wide range of alternative antimicrobial products that aim to deliver the productivity and health benefits that have traditionally been associated with antibiotics. Amongst the most popular alternatives are phytogenic product-based extracts from herbs and spices with known antimicrobial properties. Despite embracing such alternatives, the industry is still largely unaware of modes of action, their overall effects on animal health, and interactions with other feed additives such as probiotics. To address some of these issues, three phytogenic products were selected and their interactions with caecal microbiota of layers, grown under six different production systems, were investigated in vitro. Caecal microbiotas were grown with and without phytogenic products, and the changes in microbiota composition were monitored by sequencing of 16S rRNA gene amplicons. Phytogenic products and production system both significantly influenced microbiota composition. The three phytogenic products all altered the relative abundance of species within the *Lactobacillus* genus, by promoting the growth of some and inhibiting other *Lactobacillus* species. There were also significant alterations in the *Bacillus* genus. This was further investigated by comparing the effects of the phytogenic products on the growth of a commercially used *Bacillus*-based probiotic. The phytogens affected the probiotic mix differently, with some promoting the growth of *Bacillus* sp. at lower phytogenic concentrations, and fully suppressing growth at higher concentrations, indicating the importance of finding an optimal concentration that can control pathogens while promoting beneficial bacteria.

Key points
• After removal of antibiotics from animal feed, urgent solutions for pathogen control were needed.
• Alternative products entered the market without much knowledge on their effects on animal health.
• Probiotic products are used in combination with phytogens despite the possible incompatibility.

Keywords Microbiota · Chicken · In vitro · Phytobiotic · *Bacillus*

Introduction
Antibiotic growth promoters (AGPs) have been used in the livestock industry since the 1940s discovery that their addition can improve the growth of livestock, and they became a common practice in the 1950s. By 2001, approximately 70% of total antibiotics used in the USA were given to livestock (USA 2004; Tasho and Cho 2018). It became apparent that the growth-promoting effect of AGPs was related to intestinal microbiota when their administration failed to have any growth-promoting effects in germ-free animals; moreover, in germ-free animals, there were even negative effects instead of accelerated growth (Dibner and Richards 2005). It was speculated that the AGPs’ mode of action included pathogen control; this was especially beneficial for the proven ability of AGPs to control necrotic enteritis, a disease that imposes a significant economic burden on the poultry industry (Elwinger et al. 1998; Van Immerseel et al. 2004;
Visiennnon et al. (2000) and Salmonella, a severe human pathogen, often transmitted from poultry (Wilson 1989). Despite the benefits of AGP use for the industry, in terms of lowered production cost and animal welfare via decreased mortality, very soon after the introduction of AGPs in livestock feed, the first reports of antimicrobial resistance (AMR) and calls for controlled usage started to appear (Starr and Reynolds 1951).

Although the initial rise of AMR is attributed to mutations, bacteria can exchange the AMR genes and hence AMR can spread rapidly and contribute to the reduced clinical efficacy of currently used antibiotics (Bywater 2005; O’Neill 2016). Extended exposure to antibiotics gives a competitive advantage to AMR carrying bacteria which results in AMR strains rapidly taking over the environment. Currently, there is AMR to all known antibiotics, including the last generation that was intended for use in worst-case scenarios (Prestinaci et al. 2015). The World Health Organization (WHO) declared AMR a priority research area with dismal predictions that the death toll due to AMR infections may be one person every 3 seconds, and 10 million per year by 2050. If this prediction came true, the cost to the world is expected to be over 100 trillion USD (O’Neill 2016) and the current coronavirus pandemics is an eye opener to all AMR doom scenarios.

After the evidence on AMR growing escalated, so did the media and public calls for control of AGPs in livestock. As a result of the growing concerns about the escalating occurrence of AMR pathogens, the first country to stop using AGPs was Sweden in 1986, followed by Denmark’s ban of avoparcin which was soon after endorsed by the European Union (EU). This started a chain reaction of AGP removal in EU countries and raised awareness in the rest of the world. A detailed history of the ban of AGPs has been documented in Dibner and Richards (2005). Further bans on AGP use, around the globe, have accelerated with public approval and often triggered by the media coverage of extreme cases of antibiotic abuse in some countries (Darwish et al. 2013; Davies and Walsh 2018; Li et al. 2013; Viswanathan 2014).

Following the implementation of bans on AGP use, there has been a burning need for alternative products that can be used for pathogen control. The alternatives to AGPs considered are many, including enzymes, bacteriocins and antimicrobial peptides, organic acids (such as fumaric, citric, formic, lactic, and sorbic acid), probiotics used continuously and at-hatch administered (bentonite, zeolite, charcoal, and biochar), and essential oils and their individual active antimicrobial phytochemicals such as carvacrol, thymol, cinnamaldehyde, eugenol, and eucalyptol (Anonye 2016; Huygebaert et al. 2011; Tellez and Latorre 2017; Thacker 2013; Verstegen and Williams 2002). New products containing mixed phytochemicals, whole plant, or mushroom extracts are becoming widely accepted in the Australian and worldwide livestock industries and there are a variety of products available on the market, although little is known about their mode of action.

We investigated the validity of the hypothesis that the phytogenic products would influence beneficial and commercial probiotic species as well as pathogens, and that the range of both beneficial and pathogenic bacteria may have natural resistance to these products which could result in their use having the same AMR effects as the originally used banned antibiotics. We selected three commonly used products and investigated their effects on the complex microbial communities taken from the caeca of healthy layer birds.

Materials and methods

In vitro system

Enriched LYHBHI media was prepared by adding chicken feed extract (100 ml), bacterial ferment (100 ml), vitamin mix (1 ml), and trace mineral mix (1 ml) to 798 ml of LYHBHI media (brain heart infusion (37 g/l, BD Biosciences, San Jose, CA, USA), yeast extract (5 g/l, Alfa Aesar, Ward Hill, USA), cellobiose (1 g/l, BD), hemin (0.005 g/l, BD), L-cysteine (0.5 g/l, Alfa Aesar), resazurin sodium salt (5 mg/l, Alfa Aesar)) to make the final volume of 1 l. The LYHBHI media and the feed extract were sterilised by autoclaving while bacterial ferment was filter sterilised. The media was purged with nitrogen gas to remove the oxygen in the media.

Lactobacillus plantarum (ATCC® BAA-793™) and Lactobacillus rhamnosus (ATCC® 53103™) were cultured aerobically at 37 °C in 60 ml of LYHBHI. The culture was centrifuged and 50 ml of supernatant from each culture was filter sterilised to mix in the media. To prepare the feed extract, 100 g of chicken feed (Red Hen Chick premium micro starter crumbles antibiotic and anticoccidial free; Lauke Mills, Daveystone SA, Australia) was ground for 3 min (1500 W, Nutri Ninja Auto iQ Duo; SharkNinja, Needham, USA). Powdered feed was blended for an additional 3 min after adding 1 l of water. The feed extract was autoclaved and centrifuged (3220g, 5 min) and 100 ml of supernatant was added to LYHBHI.

One capsule of vitamin mix (Multivitamins and Minerals, Cenovis, Brisbane, Australia) and a capsule of vitamin K2 (Caruso’s Natural Health, Eastern Creek, Australia) were mixed in 10 ml of water and filter sterilised to use in the media. Similarly, 1 ml of trace elements mix (Youngevity, Chula Vista, USA) was also added in the media after filter sterilisation.

Caecum parent cultures

The caeca from 24 layer hens from the same commercial layer farm in Queensland, Australia, were collected, transported on dry
ice, and stored at −80 °C. The farm has its own hatchery and feed mill ensuring the same genetics and feed for the production systems used. The caeca were thawed inside an anaerobic (with N₂/CO₂ gas) workstation (A35, Whitley, Shipley, UK) at 37 °C. The caecal contents were squeezed into Erlenmeyer flasks containing 50 ml of enhanced LYHBBHI media. The caeca were then opened and transferred into the flask together with the remaining content to ensure the inclusion of mucosa-associated microbes in the parent culture. The culture was incubated at 37 °C for 2 h in the anaerobic chamber with shaking at the rate of 90 rpm (orbital shaker) and aliquoted into cryotubes with 30% sterile glycerol and stored at −80 °C. The Animal Ethics Committee of Central Queensland University approved this study under the approval number 0000020312.

**Experimental design**

Three phytogenic products were used in the study. Product 1 (Prod1) is a mix of phytochemicals including plant compounds such as carvacrol, cinnamaldehyde, and organic acids. Product 2 (Prod2) is a whole-plant extract containing caffeoylquinic acids, flavonoids, polyphenols, and pro-vitamins, and product 3 (Prod3) is another mix of phytochemicals containing 50% oregano essential oil and a food-grade silicic acid. Twenty-four parent cultures, 4 each from the six different production systems: cage, barn, organic, free-range, and 2 control enriched LYHBBHI media as well as in the same media. The caeca were then grown for 24 h, collected, and immediately centrifuged and the microbial pellet was stored for DNA extraction.

**DNA extraction**

DNA from the microbial pellets of the anaerobic cultures was extracted using the method as previously described (Bauer et al. 2019; Gangadoo et al. 2019). Briefly, the microbial cells were lysed using a bead beater with 0.2 g of 0.1-mm glass beads and 500 µl of lysis buffer. DNA was purified using a DNA spin purification column (Enzymax LLC, Cat# EZC101, Kentucky, USA) and quantity and quality of DNA was measured with a NanoDrop Micro-UV/Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

**16S rRNA gene sequencing**

The V3–V4 regions of the 16S rRNA genes were amplified using the primers ACTCTCTACGGGAGGCAGCAG (forward) and GGACTACHVGGGTWTCTAAT (reverse). The DNA sequencing library was prepared following the manufacturer’s protocol (Illumina Inc., San Diego, CA, USA) using barcodes, spacers, and Illumina sequencing linkers (Fadrosh et al. 2014). The DNA library was sequenced on Illumina MiSeq platform using 2 × 300 bp paired-end sequencing.

The upstream DNA sequence analysis was done using Quantitative Insights Into Microbial Ecology (QIIME v.1.9.1) (Caporaso et al. 2010) as this software package includes methods to deconvolute and process dual barcoded amplicons that are not available in more recent software packages. Fastq-Join algorithm was used to combine paired-end sequences allowing no mismatches within the overlapping region. The reads with Phred quality score of 20 or above were selected for the analysis. UCLUST (Edgar 2010) was used for OTU (operational taxonomic units) picking at 97% similarity while Pintail (Ashelford et al. 2005) was used for the inspection of chimeric sequences. The sequences were assigned with taxonomy against the GreenGenes database (v 2013_8) with QIIME default parameters (DeSantis et al. 2006). A rarefied OTU abundance table was used to obtain a UniFrac matrix. The OTU table was Hellinger transformed (Legendre and Gallagher 2001) for further downstream analysis and interpretation.

Downstream analysis of the data and visualisation of the result was performed using Calypso (Zakrzewski et al. 2017). The sequence data is publicly available at the MG-RAST database under a project ID mgp454253 and library ID mgl794100.

**Bacillus inhibition growth curves**

*Bacillus* probiotic product was a mix of 3 *Bacillus* species and will not be named. The probiotic product culture was grown at 37 °C overnight by inoculating the product powder into brain heart infusion (BHI) media. The primary culture was then subcultured and harvested in the exponential growth stage for use in the *Bacillus* inhibition growth curve analysis. The phyogenic product concentrations were optimised in a test run and the *Bacillus* growth curves (n = 7) were measured on a SpectroStar Omega microplate reader (BMG Labtech, Ortenberg, Germany), with ultrafast UV/Vis, absorbance spectrophotometer. Optical density was measured every hour for 20 h at 600 nm. Each data point for each sample was an average of 5 OD measurements, evenly covering all parts of the well. The 96-well plate reader was operating overnight at 37 °C and with 100 rpm plate orbital shaking. Each treatment was represented with n = 7 measurements (wells) and each product concentration had its own blank that was later deducted.

**Results**

The influence of treatments and of the production systems on microbial communities in the in vitro system were both significant, with 2-way PERMANOVA (done using Primer 7e) \( P < 1 \times 10^{-5} \) and \( P = 3 \times 10^{-4} \), respectively. There was no significant interaction between the treatments and production systems.
We tested a range of multivariate analyses for visualisation, and the control was consistently separated from more or less overlapping treatments. Discriminant analysis of principal components (DAPC plot) analysis showed the two more similar products (Prod1 and Prod3), both containing carvacrol and herbal phytogens, overlapped (DAPC plot, Fig. 1) while Prod2, a perennial edible plant extract, was slightly separated (Fig. 1). Control was distinctly separated from all three products.

Similarly, the production system DAPC plot presented in Fig. 2 shows the separation of cage and barn originated microbiota and overlap of the organic, free-range, and open range, emphasising again that organic-fed birds are also grown in free-range sheds but fed organic feed mix while the open-range birds have a much bigger outdoor range like pasture or orchards access. Each production system was represented with 16 different replicates (4 biological replicates grown under 4 phytogenic conditions). Two different sheds under open-range production system showed high microbiota similarity. Cage production system seems the most unique compared to the others. The overlap of the physically closed cage and barn systems, where the birds have no access to outdoors, was relatively tight compared to the remaining systems with outdoor access that showed more variation.

The treatments with different products did not have any major influence on the diversity measures inspected (Chao1 $P = 0.86$; Richness $P = 0.98$; Evenness $P = 0.31$; Shannon $P = 0.41$, and Simpson $P = 0.14$); however, the production system did show some significant alterations (Chao1 $P = 3.5 \times 10^{-5}$; Richness $P = 1.4 \times 10^{-4}$; Evenness $P = 2.5 \times 10^{-3}$; Shannon $P = 8.6 \times 10^{-4}$; and Simpson $P = 4.8 \times 10^{-3}$) (Fig. 3). The barn-grown birds clustered into two groups with lower and higher Chao1-based diversity. Surprisingly cage bird samples showed relatively higher diversity than the other groups; however, it should be noted that these are in vitro culture diversity measures and that they should not be confused with original microbiota of the donor animal caecal diversity as that would be strongly influenced by the community supported by the microbiological media used.

There were six genera significantly (ANOVA, $P < 0.05$) affected by the products (Fig. 4), with the overlapping effect of the three products in all genera except for Isobaculum, where Prod2 (whole-plant extract) had the opposite effect compared to herbal phytogenic mixes Prod1 and Prod3. In all other affected genera, the three products acted equally, by reducing Clostridium, unknown Clostridiaceae, Trichococcus and recently very popular poultry probiotic genus Bacillus. All three products increased the overall abundance of the genus Lactobacillus.

There were three genera significantly affected by production system: Pediococcus ($P = 0.011$, slightly higher in open range), Aeriscardovia ($P = 1.2 \times 10^{-3}$), and Isobaculum ($P = 5.9 \times 10^{-5}$) both slightly higher in the organic and in the

Fig. 1 Discriminant analysis of principal components (DAPC) showing the similarities between the treatments. Each dot represents a microbiota profile from a successfully sequenced sample.
cage. Organic and cage systems also showed more of the phylum *Actinobacteria* (*P* = 0.001). The linear discriminant analysis (LDA) effect size method (LEfSe, Fig. 5) selects the genera most likely to explain differences between the treatments and production systems microbiotas, via combining standard statistical tests with further tests encoding biological consistency and effect relevance. The analysis identifies Prod1 as the most *Lactobacillus* promoting and Prod3 as related to unclassified *Clostridiales*. Control birds microbiota was characterised with *Clostridium*, *Bacillus*, and *Trichococcus*. Similarly, the barn production system was associated with *Clostridium* and *Trichococcus*, whereas the cage system was an *Enterococcus* and *Isobaculum* represented environment (Fig. 5).

At the OTU level, there were 408 OTUs differentially abundant between the treatments (ANOVA, *P* < 0.05), 27 of those assigned to *C. perfringens* with blastn %ID up to 100%, all reduced in all three treatments as a major contributor to the reduction of genus *Clostridium* (Fig. 4). The overall increase in the *Lactobacillus* genus, by all three products, was a product of substantial rearrangement of the *Lactobacillus* species (Fig. 6), with 330 differential OTUs from this genus, 84 OTUs significantly reduced and 246 promoted by the products. This can be an indication of common multiple phytogenic antimicrobial resistance amongst some *Lactobacillus* species.

Unlike with genus *Bacillus* that contains a fewer probiotic and some pathogenic species, there are many unique species...
and strains of *Lactobacillus* used as probiotics in poultry production that, based on our data, can be either promoted or inhibited or even removed by the products we investigated. Contrastingly, the data in Fig. 4 shows the reproducible inhibition of *Bacillus* species by all three phytogenic products. We selected a widely used *Bacillus* commercial probiotic product that contains three species of *Bacillus* and performed a growth curve assay in the presence of the three products (Fig. 7). Figure 7 indicates that Prod1 and Prod3 at lower concentrations can stimulate the growth of the *Bacillus* probiotic but become inhibitory at higher concentrations. Prod3 was slightly inhibitory at the lowest concentration tested but became growth-promoting at all higher concentrations. It should be noted that the plate reader growth curves move out of the linear range after OD = 0.5 and since it is technically impossible to dilute the sample into linear range while in the plate reader, the growth curves at higher OD are “compressed” and the differences in the stationary phase would be more prominent if the growth curves were plotted using dilutions. Despite this, the differences between both 0.05 and 0.1% Prod1 and control were significant in all measurements after reaching stationary phase at 10 h (*P* < 0.01 using multiple *t* tests for each hour). There was no significant difference between the treatments and control for Prod2 and no difference between

![Fig. 4 Genera significantly altered (ANOVA, *P* < 0.05) by the three treatments](image)

![Fig. 5 Linear discriminant analysis effect size method (LEfSe) showing the genera most likely to explain differences between the treatments (top image) and production systems (bottom image) microbiotas](image)
0.1% Prod3 and control at the end of stationary phase measurements.

Discussion

With the recent prohibition of the use of AGPs in livestock feed and an overall negative public perception of using AGPs in the livestock industry, there has been a flood of products promising effective pathogen control using organic-compatible, mostly plant-based products. Here we investigated the effects of three of these products on layer bird caecal microbiota using an in vitro system and microbial community analysis. We concluded that, while both the phytogenic products and the production systems did have a major influence on microbiota, there was no significant interaction between the two; thus, the effect of the phytogenic products should be more or less reproducible across the different layer production systems.

Interestingly, despite having a significant influence on microbiota, our results suggest that the phytogenic products did not reduce richness and diversity measures using in vitro model, as opposed to the production systems where differences in richness were quite substantial. This may come as a surprise because the role of these products is to control bacteria, specifically pathogen growth and overgrowth in the gut of livestock, and based on human data on the influence of prolonged use of antibiotics on richness and diversity (Fricke 2014), we could expect a significant reduction of diversity by antibiotic-alternative products. On the other hand, antibiotic growth promoters previously used in the industry also had no major influence on intestinal microbiota and no reduction in diversity with zinc bacitracin even increasing richness in broilers (Crisol-Martinez et al. 2017); thus, their mode of action is more accurately described as preventing microbial overgrowth rather than entirely removing a range of susceptible species. The reduction of some species/OTUs below detection limit can allow for growth of other rare microbiota above the detection limit, thus compensating diversity reductions.

Apart from numerous OTUs that were similar or identical to Clostridium perfringens, that were almost entirely removed by all 3 products, our samples had no major poultry pathogens; thus, the effect of the products on Salmonella and Campylobacter could not be investigated. However, the influence on the Bacillus commercial probiotic, as well as rearrangements of Lactobacillus species, is of industrial significance. High level of resistance to multiple phytogenic products by some of the Lactobacillus species indicates that some bacteria have natural resistance to a range of distinct phytogenic products. Mechanisms of this resistance are still unknown and the possibility of phytogenic products promoting AMR needs to be further investigated.

Lactobacillus and Bifidobacterium probiotic species have dominated the livestock probiotic industry for decades with other beneficial species of Enterococcus, Streptococcus, and


Bacillus having a lesser role. However, in more recent years, Bacillus-based probiotics have become more common in the livestock market, to now become one of the most widely used. Bacillus probiotics are gaining attention in probiotics formulas for human use as detailed in a recent review (Elshaghabee et al. 2017), mostly due to their enhanced tolerance and survivability under the aggressive environments of probiotic production practices such as freeze-drying and bile and acid of the gastrointestinal tract. Bacillus probiotics have proven themselves as more stable during prolonged storage due to the spore-forming capability. Bacillus probiotic species possess the capability of pathogen exclusion, anti-oxidant, antimicrobial action, beneficial immuno-modulatory, and food fermentation abilities (Elshaghabee et al. 2017).

Despite the considerable range of Bacillus probiotic benefits, the recent rise in demand for Bacillus products in the livestock industry comes from their exceptional capacity to produce extracellular enzymes. Bacillus species are used for industrial production of food-grade amylase, glucoamylase, protease, pectinase, and cellulase (Elshaghabee et al. 2017; Kiran et al. 2018; Kuppers et al. 2014; Simair et al. 2017; Wiegand et al. 2013) as well as vitamins (Commichau et al. 2014; Mahdinia et al. 2018; Paracchini et al. 2017) and carotenoids (Xue et al. 2015; Yoshida et al. 2009) used for the production of numerous human health supplements. Bacillus production of digestive enzymes is significant for the poultry industry that uses hard to digest foods in animals that have very short food gut retention time. This is amplified during the current industry adjustment to ecological impacts of production and sustainability that involves experimenting with hard to digest unconventional foods and waste products that could be more readily available in future than poultry feedstuffs that have been traditionally used (Abudabos et al. 2017).

The recent rise in the use of Bacillus products coincides with the replacement of traditional antibiotics growth promoters with a range of phytotherapeutic and other alternative products. It is interesting that, to date, no work has been published to demonstrate their mutual compatibility, despite the fact that the farmers commonly mix them in the same ration. Our data indicate that some products at lower concentrations can significantly promote Bacillus probiotic growth but suppress it at higher concentrations; thus, it is possible to find a “sweet spot” and adjust the phytotherapeutic concentration towards promoting probiotic growth while keeping pathogens under control. This requires careful on-farm optimisation. We must note that we performed growth curves on a mixed probiotic product containing 3 Bacillus species and that each species could be differently affected. It is, however, difficult to perform competitive exclusion curves with 3 species of the same genus without strain modification, but even individual species growth curves would be useful to answer this question. The results suggest that there is likely to be value in testing the compatibility of other probiotics with the phytotherapeutic products in order to best guide their effective combinatorial use on farms.

This study has provided new insights into the action of phytotherapeutic products on intestinal microbiota of layers, using in vitro experiments on the complex caecal microbial community. However, microbiota in the actual birds is more complex than in the in vitro model and it differs between the gut sections. In vitro methodology uses only subsection of the original bird microbiota that can grow on the specific media and can survive freezing of the inoculum for storage. Additionally, there is a robust variability of intestinal microbiota between and within the flocks (Stanley et al. 2013) further complicated by complex interactions between microbes and their metabolites in these communities, so that the effect of phytotherapeutic products on the livestock cannot be easily predicted. Administration of both probiotics and alternative antibiotic products from early days post-hatch is likely to have a critical influence on the development of microbiota (Baldwin et al. 2018; Donaldson et al. 2017). More research is needed to better understand their impact on animal health, antimicrobial resistance on farms, pathogen control, and immunity.

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Data availability Sequencing data is publicly available on MG-RAST Metagenomics Analysis Server Database (https://www.mg-raft.org/) with full sample annotation under project mgp454253 and a library ID mgl794100.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animal ethics approvals were obtained from the Animal Ethics Committee at Central Queensland University with the approval number 0000020312.

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