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Zeolite food supplementation reduces abundance of enterobacteria

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ABSTRACT

According to the World Health Organisation, antibiotics are rapidly losing potency in every country of the world. Poultry are currently perceived as a major source of pathogens and antimicrobial resistance. There is an urgent need for new and natural ways to control pathogens in poultry and humans alike. Porous, cation rich, aluminosilicate minerals, zeolites can be used as a feed additive in poultry rations, demonstrating multiple productivity benefits. Next generation sequencing of the 16S rRNA marker gene was used to phylogenetically characterize the fecal microbiota and thus investigate the ability and dose dependency of zeolite in terms of anti-pathogenic effects. A natural zeolite was used as a feed additive in laying hens at 1, 2, and 4% w/w for a 23 week period. At the end of this period cloacal swabs were collected to sample faecal microbial communities. A significant reduction in carriage of bacteria within the phylum Proteobacteria, especially in members of the pathogen-rich family Enterobacteriaceae, was noted across all three concentrations of zeolite. Zeolite supplementation of feed resulted in a reduction in the carriage of a number of poultry pathogens without disturbing beneficial bacteria. This effect was, in some phylotypes, correlated with the zeolite concentration. This result is relevant to zeolite feeding in other animal production systems, and for human pathogenesis.

1. Introduction

The poultry industry is striving for production methods that reduce dependence on the use of antibiotic growth promotants and antibacterial chemicals to avoid the development of microbiota with antibiotic or antibacterial chemical resistance. Zeolite, a porous aluminosilicate mineral, is used as a dietary supplement for a number of purposes, including for its anti-microbial properties. Zeolite is available in natural and synthetic forms, being widely used across a range of industries, including as a catalyst in petrochemical refining. Natural zeolite, most commonly supplied in the form of clinoptilolite, is suitable for animal feed application. Clinoptilolite has a three-dimensional framework of silicates and aluminates forming tetrahedra linked through shared oxygen atoms (Mumpton and Fishman, 1977). The presence of channels and cavities give a highly effective surface area, and the material is characterised by exchange of cations and the adsorption of organic compounds, including mycotoxins (Mumpton and Fishman, 1977; Filippidis et al., 1996).

The use of zeolite as a feed additive to enhance performance is well established in commercial animal production (Mumpton and Fishman, 1977; Mumpton, 1999; Martin-Kleiner et al., 2001), with reports of use in rats, lambs, pigs (Pond and Yen, 1983; Heather et al., 2009) and poultry production (Fethiere et al., 1994; Papaioannou et al., 2005). For example, in egg production, egg weight and internal egg quality have been increased when laying hens were fed zeolite (Olver, 1997; Papaioannou et al., 2005). Papaioannou et al. (2005) noted that these performance effects should be impacted by zeolite type, purity, particle size and the proportion of supplementation in rations, while Wu et al. (2013) noted that production was further improved by use of chemically modified clinoptilolite (*i.e.*, by mixing with formic acid).

Zeolites may have several positive impacts in poultry production. Feed consumption efficiency may be improved through a reduction in the rate of passage of feed through the digestive system, leading to a reduction in feed intake and more complete utilisation of nutrients. For example higher egg production, increased shell thickness, and improved feed efficiency were noticed with zeolite-amended (5% w/w) compared to control diet

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(Olver, 1989). Fethiere et al. (1990) reported equivalent egg production but lowered feed consumption (and thus decreased feed conversion ratio) with use of a zeolite amended diet. The increased performance and overall health in birds indicate no significant absorption and removal of major nutrients and vitamins. However, clinoptilolite can also adsorb toxins present in the feed, carrying these materials into excreta (Goodarzi and Modiri, 2011; Oguz, 2011). Toxins so bound include both heavy metals (Zhou, 2008) such as arsenic, cadmium, lead, mercury and radionuclides (Slavata and Vitorović, 2004) and mycotoxins (Placinta et al., 1999). Clinoptilolite has a high *in vitro* adsorption index for mycotoxins (>80%, for aflatoxin B1 and G2, (Dakovic et al., 2000). The adsorption process is reported to have a high initial rate, with most of the toxins adsorbed within the first minutes (Dakovic et al., 2000), in contrast Lemke et al. (2001) reported limited binding of aflatoxin B_1 by clinoptilite based on *in vitro* adsorption studies.

Natural zeolites have been shown to have bactericidal effects. Mallek et al. (2012) reported that addition of clinoptilolite (0.5 or 1% w/w) in a broiler diet produced a significant ($P < 0.05$) reduction in total culturable microbial levels, as compared to the control group. Clinoptilolite may also be able to adsorb selective bacterial species. In an *in-vitro* study, *Escherichia coli* and *Salmonella* Typhimurium were noted to be adsorbed by clinoptilolite but there was no general bactericidal effect (Wu et al., 2013). Supplementation of broiler chicken rations with zeolite was associated with a reduction in viable counts of *Salmonella* Enteritidis and *E. coli* in the proximal and distal gut and a reduction in mortality (Olver, 1983). Al-Nasser et al. (2011) reported that supplementation of broiler feed with clinoptilolite (1 to 2% w/w) resulted in a reduction of *Salmonella* and improved performance in production parameters. Al-Nasser et al. (2011) reported that the use of 5% w/w zeolite in poultry feed resulted in a reduction of carcass condemnation due to *Salmonella* contamination. Chemical modification of clinoptilolite with organic acids (*e.g.* formic acid) was reported to result in increased hydrophobicity of the mineral surface, increased bactericidal effect against *Escherichia coli* and its toxins, and increased cation exchange capacity (Uchida et al., 1992; Daković et al., 2005).

Egg laying birds are rarely treated with antibiotics to avoid traces of antibiotic in eggs and an increased risk of antibiotic resistance, in contrast to broiler production where antibiotic growth promoters can be used. However, egg producers are mature birds compared to juvenile broilers and thus will harbour more pathogens and consequently be in greater need of efficient natural pathogen control. For example, Spotty Liver disease is an increasing issue for the egg production industry (Crawshaw et al., 2015).

The poultry industry is changing rapidly and a consumer push for antibiotic free and more natural farming practices such as free-range and organic, has seen the use of antibiotic growth promoters (AGPs) banned in Europe. The main purpose of AGPs in poultry feed is pathogen control, and alternative natural methods for pathogen control that can be used in broilers as well as in layers are urgently needed by the industry. In an earlier report (Prasai et al., 2016) we compared the effects of high (4% w/w) concentrations of zeolite, bentonite and biochar on microbial populations in laying hens. Very different effects on microbial communities were observed for the three additives, with 4% w/w zeolite strongly affecting intestinal microbiota by completely removing a number of species and at the same time allowing species that were below detection limit to achieve higher abundance. However, 4% w/w is considered a relatively high dose necessitating use of more energy and protein rich ingredients and thus higher value constituents in the feed mix to maintain feed caloric value (metabolizable energy and protein).

The present publication investigates dose-dependant effects of zeolite on common poultry pathogens and estimates the lowest in-feed concentration with good antipathogenic effect. The low (1%) and medium dosage (2%) of zeolite in feed had comparable effects on microbiota, while high dose of zeolite (4%) showed loss of anti-pathogenic properties. A number of species demonstrated significant positive or negative correlation with zeolite concentration in feed. Our results suggest that low to medium concentrations of zeolite have the ability to reduce poultry pathogens including Proteobacteria, especially Enterobacteria.

2. Materials and methods

This work was approved by the Animal Ethics Committee of Central Queensland University (A 12/06-283). Eighty Bond Brown Layer 17 week old pullets were housed in a commercial layer caging system consisting of cages each 60 × 60 × 50 cm in height, width and depth. Pullets were randomly assigned to cages, with 5 birds in each pen and 4 pens per treatment (4 pens × 5 birds/pen = 20 birds per treatment), within a randomised block layout. The treatments were imposed one week after receipt of birds allowing the birds one week for microbiota to stabilise in the new environment, and were maintained for the duration of the trial (23 weeks). Multiple pens per treatment, pen randomisation and balanced pen and overall design with the 1 week adaptation period were used to control the caging effect on microbiota. The treatments involved a control ration and zeolite rations amended with zeolite at 1, 2, 4% w/w (n = 20 each). The commercial layer ration (control ration) consisted of 90.4% dry matter, metabolisable energy 11.75 MJ/kg, crude protein 17.9% and calcium 4.2% by dry weight, supplied as crumble mixture.

Cloacal faecal samples were collected using a sterile swab, 23 weeks after the diets were introduced. Samples were immediately transferred to a sterile vial and then stored at −80 °C. Total DNA was isolated with Bioline ISOLATE Faecal DNA Kit (#BIO-52038) using the manufacturers protocol. DNA amplification was performed using primers selected to amplify V3-V4 region of 16S rRNA gene as previously described (Wilkinson et al., 2016). Sequencing was performed on an Illumina MiSeq system (2 × 300 bp) using the dual-indexing method of Fadrosh et al. (2014). The analysis was performed in QIIME 1.9.1 software (Caporaso et al., 2010) using QIIME default parameters and discarding sequences with Phred quality threshold lower than 20. The Uclust algorithm (Edgar, 2010) was used for operational taxonomic unit (OTU) picking at 97% sequence identity and chimeric sequences were inspected using Pintail (Ashelford et al., 2005). Taxonomy was assigned with blast against the GreenGenes database (DeSantis et al., 2006). The GreenGenes database with default QIIME arguments was used only in initial data inspection with additional deeper taxonomic assignment done for taxa of interest with blastn (Altschul et al., 1997) against the NCBI 16S Microbial database. Samples with low sequence numbers were discarded during rarefaction to 1850 reads per sample and a number of birds successfully sequenced and analysed for the 4 conditions were 10, 13, 14 and 15 per treatment. The complete dataset for this experiment is publically available on the MG-RAST server (http://metagenomics.anl.gov/) under project ID mgp17623 and metagenome ID 4693703.3. Some data were visualised using Calypso (http://cgenome.net/calypso/).

3. Results

3.1. Animal performance and productivity

There were no significant differences in bird weights on day 0, *i.e.* prior to introduction of the diet treatments (Fig. 1), nor at the end of the 23 weeks supplementation period. Egg production was higher in all zeolite treatment groups than in the control group, but not significantly different between levels of treatment and thus it was dose independent. Feed Conversion Ratio (FCR) was better in the