Exploring the Potential to Improve the Gut Microbiome of Broiler Chickens using Selenium Nanoparticle Supplements

by

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Abstract

The increasing demand for food in the form of chicken meat and eggs in the poultry industry has led to an increase in research interest in enhancing growth rate, feed efficiency, and reducing pathogens in poultry birds. Antibiotics and feed additives, including nutrient and vitamin supplementations, have been incorporated in poultry diets for years to ensure the maintenance of poultry health with a focus on the control and reduction of zoonotic pathogens. In the last few years, however, key issues surrounding the antimicrobial resistance of antibiotics have urged for a replacement in combating and reducing pathogenic bacteria in poultry, while still maintaining the ability to provide the necessary nutrients to the body.

Nanoparticles (NPs) are materials of a nano-size range (< 100 nm) which have been used in a vast area of applications, including medical, optics, electronics, and nutrition. NPs have a higher surface area to volume ratio than their macro- and micro-counterparts and have been thoroughly used in poultry feed as a vehicle for delivering compounds such as vaccines and nutrient supplements. Studies have demonstrated their superior ability to enable the absorption of compounds across the intestinal wall and the direct transportation of these compounds to target multiple organs and systems. They are also able to avoid fast degradability as they can bypass the body's metabolic system and defence barriers. Most of the current research has been focused on reducing zoonotic pathogens, leaving a wide-open space for research to be conducted on the ability of NPs to modulate the gut microbiota and exercise their health impacts.

NPs of silver and other metals have been heavily used in the poultry industry to improve the growth and performance of birds. Whilst successful, metal NPs exhibited higher toxicity due to the higher surface to volume ratio, especially with the use of silver. This study proposes the use of NPs of essential metals and natural compounds to safely deliver nutrients, resulting in positive impacts on health and productivity with little to no toxic effects. Selenium (Se) is an essential mineral, required for the proper functioning of the immune system and is an important element in the first cell line of defence in the body. The work described in this thesis explores the ability of Se NPs in improving the health and growth of broiler chickens by modulating their gut microbiome and metabolome, without the toxic effects observed with silver.

Materials & Methods

In study 1 (Chapter 2), Se NPs were synthesised using a bottom-up approach, where Se metal is reduced to Se “seeds” and a protecting agent is added to provide an electrical bilayer around the particle. This electrical layer results in a surface charge and contributes towards the stability and dispersity of the NPs, where the repulsion of the NPs leads to less agglomeration and aggregation. The NPs were characterised using various techniques and instruments, confirming the size, shape, crystallinity, elemental composition and dispersion. The Se NPs were then added to poultry feed in study 2 (Chapter 3) of this thesis, which entailed an animal trial of two commonly used Se additive, sodium selenite (inorganic Se) and selenomethionine (organic Se) and three different concentrations of the Se NPs. The animal trial comprised of 10 birds in each of the five experimental groups. The birds were fed and weighed daily for 4 weeks. The birds were then euthanised, where tissue samples from various organs were taken for histological and toxicological analysis. Faecal and caecal extracted-samples were also collected for DNA sequencing and short-chain fatty acid (SCFA) metabolite analysis, using Gas-Chromatography-Mass-Spectrometry (GCMS). The faecal and caecal samples compared the different microbial and metabolite signatures of the different groups. In study 3, the tissue samples were prepared for histological assessment using haematoxylin and eosin dye, performing a typical acid digestion and ICP-MS instrumentation to examine the tissue for uptake and thus toxicity. In study 4 (Chapter 5), Se NPs were used to modulate caecal samples in an anaerobic environment at one concentration to improve beneficial gut bacteria, increase SCFA and reduce pathogen such as *Enterococcus* species.

Results

In study 1, a ultraviolet-visible spectroscopy (UV-Vis) measurements were carried out at a medium scan rate in the visible region, 200 to 800 nm, confirming the presence of Se NPs generating a typical surface plasmon resonance band at 262 nm. Dynamic light scattering (DLS) spectroscopy was used to determine the particle size and size distribution of the particles, showing an average size of 50 nm with a polydispersity value of 0.04, confirming the NP solution to be monodisperse. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to determine the shape, morphology, and size, confirming the NPs to be spherical. X-ray

diffraction (XRD) was conducted to confirm the crystallinity of NPs, with two peaks confirming the presence of Se, however, the peaks exhibited low strength confirming a mix of amorphous and monoclinic Se. Energy dispersive spectroscopy (EDS) was performed to confirm the conversion of Se ions into elemental Se at the representative peaks.

In study 2 – The intermediate concentration of Se NPs (0.9 mg/kg) performed best improving the gut health by increasing the abundance of beneficial bacteria, such as *Lactobacillus,* as well as SCFA ($P<0.01$). Additionally, *Faecalibacterium* was strongly correlated with Se NPs ($P<0.001$, $r=0.63$). Se NPs, had no significant effect on live weight gain or abundance of potentially pathogenic bacteria.

In study 3 – Se NPs were not toxic to various tissues sampled, including breast, liver, spleen, brain, duodenum, and ileum as seen by histopathological assessment. An increased concentration of Se was observed with the breast ($P<0.01$) and duodenum tissues ($P<0.05$) of the NP supplemented group, exhibited in the ICP-MS data.

In study 4 – Se NPs showed a significant reduction in the abundance of an emerging poultry pathogen, *Enterococcus cecorum* but no significant effect on the gut microbiota was observed.

Conclusions

NPs of Se were successfully synthesised using a bottom-up approach and a detail characterisation was carried out to ascertain size, morphology, and dispersity. The *in vivo* and *in vitro* experiments, anaerobic culturing and animal trial respectively, demonstrated the ability of Se NPs to improve the gut microbiota. They additionally increased the concentrations of healthy gut metabolites in the animal trial and did not instigate any pathological damage to tissues of various organs.

Declaration of authorship and originality of thesis

By submitting this thesis for formal examination at CQUniversity Australia, I declare that it meets all requirements as outlined in the Research Higher Degree Theses Policy and Procedure.

By submitting this thesis for formal examination at CQUniversity Australia, I declare that all of the research and discussion presented in this thesis is original work performed by the author. No content of this thesis has been submitted or considered either in whole or in part, at any tertiary institute or university for a degree or any other category of award. I also declare that any material presented in this thesis performed by another person or institute has been referenced and listed in the reference section.

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(Original signature of Candidate) Date

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List of Abbreviations

Ag = Silver

ADFI = Average daily feed intake

ADG = Average daily gain

ADW = Average daily weight

Al_2O_3 = Aluminium oxide

ALB = Albumin

ALT = Alanine transaminase

ANOSIM = Analysis of similarities

ANOVA = Analysis of variance

ASP = Asparagine transaminase

AST = Aspartate transaminase

ATP = Adenosine triphosphate

ATP1A1 = Na^+/K^+ transporting ATPase

Au = Gold

BBB = Blood brain barrier

BWG = Body weight gain

CAT = Erythrocyte catalase

CBH = Cutaneous basophilic hypersensitivity

CeO_2 = Cerium (IV) oxide

CNP-Cu = Copper-loaded chitosan

CO_2 = Carbon dioxide

CTC = Chlortetracycline

CVP = Chemical vapour deposition

Cys = Cysteine

DHA = Docosahexaenoic acids

DLS = Dynamic light scattering

EDS = Energy dispersive spectroscopy

EDX = Energy dispersive x-ray

EI mode = Electron ionisation mode

EPA = Eicosapentaenoic acids

FCR = Feed conversion ratio

Fe_3O_4 = Iron (II) oxide

FGF2 = Fibroblast growth factor 2

GC-MS = Gas chromatography mass spectroscopy

GSH-Px = Glutathione peroxidase enzyme

HP = Heat production

Hyp = Hydroxyproline

H&E dyes = Haematoxylin and eosin dyes

IBA = Isobutyric acid

IB = Infectious bronchitis

IBD = Infectious bursal disease

ICP-MS = Inductively-coupled plasma mass spectroscopy

In ovo = in the egg

In vitro = Biological and microbiological experiments performed outside of the normal biological context

LOD = Limit of detection

LOQ = Limit of quantitation

M-cells = Mononuclear cells

MDA = Malondialdehyde

MBC = Minimum bactericidal concentration

MgO = Magnesium oxide

MIC = Minimum inhibitory concentration

MMT = Million metric tonnes

MRSA = Methicillin-resistant *staphylococcus aureus*

MyoD1 = Myogenic differentiation 1

NP = Nanoparticle

ND = Newcastle

NDV-CS-NPs = Newcastle disease virus vaccine encapsulated in chitosan nanoparticles

NIST library = National institute of standards and technology

O_2 = Oxygen

OMP = Outer membrane proteins

OTUs = Operational taxonomic units

PAS stain = Periodic acid-schiff stain

PBCA = polybutylcyanoacrylate

PBMAD = poly(butadiene-maleic anhydride-co-L-DOPA)

PCL = Poly-ε-caprolactone

PCNA = Proliferating cell nuclear antigen

PCoA = Principal coordinates analysis

PCV = Packed cell volume

PDI = Polydispersity index

PERMANOVA = Two-way permutational multivariate analysis of variance

Pd = Palladium

PLA = Polylactic acid

PLGA = Poly-D, L-lactide-co-glycolide

PSSS = Poly(sodium 4-styrene sulfonate)

PVP = polyvinylpyrrolidone

RDA = Multivariate redundancy analysis

SCFA = Short-chain fatty acid

Se = Selenium

$SeCl_4$ = Selenium tetrachloride

SEM = Scanning electron microscopy

SeNP/nanoSe = Selenium nanoparticles

SGOT =Serum glutamic-oxaloacetic transaminase

SOD = Superoxide dismutase

SRBCs = Sheep red blood cells

TEC = Total erythrocyte count

TEM = Transmission electron microscopy

Thr = Threonine

TP = Total protein

TRI = Translational research institute

VEGF = Vascular endothelial growth factor

VEGFA = Vascular endothelial growth factor A

YS = Yolk sac

Zinc = Zn

Zn-ZCP = Zinc bearing zeolite clinoptilolite

ZrO_2 = Zirconium dioxide

List of Publications Arising from Thesis

Peer-reviewed publications

1. **Gangadoo, S.,** Stanley, D., Hughes, R. J., Moore, R. J., & Chapman, J. (2016). Nanoparticles in feed: Progress and prospects in poultry research. *Trends in food science & technology*, *58,* 115-126.
2. **Gangadoo, S.,** Stanley, D., Hughes, R. J., Moore, R. J., & Chapman, J. (2017). The synthesis and characterisation of highly stable and reproducible selenium nanoparticles. *Inorganic and Nano-Metal Chemistry*, *47*(11), 1568-1576.
3. **Gangadoo, S.,** Dinev, I., Chapman, J., Hughes, R. J., Van, T. T. H., Moore, R. J., & Stanley, D. (2018). Selenium nanoparticles in poultry feed modify gut microbiota and increase abundance of Faecalibacterium prausnitzii. *Applied microbiology and biotechnology, 102*(3), 1455-1466.
4. **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. 5*(4), 424-431.
5. **Gangadoo, S.,** Dinev, I., Willson, N., Moore, R. J., Chapman, J., & Stanley, D., (2019). Nanoparticles of selenium as high bioavailable and non-toxic supplement alternatives for broiler chickens. *Environmental Science and Pollution Research,* 1-8.

Conference abstracts

1. **Gangadoo, S.,** Moore, R. J., Hughes, R. J., Stanley, D., & Chapman, J. (2018). The Influence of Selenium Nanoparticles (NP) on Gut Health and Performance. *Proceedings of the 2nd International Conference of Theoretical and Applied Nanoscience and Nanotechnology (TANN'18). Niagara Falls, Canada. DOI: 10.11159/tann18.133*.

List of Further Publication Contributions During Candidature

1. **Gangadoo, S.,** Chandra, S., Power, A., Hellio, C., Watson, G. S., Watson, J. A., Green, D. W., & Chapman, J. (2016). Biomimetics for early stage biofouling prevention: templates from insect cuticles. *Journal of Materials Chemistry B, 4*(34), 5747-5754.
2. Wilkinson, N., Dinev, I., Aspden, W. J., Hughes, R. J., Christiansen, I., Chapman, J., **Gangadoo, S.,** Moore, R. J., & Stanley, D. (2018). Ultrastructure of the gastro intestinal tract of healthy Japanese quail (Coturnix japonica) using light and scanning electron microscopy. *Animal nutrition, 4*(4), 378-387.
3. Rajapaksha, P., Elbourne, A., **Gangadoo, S.,** Brown, R., Cozzolino, D., & Chapman, J. (2019). A review of methods for the detection of pathogenic microorganisms. *Analyst. 144*(2), 396-411.
4. Bauer, B. W., **Gangadoo, S.,** Bajagai, Y. S., Van, T. T. H., Moore, R. J., & Stanley, D. (2019). Oregano powder reduces Streptococcus and increases SCFA concentration in a mixed bacterial culture assay of chicken. *BioRxiv,* 625152.
5. Truong, V. K., Dupont, M., Elbourne, A., **Gangadoo, S.,** Rajapaksha, P., Cheeseman, S., Chapman, J., & Cozzolino, D. (2019). From Academia to Reality Check: A Theoretical Framework on the Use of Chemometric in Food Sciences. *Foods. 8*(5), 164.
6. Elbourne, A., Truong, V. K., Cheeseman, S., Rajapaksha, P., **Gangadoo, S.,** Chapman, J., & Crawford, R. J. (2019). The use of nanomaterials for the mitigation of pathogenic biofilm formation. *Nanotechnology, 46,* 61.
7. Chapman, J., Elbourne, A., Truong, V. K., Newman, L., **Gangadoo, S.,** Rajapaksha, P., Cheeseman, S., & Cozzolino, D. (2019). Sensomics-from conventional to functional NIR spectroscopy-shining light over the aroma and taste of foods. *Trends in Food Science & Technology.*

Conferences Attended

1. Poultry CRC Ideas Exchange. (2015). *Gold Coast, Queensland, Australia.*
2. Poultry Information Exchange. (2016). *Gold Coast, Queensland, Australia.*
3. Symposium 207 – Gut Microbiome and Mucosal or Systemic Dysfunction: Mechanisms, Clinical Manifestations and Interventions. (2017). *Brisbane. Queensland, Australia.*
4. Poultry Grad Conference. (2017). *Armidale, New South Wales, Australia.*
5. TANN'18 – 2nd International Conference of Theoretical and Applied Nanoscience and Nanotechnology. (2018). *Niagara Falls, Canada.*
6. Second Postdoctoral Methods Symposium: From Single Cells to Molecules. (2018). *Melbourne, Victoria, Australia.*

Presentations

1. Oral Presentation - TANN'18 – 2nd International Conference of Theoretical and Applied Nanoscience and Nanotechnology. (2018). *Niagara Falls, Canada.*
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"It is the mark of an educated mind to be able to entertain a thought without accepting it"

- Aristotle

*

"Disappointments are new and wonderful beginnings"

- The Coincidence Makers

*

"I fear not the man who has practiced 10,000 kicks once, but I fear the man who has practiced one kick 10,000 times"

- Bruce Lee

DECLARATION OF CO-AUTHORSHIP AND CONTRIBUTION

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Nature of Candidate's Contribution, including percentage of total

SG was involved in the manuscript preparation and writing of this scientific article (60%).

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All co-authors assisted with the conception and revision of the article. DS (15%); RJH (5%); RJM (5%); JC (15%)

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)

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Chapter 1

Nanoparticles in feed: Progress and prospects in poultry research

Chapter 1 introduces the current use and focus of nanotechnology in the poultry industry. The growing demand for chicken meat and eggs in the last few years has driven the industry in manipulating feed formulations and supplementations to achieve market weight with lower feed requirements, at a faster pace. The problem with current additives and supplements used, include fast degradability and antibiotic resistance, in the case of antibiotics used to reduce poultry pathogens. Hence, NPs have been introduced as an alternative platform to increase transportation and absorption of vitamins and minerals, and to reduce pathogenic bacteria. While silver has been the most commonly used NPs and have shown success in increasing growth and reducing harmful bacteria, several study cases have reported induced toxicity of silver in the birds. This has led further research to explore the benefits of other NP alternatives, such as other metal, polymeric and natural products, while evading the toxic effects that were observed with silver. This chapter also discusses the superior physicochemical properties of NPs and how it enhances the uptake and absorption routes of products.

This chapter provides an overview of the body of knowledge for the thesis and has been published as a literature review in Trends in Food Science & Technology journal, with an impact factor of 8.519. This review has been cited 24 times.

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Review

Nanoparticles in feed: Progress and prospects in poultry research

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ABSTRACT

The global poultry industry has great ly expanded due to an increase in demand for chicken meat and eggs. G row th of the industry was followed by growth in research whic h resulte d in improv ed growth rate, fee d efficiency , health status. and reduced carriage of pathogens. However, major research focus was improvement in productivity. It is possible to manipulate feed formulations to improve the feed conversion ratio (FCR), which results in a lower feed requ ire me nt to achieve market weight. Feed add i ti ves, conta ining vitamins and minerals. are commonly added to typical diets to support rapid growth and favourable FCR. Nanoparticles can be added to feed and provide an excellen t platform to incorporate in various compo und s, such as vaccines and nu tr ient supplements, due to large surface area to volume ratio and hig h absorption in the bod y. Nanopart icles can enable di rect trans portation of compounds to targeted organs or systems while avoid ing fast degradability often seen with antibiotics and can encourage mu lti ple health benefits. Silver, cu rre ntl y the most common nanopa rticle investig ated for use in chicken fe ed, has been shown to improve the microbiota of chickens . However, the positive results are tempered by the finding that silver nanoparticles have relat ively high toxicity in birds. The question the refore arises as to whether other nanoparticle forms of essential metals and natural compounds can be safely de li vered to provide positi ve impacts on healt h and productivity without the toxic side effects that can be see n with s il ver nanoparticl es. He re, we review the current state of nanopa rticle use as a poultry feed supp lement - the successes and pitfalls of nano-feed as reported by researchers across the world.

1. Introduction

Nano-d i mension ed (<100 nm) materials are being widely invest ig ated for use in an array of different applications across many diffe rent industries. Nanoparticles (NP) have been introduced into poultry feed to increase absorption and efficient use of feed that would otherwise be poorly digested and/or secreted without retention of all the available vitamins and minera ls . Poultry is one of the fastest growing industr ies within the agricultural sector, with immense inte rest in animal nutrition and research and develop - ment focused on i mp roving health, disease resistance and productivity (Chadd, 2007). As a result of continual research and development today's commercial broilers are 4 times larger at the same age and requ ire a third of the food to achieve market weight compared to the broilers of 60 years ago (Havenstein , Ferket , & Qureshi, 2003). Naturally, this significant improvement has generated positive impacts; greatly reducing the cost of poultry products to the consumer, reducing the environmental footprint (grain, water and land requirement) per unit of production, and hence improving global food securi ty. The growth of the poultry industry accele rated in t he 1990's when the world's chicken meat production was - 29 million metric tonnes (M.MT). Estimated growth in the 1990's was 2 MMT increase per year (Bell, 2002). During that period , $40 billion was invested into the poultry industry and propelled its continual growth, which continues today (Bell, 2002).

In the broiler industry, the growth and pe rforman ce of a flock remains a farmer's top priority (Stanle y et al., 2012). Interest in reducing poultry based pathogens is large, because of a desi re to

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increase the health status of the chicken and the need to reduce carriage of zoonotic human pathogens such as *Campylobacter jejuni* and *Salmonella* sp., as chickens are potential sources of bacterial strains resistant to antibiotics (van den Bogaard & Stobberingh, 2000). There are a number of performance measures used across the industry, however, feed conversion ratio (FCR), body weight gain (BWG) and the time to achieve market weight are commonly used in research and in commercial practice. FCR is the ratio of consumed feed to gained weight and indicates the ability of animals to convert feed to body mass. Birds with the lowest FCR, that require the smallest amount of feed per kg weight gained, are regarded as the top performers in the flock. In addition, FCR-based flock uniformity is also important when automation is used to slaughter and process birds for human consumption. FCR is influenced by many variables other than animal health, such as breed, genetics and sex (Benyi, Tshilate, Netshipale, & Mahlako, 2015; Korver, Zuidhof, & Lawes, 2004). However, one of the main contributors is the diet, for example protein (Fancher & Jensen, 1989) and fat sources (Skrivan, Skrivanova, Marounek, Tumova, & Wolf, 2000), or digestibility (Walk, Bedford, & McElroy, 2012), that can be manipulated with specialized feed additives to maximise performance with the most cost effective feed ingredients. It is well known that feed particle size has a significant influence on the ability of birds to assimilate the energy from feed as well as on overall performance and digestive tract physiology (Amerah, Ravindran, Lemle, & Thomas, 2007). Poultry feed requirements depend on the purpose the chickens are being grown for; a typical feed consists of a number of energy and protein sources and a small proportion of specific additives to provide vitamins, minerals and essential amino acids (Kratzer et al., **1994**).

Recent advances in sequencing technology has brought the intestinal bacterial communities into the research spotlight. The gastrointestinal tract of chickens is home to hundreds of microbial species that play significant roles in the ability of the bird to absorb energy from food and use it towards growth as well as in helping the host resist pathogen invasion (reviewed in D. Pan & Yu, 2014; Stanley, Hughes, & Moore, 2014). When fully developed, intestinal microbiota contributes to a bird's health, maintains intestinal homeostasis, guides development of the immune system, helps digestion, produces short chain fatty acids and numerous health promoting metabolites (D. Pan & Yu, 2014; Stanley et al., 2014). When this harmonious state of bacterial and host-bacterial interaction is disturbed, many health consequences can follow. Some of those include: gastrointestinal diseases and poor health and performance (Shin, Whang, & Bae, 2015). Such an unnatural state of intestinal microbiota is called dysbiosis and can be reduced by proper diet in poultry, which helps control and shape a healthy intestinal microbiota. Through pre-processing in the upper digestive tract, feed consumed by the host can act as a growth media to beneficial bacterial species, which can reduce colonisation of pathogens by competitive exclusion. Even small changes in host diet can lead to major microbiota shifts (D. **Pan & Yu. 2014**). Thus, feed additives can influence the host's health and wellbeing directly through interactions with host metabolism, as well as indirectly by changing the intestinal microbiota community and consequently the metabolites and other products supplied by the microbiota to the host.

The world's population is set to grow to an estimated 8 billion people by 2025 and 9 billion by 2050; global agricultural productivity and efficiency must increase to feed this rapidly growing population. The Food and Agriculture Organisation of the United Nations predicts that annual meat production of200 million tonnes will be required by 2050 to respond to this human increase (Ghasemzadeh, 2012). Given these increases. modern technologies, such as nanotechnology in the agricultural and food domains, will be used to improve health, performance, efficiency and unit production. Nanotechnology has a potential role to play in helping to facilitate the necessary productivity gains to revolutionize agriculture and, for this review, the poultry industry.

2. Nanotechnology and nanomaterials

The nanoscale refers to a size range typically between 0.2 and 100 nm where at this scale, properties of the material begins to differ with respect to their physical, chemical and biological properties from those at the larger or ionic scales. Nanotechnology refers to the understanding and control of matter at the nano scale where new and unique features enable novel applications, delivery mechanisms and fundamental properties to be explored.

NP use has spread across various application bases, including chemistry, physics, biology and medicine; NPs are being used in diagnosis and detection of pathogens or proteins (Kaittanis, Santra, & Perez, 2010), fluorescent biological labels (Fan et al., 2008), drug and gene delivery (Shi, Votruba, Farokhzad, & Langer, 2010), tissue engineering for bone reconstruction or replacement (Gangadoo, Taylor-Robinson, & Chapman, 2015), cancer therapy (Salata, 2004) and electronics (Komatsu & Ogasawara, 2005). NPs are also used in environmental issues such as the removal of toxic pollutants in the form of ions or organic compounds from the environment (Kaur & Gupta, 2009). Furthermore, NPs are used commercially in products such as cosmetics (Katz, Dewan, & Bronaugh, 2015), antimicrobial agents and food preservation and packaging (Duncan, 2011) also making them a target of investigation as food additives in agricultural industry for their ability to reduce certain pathogens and improve growth rate of animals and plants (Mousavi & Rezaei, 2011).

Unknowingly, NPs have been around for centuries, where they naturally occur in forms such as; dust particles formed from volcanic eruptions and wildfires, biological remains processed from microbial populations and, of late, through bottom up synthesis. The development and emergence of nanoscience did not come to prominence until studies demonstrating light interactions with metal NPs using synthesized colloidal gold NP dispersions by Faraday were conducted (Edwards & Thomas, 2007). Following this event, scientists have focused their interest on developing applications and also characterisation techniques to study the quantum size effects of NPs where they were defined as particles with sizes varying from 1 to 100 nrn. Observations have been emphasised on greater surface area to volume ratio properties of NPs as well as chemical and physical properties differing from their bulk counterparts (Roca. 1999). This allowed for various routes and approaches of nanoparticle synthesis, which consequently broadened their application ranges. Studies showed NPs can influence absorption, retention and efficacy rates of drugs and other compounds delivered to the body (Heiligtag & Niederberger, 2013; Roca, 1999).

3. Adsorption pathways and uptake

NPs are taken up and therefore induce different effects in organisms in various ways. NP exposure can occur orally, topically, inhaled or through injection and via in *ovo* administration. Therefore, size and surface charge of NPs have been identified as important parameters for changing the property of the material. The added challenge is to know how an organism responds to these changes. For example, polystyrene is a polymer used in cell culture routinely, however, when the rnaterial is in a nano-form, accumulation and toxicity can be experienced (Mayer et al., 2009). Metal NPs, when exposed to flies, mussels U.-F. Pan & Wang, 2004), fish and some plants produce lower accumulation levels when

compared to laboratory animals where accumulation of these NPs were found to aggregate in the liver , kidneys and spleen (Fro hlich & Ro blegg , 2 012).

3.1. Uptake of NPs

Particles are able to enter the body of an animaJ through various routes of entry such as inhalation, ingestion and skin contact, although the latter is less likely due to the amount of protective layers obtained from skin and tissues. Slightly larger NPs , microparticles (less than 300 nm) have been observed in the bloodstream while nanoparticles (<100 nm) can be seen distributed among various organs and tissues via diffusion. Microparticles and NPs are both able to permeate through gastrointestinal and mucosa! barriers in the body where NP adsorption is 15-250 times higher than that of microparticles (Desa i, l..abhasetwar, Amidon , & Le vy, 1996). Uptake of NP across these defensive barriers occurs through various transport mechanisms including receptor-mediated endocytosis and adsorptive endocytosis (Moha nraj & Chen, 2007).

Route of entry via ingestion occurs through the mouth, where they are moved down the oesophagus by means of saliva produced from the mouth. The cro p, used for food storage, is followed by the proventrirnlus which starts to break down and digest food with the help of acid and digestive enzymes in the form of hydrochloric acid, pepsin and gastrin. Particles pass through to the gizzard, involved in the mechanical grinding of food, before reaching the small intestine, the main site for digestion and absorption of nutrients. Absorption occurs through small finger-like structures called villi and microvilli which have a large surface area to absorb nutrients efficiently. Reineke et aJ. (2013) have shown that coatings with a PBMAD polymer can increase uptake ofNPs due to its bioadhesivity which adheres and interacts efficiently with the mucus (Re ineke et al., 2013). Most digested foods and nutrients are absorbed through the small intestine and transported to the liver through the hepatic portal blood supply. A study showed 2- 40 nm gold NPs accumulating in macrophages in both the spleen and Kupffer cells of the liver, where these specific cells are believed to be significant in NP elimination (Sada uskas et aJ., 2007). It was suggested that NPs entered cells by transcytosis, where the particles are engulfed by these Kupffer cells, of sizes 50- 100 nm, resulting into smaller NPs, 2 nm to be filtrated out of the liver via the kidneys while bigger NPs, 40 nm were retained in the Kupffer cells (Sadauskas et **al.,** 2007). Undigested food moves through the large intestine to the cloaca. The organs involved in ingestion and digestion above offer protection against foreign substances through acellular and cellular barriers and digestive enzymes (Bo dy Systems, 2016).

I nhalat ion is the most probable route of entry for NPs where they can easily travel through the mouth and nostrils, either reaching the central nervous system and/or the lungs. Protection from NPs is achieved through mucosa! layers along with suspended macrophages in the lungs and airways while the brain utilises the blood-brain barrier as its main defensive strategy against foreign materials (Elsaesser & Ho ward , 2012 ; Yacobi et al,, 2010).

3.2. Mucosa/ layers

NP uptake proceeds through an electrostatic repulsion principle, where the net charge of the surface of the NP plays a highly important role in enabling diffusion and passage through the mucous layer. Mucosa! layers are found in the mouth, oesophagus, proventriculus , small intestine and lar ge intest ine. Some viruses such as the Norwalk and the papilloma virus of sizes 38 nm and 55 nm , respectively avoid mucoadhesion and penetrate mucus due to their net surface charge (Olmsted et aL 2001). Neutral and positive surface charge NPs have been observed to diffuse at a faster rate through the mucus membrane than negatively cha rged NPs (Dawson , Wirtz. & Hanes , 2003). The size of NPs also affect transportation across cells and t iss ues ; the uptake of 14 nm latex nanoparticles was about 15 times faster than microparticles, 415 nm (S zent ku ti, 1997). Surface charge, however, is the primary factor affecting absorption and uptake in the body where one study observed 200 nm NPs of positive charge crossing the mucous membrane fasterthan <100 nm NPs of negative charge U- J. Wang, Sande rson, & Wang, 2007). The pH of the surrounding matrix can also influence NP migr at ion where acidity or alkalinity can induce a change in NP surface charge and size and prompt agglomeration of the NP matrix (Frohlich & Ro blegg , 2012).

3.3. Gastrointestinal tract

Digestive enzymes are a primaty barrier against NP delivery. Protection against enzymatic a.nd hydrolytic degradation can be achieved through the coating of polymeric onto the surface of the NPs (Maha patra & Singh, 2011). The surface of enterocytes and mononuclear (M- cells) cells display cell-specific carbohydrates where this barr ier can be overcome by introducing appropriate ligands and target strategies to colloidal drug carriers which will improve interact.ion of the NPs with adsorptive enterocytes and M-cells and can therefore allow crossing of NPs through the cellular layer (Bu nglavan, Garg, Dass, & Sameer, 2014).

3.4. Blood- brain barrier (BBB)

Crossing NPs through the BBB can occur via various pathways including endocytosis, transcytosis and opening of tight junctions (Barbu. Molnar. Tsibou kJi s , & Gorecki, 2009). Encapsulation of NPs with various polymers and materials are commonly studied for delivery strategies due to their biodegradability and protection against enzymatic degradation. Coating NPs with polymers such as polybutylcyanoacrylate (PBCA) allows NPs to adsorb to apolipoprotein which interacts with the brain capillary endothelial cells allowing the NPs to □-oss the BBB (Mic haelis e t al., 2006). Solid liquid NPs of sizes 33- 63 nm successfully crossed a BBB model using a transcellular pathway in *vitro* (Montene gro et al., 2011).

In summary, multiple absorption routes exist for NP uptake . The efficacy of NPs wilJ therefore be governed by its ability to engage and penetrate various protective barriers placed in the animal's body whiJe readily biodegrade to avoid toxici ty.

4. Nanoparticles in feed

Integration of NPs as possible feed supplements for poultry is currently emerging as a way to further improve overall health and feed conversion ratio.

NPs have been used in poultry feed to decrease numbers of harmful bacteria in the chicken microbiota while other NP types have been shown to stimulate growth of beneficial bacteria (Mah moud, 2012) and hence can potentially be used to improve growth and performance. Despite the growing body of research in this area, nanoparticles remain underutilised, not well characterise d, and not well understood as a poult1y feed additive. Here , we provide an overview of the research on the potential use of nanoparticles as poultry feed additives and propose novel strate - gies in nanopart icle use in agricu lt ure or animal nutritio n.

The following section is a summary of different types ofNPs and couplings , including various metaJs, natural products and bacteria, injected and /or fed to poultty to either exercise a positive or negative effect. Focus is divided between various aspects such as overall health and immunity, growth and performance and ant i-bacterial prospects of NPs in poulyJ .

5. NPs in poultry production

5.1. Silver

Silver has bee n routinely used as colloid al NP solution in pou lt ry studies due to it s original antimicrobial proper ties. It has bee n hypothesise d that silver nanoparticles (Ag NPs) are able to directly target specific cell types and interact wit h a cell's structure an d fu nctio n to successfully eliminate bacter ia due to the nano size range (Rai , Yadav, & Gade , 2009). Nanoparticles of Ag and Ag coupled with an energy mole cule, adenosine triphosphate (ATP), of sizes 2-35 nm at 50 ppm effectively acted as can·iers for ATP, facilitating its dis tribut ion in cells within eggs. Th e result s showed increased pectoral muscle cell st ruct ure as well as maturation and d ens ity of myofibers, and mus cle cells with multiple nucleus (F. Sawosz et al , 2012). This stu dy showed NPs could potent ially i mprov e muscle morphology without affect ing broile r performance and embryo growth. The Ag NPs also increase d gene expression of fib roblast growth factor 2 *(FGF2),* vascular endo thelial grow th factor (VEGf) and *ATP1A1* involved in muscle cell proliferation mechanisms and gene express io n of *MyoDl,* i nvolv ed in muscle cell differentiation. Ag NPs have been show n to promot e growt h an d develo pment of muscle cells, which can further increase body we ig ht gain due to the expansion of breast muscle (F. Sawosz et al., 2012; Sawosz et al , 2013). Additionally smaller sized Ag NPs , 2- 6 nm , posit ively influenced *FGF2* and vascular endoth elial growth facto r A (VEGFA) gene expression at both mRNA and p rotei n levels in broilers (Hotowy et **al.,** 2012). Both factors are also involved in ea rly embryo devel opment. Ag and Ag coupled with hyd roxyp roline NPs injected in embryos also showed sig nifican t u p-regula ted ex pression of *FGF2* at mRNA and protein levels as well as a sig nifican t in crease in blood vessel size, cartilage collagen fibre latt ice size and bund le th ickness (Bec k et al.. 2015). In one study, injec tion of physiologica l saline in chicken embryos induced hyper tro phy of he patocytes (en largeme nt of th e liver cells) an d 50 ppm silver and palladium (Ag /Pd) NPs s howed an ability to reverse these effec ts (Studnicka, 2009). ln jectio rlS p e rformed on the embryos could have trigge red oxida.tive stress resultin g in the in flam mation of cells, furthermore causing cell enla rgeme nt. Ag NPs of 50 ppm concentration with a size range of 2-7 nm have bee n coupled with amino acids, Th reo nin e an d Cys teine an d showed i mproved imm une-competence as well as in n ate and adaptive i m mun ity in both embryos and chic ke ns. Liver weight was red uced as we ll as mois tu re lo ss, while 02 consumption i n eggs was impro ved (Bhanj a et al., 201 5).

NPs are add itiona lly st ud ied as nutritional su pplemen ts in diets for the improvement of broiler health and performance since they are able to carry nutrients direct ly to cells. Birds were fed diets containing Ag NPs of 2, 4, 6, 8 and 10 ppm concen tra tio ns and showed increased body weight gain and to tal serum antioxidant with se rum total protein and cholesterol decrease d in broile rs. Birds fed with Ag NPs at 4 ppm showed the best performance than those fed at a higher rate of **10** ppm, showin g the worst performan ce based on FCR. Res u lt s indic at ed a decreased number of harmful bacteria such as *Escherichia coli* but Ag NPs had no effe ct on the *Lactobacillus,* which could be d ue to th e thick peptidog lycan layer common ly found in gra m- positive bacteria (Elklo ub, Mo usta fa, Ghazala h, & Rehan, 2015). Ag NPs of concentration 0, 300, 600 and 900 ppm were ad ded to broile r feed observing growth and diet performance. Higher concentrations of 900 ppm Ag NPs administered to feed showed sig nificant in c rease of broile r weight as well as highest feed intake and best FCR values with the low es t feed inta ke achieved from 600 ppm in birds U- Ahm adi, 2009). Inclusion of Ag NPs also observed an increase in nap brus h borders he ight which could suggest a more effe ctive absorption of nutr ients in the intesti ne or liver and bet ter conversion coefficien t U- Ahmadi, Irani, & Choob chian, 2009). Ag NPs of sizes 2-35 nm included in wa ter in tak e at 10 ppm showed a higher N intake and rete nt ion as compa red to contro l co ntaining no nanoparticles but had no effe ct on feed int al<e , body weight and FCR (Pineda. Chwalibog, Saw osz, Lau ridsen, et al., 2012). Ag NPs t hroug h water intake could also prove to be a poten tial coccid iost at to treat coccidiosis, a main par asi tic poultry diseas e caused by apicomplexan protozoa n *Eimeria* (Dallou l & Lillehoj , 2006). Ag NPs reduced oocyte ou tpu t in faecal samples by about 50%; t here were no sig ni ficant differences between the nano particles and the coccidiostat trea tment, Baycox (Cha uke & **Siebrit s**, 2012).

While the above stud ies have demonstrated the pos it ive influence of Ag NPs administered to broiler chicks, other studies presen ted conflicting res ults reporting negative effects of Ag NPs on bird health and performance. For exam ple , a size range of 2 - 35 nm Ag NPs injected in egg embryos demo nstrated a negat ive influ en ce on metabolic rate with a decrease in fat u ptake, lower 02 consumption an d CO2 prod uctio n rate at 50 and 100 ppm (Pin e da, Chwa libog , Sawosz, Ho to wy, et al., 2012). Die t s supp lemented wit h Ag NPs of 4, 8 and 12 ppm observed a significa nt nega tive impact on broiler performa nce with a dec reased feed i nta ke and body weight while FCR value and mortality rate i ncreased s ig nificantly. Some authors have speculated tha t th is effect may have occurred due to the antimicrobial effec ts of Ag d is tu rbi ng the balan ce of usefu l g ut mic robiot a shifting from good to bad bacteria. Silver retention significantly increased with 4, 8 and 12 ppm in edible carcass parts of birds such as liv e r, breast and fem ur muscle (F. Ahmadi & Rahimi, 2011). The weight of th e small intestine was also shown to have increase d with these concentrations (F. Ahma di et al., 20}3; F. Ahmadi & Rah imi, 2011 } as we ll as 20, 40 and 60 ppm (F. Ahmadi , 2012) an d Ag NPs co upled with inorganic sele nium includ ed in diets (Fele hgar i, Ahmad i, Rok hzad i, Kw-destany, & Khah, 2013). Oth er organs such as liver also had increase d weight (F. Ahmadi & Kurd es tany, 2010; Felehgari et al., 2013) sugges ti ng Ag NPs could have caused inflam mation. Unlike th e liver and small int es ti ne, th e negativ e effects of Ag NPs decrease weight of bur sa of Fabricius (F. Ahmadi , 2012; F. Ahmadi et al , 2013 ; F. Ahmadi & Kurdestany, 2010) with lymph foLlicles of bu rsa shown to de generate with only 10 ppm Ag NPs include d in broiler diets (Grodzik & Sawosz, 2006). Bursa of Fabricius is linked to an tib ody-med iated immunity in youn g broiler chickens (Glick, Chang, & Jaa p, 1956) which further suggests that Ag NPs *can* sign ificantly affect immunity of broile rs. Oxida tive stress le vels were also ele vated d ue to an increase in malondia ldehyde le vels in red blood cells (F. Ahmadi , 2012; F. Ahmadi & I<urdestany . **2010).** Malondialdehyde is often use d as a marker for oxida tive stress as an i ncrease i n free rad ic als in the body is linked to its overp roduction . NPs, in the body, are believed to stimulate fat peroxidation which contributes to release of free radicals that damage cell components such as the mito - chondria. These free radicals also cont ribute towards increased cholesterol and tri g lycerides as well as de terioratio n of some bloo d parame te rs seen in reduced ALT, AST and ALP e nzymes (F. Ahmad i. 2012; F. **Ahma di** et al., 2013). Alanine tra nsamin ase (ALT), Aspartate tra nsam inase (AST) and As paragine transamina se (ASP) are clinically used as bioma rke rs for liver healt h and diseases involving other organs such as th e heart . An increase in these enzymes common ly sugges ts damage was done to the liver and leakage of th e he pat ic enzymes into the blood has occurred, whereas reduced level s us ua lly indicates health y livers . In some cases, how eve r, low lev els could still show some liver damage. Ag NPs of size ran ges of 18 nm of low concentrat ion, 4 ppm , in drinking water demonstrated necros is and apoptosis of liver cells in broilers us ing common histo logy met hod s (Log hman, !raj, Naghi , & Pejman . 2012). Ag NPs were also found to negatively induce cardiac stTucture and

function of the heart by decreasing cardiac contractility (Raies zadeh. Noaman. & Yadegari, 2013). Table 1 summarises Ag NPs utilised in broiler studies and their effects on several aspects of health, growth and pe1formance.

5.2. Selenium

Selenium NPs, in contrast to silver, have only recent ly emerged as supplements for poultry. Selenium is important as a co-factor for the production of glutathione peroxidase enzyme , GSH-Px. This essentiaJ metal is required in the first line of cell defence in the body and, along with Vitamin I(, eliminates reactive oxygen species, thus reducing oxidative stress in the body.

NPs using selenium in concentrations ranging from 0.15 to 1.20 ppm have been administered to poultry and were found to ino·ease average daily gain of broilers as compared to 0.30 ppm NPs of sodium selenite decreased the daily weight gain of broilers, demonstrating that selenium, in the form of the nanoparticle had higher effects on performance than inorganic sele nium. More efficient retention of treatment in the whole body was achieved by selenium NPs except in the intestines. This shows the NPs exhibit a positive influence in the body of the chicken with reduced toxicity achieved at low concentrations, as compared to inorganic selenium (Hu et al., 2012). Other studies supported these claims by demonstrating best performance of broiler achieved with 0.3 ppm (Moha parra et al., 2014) and 0.5 ppm (Bag her i, Go lchin-Ge leh doo ni, Mohamadi, & Tabidian, 2015) selenium NPs as compared to both organic and inorganic selenium sources. Antioxidant functions were found to be enhanced at levels of 0.15- 1.2 ppm as well as overall immunity at 0.6-1.2 ppm of selenium NPs (Fuxiang et al., 2008). It was proposed that the concentration of selenium NPs fed to broiler chickens not to exceed 1.0 ppm with optimal levels bei ng 0.3-0.5 ppm(Cai et al.. 2012: Selim. Radw.m, Youssef, Eldin, & Elwafa, 2015) as higher levels could lead to some severe pathological changes to liver histology. Cai et al. (20 12) observed enhanced antiox idant activity at 0.3 ppm of se len iu m NPs while 2.0 ppm concentration lev els showed deterioration of immune fun ctions. Observations also included increased levels of lgG anti - bodies, important in control.ling infectious tissues and lgM antibodies, significant for a quick response agairrst antigens. Wang (2009) contradicted all the above studies reporting that sodium selenite and selenium NPs , fed to avian broile rs, showed no significant differences among each other, but did increase daily we ig ht gain, survival rate and improved feed conversion ratio as compared to control. In conclusion selen ium NPs can enhance the performance, growth and overall health of poultry due to being a non-foreign . essential, metal in the body and its importance as a co-factor to the enzyme glutathione peroxidase, which is crucial in developing immunity. Table 2 below shows a summary of selen ium nano part icles, as well as othe r nanoparticles of metal, natural products and polymers and their effects on broiler performance , growth and health.

5.3. Copper

Co pper NPs supplemented to feed has had mixed outcomes, where similar concentrations of the nanoparticle could be either beneficial or detrimental to poultry hea lt h. A concentration of 50 ppm of sizes 2-15 nm showed a decrease in metabolic rate with regard to 0 2 consumption and heat production (HP), two important reg ulators in the developmental stages of an egg , thus a decrease in these factors resulted in a decrease in embryonic development . NPs did not affect immunoglobulin concentrations or humoral responses (Pineda . Sawosz. Vadalasetty , & Chwalibog, 2013). The same concentrat ion (50 ppm) with larger NP size distributions of, 15-70 nm was reported to increase body weight while improving feed conversion ratio. Both breast and leg muscle increased along with a decrease in mortality in broiler chicks upon inclusion of copper NPs in feed (Mroc zek-Sosnows ka, Lu kas ie wicz, eta!., 2015). The opposing results could be due to a number of variables including both exper imenta l factors and external factors, such as housing, feed , mkrobiota etc.

Copper-loaded chitosan nanoparticle (CNP-Cu), at 100 ppm, showed similar responses to a lesse r 50 ppm concentration of a commonly used an tibitio t ic. chlortet racycl ine (CTC); with improved growth and immunity performance and caeca.I m icrobiota while resu lt s also ind icated an increase i n prote in synthesis (C. Wang, Wang et **al.,** 2011). Copper silicate NPs, at 2 ppm, were found to significantly improve intestinal microbiota of Yellow Chicken, shown as increased counts of *Lactobacillus* species and decreased counts of *E.coli* (Ming lei , Zheng, Xiaoye, & **Xiu'an,** 2013). Coppe r NPs, at 50 ppm, were also found to exhibit pro-angiogenic properties at the systemic level with the promot ion of blood vessel development (Mroczek-Sosnowska, Sawosz, et al., 2015). Essentially Cu NPs at smaller concentrations can demonstrate positive responses in the microbiota of broilers while promoting growth and per formance .

5.4. Gold

Gold NPs have shown litt le to no influence in poultry perfor ◦ mance and healt h in several papers using either Au NPs alon e (A. Zielir'tska, Sawosz , Grodz ik, Chwalibog, & Kamaszewski, 2009) or couplings of Au NPs with ATP (Hotowy et al., 2012) and ta.urine (M. Zielinska et al., 2012) possibly due to the inertness of the metal itself. Gold nanocarriers of sizes 2- 29 nm , at a concentration of 50 ppm, were coupled with ATP and injected *in ova,* but showed no effect in growth development and meta bolic rate. One study observed nuclei and myocytes increased in the muscles of broiler embryos with the i nclus ion of a complex synthesised from 50 ppm gold NPs coupled with 0.032 mg/L heparin sulphate (M. Zielifu;ka et **al.,** 2011). This impact is positively significant towards the needs in boosting the muscle mass of broilers and therefore increase the market weight.

5.5. *Other metal* oxides

Va rious other metals have been synthesised as NPs and supple - mented to poultry feed. For exam ple, zinc oxide NPs have been synthe sized and coupled onto a loaded active calcium alginate film. The NPs were tested for toxicity and successfully reduced the viability of *Salmonella typhimurium* and *Sta phylo coccus aureus in* vitro (Akbar & Anal, 2013). Other metal oxide NPs have shown similar antibacterial activity; A)i0 3, Fe30 4, Ce0 2, Zr0 2 and MgO, where maximum antibacterial activity against *Salmonella* sp. was obtained using Zr0 2 at a concentration of 2.5 µg/ml (Ravikumar & GokuJakrishnan, 2012). Platinum NPs, 1 - 20 flg/mL concentration reduced cell numbers and increased the apoptosis process of cells in brain tissue. There was no effect observed on the growth and development of the embryos (Prasek et al., 2013). The investigation of the effects of these metal NPs has mostly focused on reducing antibacterial activity rather than their influence on performance and growth of poult ry, representing an opportunity for future research.

5.6. Functionalised NPs

Nanopart icles can be made to act as carriers to ensure protection and efficient delivery of antibiotics and vaccines that are otherwise easily degraded in the body by th e gastrointestinal tract. Annamalai et al, (2013) synthesized biodegrada ble and biocompatible poly

Table 1
Studies showing effects of Ag NPs on growth, performance and other aspects in broilers.

Particle size & NP (formulation)	Treatment plan	Effect	Reference
2-35 nm Ag and Ag/ATP	- In ovo at 50 ppm - Incubated for 20 days	- Improved structure of chicken embryo pectoral muscles - Manired and more dense myofibers of muscle tissues	(F. Sawosz et al. 2012)
2-35 nm Ag, Ag/ATP	In ovo at 50 ppm Incubated for 20 days	- Gene expression of FGF2. VEGF and ATP! AI (j). improving muscle cell proliferation - MyoD I (i), thus improved cell differentiation	(Sawosz et al. 2013)
2-35 nm Ag, Ag/Glu	- In ovo at 50 ppm - Incubated for 20 days	- mRNA expression of genes *FGF2* and *VEGFA* (1) - Expressions of MyoDJ and *ATPIA1* (j)	(F. Sawo S?. et al., 2012)
2-6 nm Ag	In ovo at 10 & 20 ppm - Incubated for 21 days	- Improved gene expression of *FGF2* and *VEGFA* genes on the mRNA and protein levels in growing chicken - Influenced *FGF2* (!) and *VEGFA* (i) expression at a protein level in the heart only	(Hmowy et al., 2012)
Ag, Ag/Hyp	In ovo - Incubated for 20 days	- Expression of *FGF2* (TI by Hyp and Ag separately ($p < 0.05$) while only slightly (i) with Hyp /Ag combination but result not sig. ($p < 0.1$) - Ag/ Hyp sig. (1) blood vessel size, cartilage collagen fibre lattice size and bundle thickness ($p < 0.01$)	(Beck el al., 2015)
2-7 nm Ag	- In ovo at 50 ppm Incubated for 20 days	- Silver and palladium (Ag/Pd) nanoparticle solution showed the ability to reverse the negative effects of injecting chicken embryos with physiological saline, which causes hypertrophy of hepatocytes in the embryos	(Studnicl ca, 2009)
2-35 nm Ag, Thr/Ag, Cys/Ag	- Irr ovo at 50 ppm - Incubated for 20 days	- Improved immunocompetence - Potential agents for enhancement of innate and adaptive immunity in chicken - Liver weight and moisture loss (!) - O_2 consumption and EE sig. (i)	(Bhanji er ..11., 2015)
Ag	- 2, 4, 6, 8, 10 ppm - Included in diets	- All levels (I) body weight and body weight gain, with 4 ppm performing best results while 10 ppm had lowest body weight -4 ppm showed overall best performance in broiler - Serum total protein (r), 10 ppm had highest value of albumin while 4 ppm had lowest value - Cholesterol sig. (!) with 2. 4 & 5 ppm ($p < 0.05$) - Total serum antioxidant activity sig. (l) with 4 ppm recording the highest value ($p < 0.05$) - Number of harmful bacteria, E.coli, (I) but no effect on microflora, lactobacillus	(Elltloub et al., 2015)
Ag	- 300, 600 and 900 ppm - Included in diets	- Sig. presence of polymorphonuclear cells - Height of nap brush border (1), potential absorption and conversion coefficient improvement	U- Ahmadi et al., 2009)
Ag	-300, 600,900 ppm - Included in *diets* -56 days	-900 ppm (i) weight sig. with a lower FCR value compared to control and highest Feed intake ($p < 0.05$) - ALT levels in blood (*L*)	U. Ahmadi, 2009)
2-35 nm Ag	-10 & 20 ppm Included in water *incake* - Day 7 to 36 post-hatching	- Higher Nitrogen intake and retention with 10 ppm	(Pineda, Chwalibog, Sawosz. Lauridsen. et al., 2012)
Ag	15 ppm - Included in water *intake* -2 trials: 14-27 days and 14-34 days	- Coccidian oocyst counts in the excreta of the chicks at day 7 after the challenge were 408 000 for Ag NPs, 364 000 for the coccidiost at group and 788 000 for unmedicated group, which suggest that the silver treatment also (!) oocyst count by about 50% compared to the untreated group	(Chauke & Siebrits, 2012)
2-35 nm Ag	- In ovo at 50, 75 and 100 ppm - Incubated for 21 days	- Fat uptake (!) with lower rate of O_2 consumption and CO_2 production with 50 and 100 ppm - Negative effect on metabolic rate -75 ppm (t) fat uptake but metabolic rate not significant from control	(Pineda, Chwalibog, Sawosz, Hotowy, et al, 2012)
Ag	-0, 4, 8, 12 ppm - Included in diets - 42 days	- Sig. negative effect on FI, LBW, FCR and mortality than control ($p < 0.05$) - Repletion of lymphoid cell from follicles of bursa - Visceral organ weight (1) sig. ($p < 0.01$) - Retention of silver in edible carcass parts - Silver in faeces excretion (i)	(F. Ahmadi & Rahimi, 2011)
Inorganic Se + Ag NP	-0.2 mg ISe 0.4 mg ISe Either + 25 mg or 50 mg nano Ag - Included in *diets* -21 days	- Body weight and feed intake (t) compared to control - Relative weight of liver and small intestine sig. (i) ($p < 0.05$) -50 mg Ag NP had highest relative weight of mention organs	(Felehgari er al.. 2013)
Ag	-4. 8 and 12 ppm - Included in *diets* -21 days	- Sig. (1) in liver and small intestine weight ($p \leq 0.05$) - Size of Bnrsa of fabricius(L) - TG. VLDL and LDL and uric acid levels sig. (T) ($p \leq 0.05$) - HD sig. (*l*) ($p \leq 0.05$)	(F. Ahmadi et al., 2013)
Ag	-20, 40 & 60 ppm - Included in *diets* -42 days	- Small intestine weight and abdominal fat (l) - Bursa offabricius sig. (*L*) ($p < 0.05$) (affected microbial population and changed balance between pathogen and non-pathogens of organisms in the gut) - Total protein. albwnin. globulin, TG (!)	(F. Ahmadi, 2012)

Table 1 (continued)

Particle size & NP [formulation]	Treatment plan		Reference
		- Cholesterol sig. (↑) with 60 ppm ($p < 0.05$) - ALT, AST and ALP sig. (↑) [oxidative stress that caused peroxidation of fat and release of free radicals in the body where mitochondria might have been negatively affected by Ag NPs ($p < 0.05$) - CAT levels and GPx sig. decreased, SOD and MDA sig. (↑) ($p < 0.01$)	
Ag	- 5, 15 and 25 ppm - Included in *diet* - Till 42 days	- Erythrocytes activities and MDA levels sig. (↑) ($p < 0.05$) - Sig. (↓) relative weight of bursa ($p < 0.05$) - Spleen weight (↑)	(F. Ahmadi & Kurdesrany, 2010)
18 nm Ag	--4. 8 and 12 ppm - Included in *water intake* - Day 42	- Hepatocytes damage (↑) in liver tissues with (↑) Ag NP concentration - Sig. (↓) of apoptotic cells in treatments of 8 & 12 ppm Ag NP ($p < 0.01$)	(Loghman et aL, 2012)
Ag	- 10, 20 ppm, 30 ppm, 50 and 70 ppm - Included in *water and in meal* - Day 26 of age	- Negative effects of high close silver on cardiac structure and function. - Cardiac contractility (↓) due to toxicity with high dose of Ag NPs	(Raiszadeh et al., 2013)

(lactide -co-glycolide) nanoparticles and encapsulated them into outer membrane proteins (OMP) of C. *jejuni* as a vaccination strategy against *Campylobacter jejuni* populations in poultry. Poultry are known to be the main source of *C.jejuni,* one of the main bacterial causes of human gastroenteritis (Lin, 2009). Chickens were vaccinated with different doses of the vaccine with and without NP encapsulation. Results showed 125 flg of encapsulated NPs demonstrated the better results with low detection levels of C. *jejuni* in both caecal and cloaca) samples but had no significant difference between the two treatments suggesting not a significant effect exerted by the NP encapsulation.

Polymeric NPs, as mentioned before, are extensively studied for targeted drug delivery in the body due to its high bioavailability and biodegradability. Nanoencapsulation enables for highly efficient absorption and retention of drugs in cells and tissues with protection against early degradation. Polymers include poly-D, L- lactide-co-glycolide (PLGA), polylactic acid (Pl.A), poly-c-caprolactone (PCL), gelatin and chitosan which are broken down in the body producing biodegradable metabolites. These polymers are used for encapsulation of multiple drugs including anticancer, diabetes, and psychotic drugs among many more (Kumari, Yadav, & Yadav, 2010). Live Newcastle Disease Virus vaccine encapsulated in chitosan nanoparticles (NDV- CS- NPs) showed improved immune responses against a highly virulent NDV strain as compared to live and inactivated NDV vaccines. NDV-CS- NPs demonstrated increased lgG and IgA, vital component of mucous membranes' immune response, concentration levels and a higher T-cell immune response. T-cells are white blood cells found in circulation scanning for infections and abnormalities (Janeway et al.. 2001). The NDV-CS-NPs were synthesised by ionic cross linking method resulting in spherical nanoparticles with a size distribution of 147.72- 594.4 nm (Zhao et al., 2012). The bioavailability and retention ability of the chitosan to mucus is achieved by the presence of amino and carboxyl groups in chitosan molecules which form a hydrogen bond with glycoprotein in mucus resulting in adhesion 0-J. Wang, Zeng et al., 2011). A similar study utilised PLGA instead of chitosan and found the NPs to demonstrate high cellular and humoral immune responses. PLGA - plasmid DNA nanoparticles (pFNDV-PLGA-NPs) were synthesised using a water/oil/water double emulsion-solvent evaporation method. The shape of pFNDV- PLGA - NPs were round and had an average size of 433.5 ± 7.5 nm. Results demonstrated high level of lgA and lgG antibodies as well as an increase in mucosal immune response, the latter being an important defence against ND prevention which is primarily taken in the body by inhalation and ingestion (Zhao et al., 2013).

PLGA NPs are mostly applied to broiler chickens in an encapsulated form used for vaccines and drug delivery rather than integrated in feed. The NPs have provide excellent attachment and penetration through defensive barriers of the animal body such as mucosa) layers and various immunity responses. Further studies should consist of exploiting these biodegradable and bioavailable Pl.GA NPs in improving the FCR ratio and growth performance of broilers.

5.7. Natural products

Other methods for functionalising NPs have used polymers and or extracts of natural antibacterial products such as certain herbs and oils. Similarly, a study involved a turmeric extract enclosed in a nanocapsule was found to improve meat quality without affecting performance as a feed additive for regular broiler feed. Treatment was made by mixing three components - turmeric extract, chitosan and sodium tripolyphosphate solution. The mixture was filtered, dried and ground to produce turmeric extracted/nanoparticle. Results showed 0.2% turmeric nanocapsule to be the optimum level, producing the best FCR value while 0.4% of the nanocapsule significantly decreased Uverocliolesterol and subcutaneous fat. The study concluded maximum level of the treatment to not exceed 0.4% as higher concentrations showed reduced growth among birds (Sundari, Yuwanta, & Martien, 2014). Clay minerals of nano-suspensions were found to improve antibody titre protection against most infectious diseases such as Newcastle disease, infectious bronchitis and bursa) disease. Solution of nano-suspensions were synthesised by physical approach with mechanic wet grinding of clay minerals to a nano range and later added to potable water to make three concentrations, 1%, 1.5% and 2%. 1% and 2% of the suspension were fed to broiler chicke ns where feed conversion ratio, body weight and body weight gain significantly improved (Elshuraydeh, **Al-Beitawi & Al-Faqieh, 2014**).

One study synthesized and introduced silver NPs in a zeolite framework, forming a diameter of 2.12 - 3.11 nm, d1rough an ion-exchange path with addition of a reducing agent, sodium borohydride. The silver-zeolite nanoparticle framework, at concentrations 1, 2 and 5 g/100 g zeolite, showed positive antibacterial activity against gram -negative bacteria, *E.* coli, *Shige/la dysentriae* and gram-positive bacteria, *Stap/1ylococcus aureus* (Shame U, Ahmad, Zargar. Yunus. & Ibrahim, 2011). Due to the approach of adding nanoparticles in feed is relatively new, there have not been many studies investigating zeolites nanoparticles in poultry. Zeolites, have been reviewed and extensively researched in the poultry industry with recent work including zeolites used as a means of both

Table 2
Su mm ary of meta l, natural products, polymeric and other nanopar ticles and the,r effects on broiler perfo rmance, growth and health.

Particle size & NP	Treatment plan	Effect	Reference
Selenium Nanoparti cles in Poultry			
20- 80 nm, average is 60 nm Se	Experiment 1 -0.15, 0.3, 0.6 & 1.2 ppm - In cluded ,n *diets* -49 days Experiment 2 -0.1 mmol/ L sodi u m sele nite *vs* Se NP investigati ng intes tin al re ten tion of Se Experime nt 3 -0.I mmol/ L sod ium sele ni te vs Se NP invest igati ng intes tin al tr ansport of Se	Si g. imp ro ved grow th perform ance. serum CSH- Px activity & tissue Se concentr atio n ($p < 0.05$) Si g. (I) ADG, gain/feed and survival ratio *(p* < 0.05) - Si g. (r) concentratio ns or Se in serum. li ver and Dreast m uscle ($P < 0.05$) - Se NP Dette r retaine d in the body than sod ium seleni te - Transfer of Se N P from intestinal lumen to the body higher than selenite - In testinal retention of Se NP lower tha n selenite	(Hu et al,, 2012)
50 - 100 nm, average 80 nm. Se	-0.07 5, 0.15, 0.3 & 0.6 ppm - included in *diets* -8 weeks	- Si g. (i J final weight significantly and im proved FCR ($p < 0.0$ 5) - Sig. (T) serum glucose, total protein , globulin, SCOT and urea levels ($p < 0.05$) - Si g. *(l)* serum chol esterol, triglyceride, A/C ratio and ALP *(p* < 0.05) - AntiDody titres against SRBCs im mun ization and CBH sig. higher ($p <$ 0.05) Improved erythrocyte catalase, gl urath ione peroxidase and sn p er oxide dismu taseactiv ity - Haemog lobin content, TEC and PVC val ues sig. higher ($p < 0.05$) Sel eniu m levels in ser um, liver, breast muscle, pancre as, kid ney and feathers ( )	(Mo hapatTaet ,al 2014)
20 - 8 0 nm, average 60 nm Se	-0.2 & 0.5 ppm - Includ ed in *diets* -42 days	Si g. im pro ved ADW, ADFI, ADC an d FCR w i th 0.5 ppm sh owing Dest resu l ts - Final liver and abdom inal cavity fat weight sig. (J ($p < 0.05$) - Sig. improved total antioxida nt activities (($p < 0.01$) and im proved immunity system	(ltaghen er a1.. 2015)
Average si ze 80 nm Se	-0.10, 0.25 & 0.4 ppm - Incl uded in *diets* -45 weeks	- Sign ific an tly (l) egg productio n percentage ($p < 0.001$ J and egg mass ($p < 0.001$) at 0.25 ppm - Im proved feed conversion ratio sig. ($p < 0.01$) -0.4 ppm produced highes va l u e of Yolk index - Si gni fi cantly (l J tot al lipid s, total cholest ero l ($p < 0.05$) and LDL-cholesterol ($p < 0.0$ I) while (T) HDL-c.holesterol with 0.25 & 0.4 ppm (not .sig .) - Sign ifi can tly (i) Se content in the album en ($p < 0.05$) and egg contents ($p <$ 0.001) - Sig. ( ) CSH-Px activity ($p <$ 0.001) w it h a decrease in M DA content in yolk ($p-< 0.05$) - Conce ntratio n of long chain polyunsatur ated fatty acids and monou nsarura t ed fatty acids (Tl wh il e saturated fatty acid s in yolk (l J Some hi stopat hological fin d in gs show fat ty live r wit l1 focal aggregation of inn am ma tory cells and congestion of blood vessels in spleen	(Raaw an, ElIdi n, El.-Zaial , & Mostaf . 20 l5)
Se	-0.15, 0.3, 0.6 & 1.2 ppm - [ncluded in *diets* -6 weeks	- Serum antibody tit res against Newcastle diseasevirus (i J - Immune organs index increased Imm une functions im proved with antioxidant an production of glutathi one peroxidase in creased	(Fuxiang ec al., 2008)
Average si ze 80 nm Se	-0.15 & *03* ppm - Included in 2 treatments: *diets* and *water intake* -40 days	Si g n ifi cant improv ement of some haematologica l parameters ($p < 0.0$I), cellnlar immunity and antioxidant status ($p < 0.05$), GSH-PX activity - Concentration ofT3 hormonewith 0.3 ppm (T) - Severe hi st opath ologica l changes wi th 0.3 ppm as focal lar ge area or necrosis w ith large area or in flammato ry cells	(Selim el al.. 2015)
10- 150 nm. average 80 nm Se	-0.3, 0.5, 1.0 and 2.0 ppm - Included in *diets* -4 2 days	lgC and lgM levels sig. (l) *(p* < 0.0 I) with 0.3 ppm sh owin g highest levels - Clu tat hione peroxid ase activity, glurarhione levels and free radical in hibition sig. (i) ($p < 0.01$J while malondialdeh yde levels sig . (.i J ($p <$ 0 .01) with 0.3 ppm demonstTating best effect w hi le exceedi ng 2.0 ppm showed poorer levels	(Cai ei al.. 2012.)
60 - 80 nm, average 68 nm Se	-0.10, 0.30 & 0.50 ppm - Included in *diets* -90 days	-0.3 and 0.5 ppm (t) final daily weight gain and Dody weight and improve d FCR - Se conte nt (j) in hepatic and muscle contents - Drip loss of breast meat CL) sig. ($p < 0.0$ I} CSH- Px acti vities sig . (rJ in serum *(p* < 0.0 1) and liver ($p < 0.05$)	(Zhou & Wang, 201l)

Table 2 *(cont in ued)*

Part icle size & N P	Treatment plan	Effect	Reference
10- 45 nm Se	- 0 .3 p pm - In c luded in *diers* -6 weeks	- Sele ni um NPs found to *U)* ox id ative stress when induc ed w it h a stressful condition - Un d e r non -oxida tive cond i t io n, choleste rol in circulatory was ele vated.	(Boostan i. Sadeghi. Mousav i. Chaman & Kashan. 2015)
30- 80 nm Se	-0 .2 & 0.5 p pm - In cluded in *diers* -4 2 days	- Sig. i mproved d ai ly we ight ga i n , su rviv al rate while red uci ng FCR ratio *(p* < 0.05) - Se content in muscle and liver tiss ue (il sig. *(p* < 0.05) - GSH- Px act ivit ies (1) sig . in bo t h sen,m and liver (p <, 0 05}	(Y. Wang, 2009)
Other Nanoparticle s in Poultry			
2- 15 nm Cu	-50 ppm - *rn DVD* - Day **19**	- 0 $_2$ consumption *(p* < 0 .001) and 1-fp of em bryos (p < 0.01) s ig. (J) - Resi du al YS we ig h t s ig . Ct) *(p* < 0.00 I) - Liver, i ntest i ne *(p* < 0.00 I) and heart we ig hrs sig. O) *(p* < 0.01)	(Pineda et al., 20 13)
15 - 70 nm. average 37 nm Cu	-50 ppm - *In DVD* -19 days	- Bo dy weight sig. (t) w it h i mpro ved FCR r atio *(p* < 0.01) - Carcass content of breast muscle (t) (p < 0.01) - pH in breast and leg m uscle (!) *(p* < 0.05)	(Mroae k-Sosnowska, Lukasiew icz. et a l., 2015)
Ave rage s i ze 95 nm Cu	-50. 100 & 1 50 ppm - In clude d in *diets* -4 2 day s	- Ave rage daily gai n s ig. (t) *(p* < 0.05) - Haematologica l character is tics ofTP and ALB i n serum (i) - Th ym us, spleen and bursa index es (i) *(p* < 0.05) - Concentrations of lgA , lgG, lgM (p < 0.01), 0 , C4 and lysozyme *(p* < 0.05) (i) in se rum - (t) bacte rial co unts of Lac cobac illus, Bifidobacterium wit h (l) co un ts in coliforms	(C. Wang, Wang et al., 2U I I)
Copper sili cate nanop an icles	- 2 g/kg - I nc luded in *diers* - 50 da ys	- Total caeca l aero b ic b ac te r ia and *E.* coli sig. Ct) *(p* < 0.05) - Total caeca l a nae ro bic bacteria and *Lactobacil/115* s ig. (r) (p < 0.05) - Serum xanthine ox iclase ac ti vi ty and stool urea ni troge n s ig. (l) (p < 0.05)	(Minglei et al., 2013)
15- 70 nm. Averag e 37.3 nm Cu	-50 ppm - *rn DVD* -20 days	Cu NP showed more int e nse of blood vesse ls de ve lopment; n umb er of branches and le ngt h of vesse ls we re sig. greater *(p* < 0.00 **I**) - Gene express io n s o f VEGF-A, FGF2, PCNA , MyoDl, RNA, Pax7 sig. (i) *(p* < 0.001), therefore innu enc i n g embryo breast musc le mRNA sy nthesis	(Mroezek-Sosnowsk a. Sawosz. el.al., 2015)
[size nor ment ioned) Au	- 50 ppm - *In DVD* - 20 days	- Nu mber of nuclei in breast muscle sig. *(j)(p* < 0.01)	(M. Zielinska et al., 2011)
Meta .I Oxides NPs: Al_2O_{1-3} <50 nm Fe, 0_4 - 9- 1 1 nm CeO_2 - <25 nm ZrO_2 - < 100 n m MgO - <3 0 n m	-2. 5, 5, l 0, 15 and 20 gg / ml - Min i mum Inhib itory Concentration Min im um Bactericidal Concentrat ion - T ime kill as say	- All tes ted pat hogens sens iti ve against all nanopart icles MIC an d MBC showe d ZrO_2 nano par ticles a re highly se nsitive agai ns t *Salmonella* sp. and *E.coli* Al_2O_3 and MgO nanopa rticles s howed some sensitive a gainst the above bacteria - Time kill assay reveals that growt h of *Salmonell a* sp . was i nhib ited from the 1st to 12th hour wit h ZrO_2 nanoparticles	(Ravik limar & Go kulal<rishn an , 2012)
2- 19 nm Pt	- **1.** 5, l 0 . 15 and 20 flg / m l - *In DVD* - 19 days	- Weight of live r sig. (0 *(p* < 0.05) - Patho logical c hanges in brain st ructu re in embryos showi ng mode ra te deg rada tion - De grada tio n of mito cho nd ria, rounded nu cle i w ith dis pe rse d chroma ti n and vac uoles in cy topl as m in brain t iss ue - PCNA- posirive nuc le i s ig . (*L)* (p < 0 .05)	(Prasek et a l., 2013)
< 100 nm Tur meric Nano cap s ule	- 0.2%, 0.4 %, 0.6% & 0 .8 % - In c luded in *diets* -6 weeks	-0.2 % demo nst rated to be the best concentrat io n w i t h best FCR value and s ig. (j) i n body weight whil e concentration exceedin g 0 .4% show e d to be poiso no us - Abdomina l fat. meat EPA (0 .6 % and 0 .8 % on ly) and DHA (0.4 %- 0.8 %) sig. (i) *(p* < 0.01) - Subcutaneous fat (0.4% only) *(p* < 0.05) an d rota.I chole s tero l of li ver (0.4%- 0 .8 %) sig. (I) *(p* <0.05)	(Sunda ri et al., 2014)
Clay mineral Nano sus pens io n	- **1%,** 1. 5 % & 2% (w/v) give n one ri me per ei th e r one or two weeks - I nc luded in *diecs* - 36 da ys	-1% one time per two weeks impro ved FCR - 1.5 % gi ven one t i me per 2 weeks (1) ant i bod ies ag ai ns t ND w hile sam e co ncentration g iven one time per one week [i) ant ibodies against 1B - **1%** given once per I week (1) a nti bodi es against IBD	(Elshuraydeh et a l., 2014)
2.12- 3 .11 nm Ag N0 $_3$ /zeo lite nan ocom posit es	- **1%,** 2 % & 5 % (g/g zeo Lit e)	- Hjgh antibacterial acti vi t y aga in st Gr am- negative and gram - positi ve bacter i.a, Ag N0 $_3$ /zea lite s how ed great in- hibition against *E.* coli. *S. dysenrriae , S. aure11s* and meth ic illin-res ista n t *S. aureus*	(Shameli er al .. 201 i)
Zn - bear ing zeolit e cl inop tilo li te	- 80 ppm - In cl uded in *die*rs -21 days	Improved growth and Feed int a ke rat io s ig. *(p* < 0.05) - Fres h we ig hts of tibia a nd pan crea s sig. (il *(p* < 0.0 5) I mproved mRNA levels o f Meta lloth io nein , a key regulato r fo r zinc absorpt ion and tr an sportat io n (p < 0.05)	(Tang et al., 2015)

[conti nued on nexf page)

Table 2 (continued)

Particle size & NP	Treatment plan	Effect	Reference
317.1 nm Newcastle virus vaccine encapsulated in NP	- In ovo	- Induced high T cell immune response - IgA antibody (i), important in immune function of mucous membranes ($p < 0.01$)	(Zhao el aL 2012)
433.5 nm Eukaryotic expression plasmid DNA that expressed the f gene of Newc.1stle disease vaccine encapsulated in PLGA NPs	- In DVD	- Immune responses of cellular, humoral and mucosal activity (1) - IgA and lg G antibody levels sig. (J) ($p < 0.01$)	(Zhao et al., 2013)

transport and supplement for nutrients such as zinc (Zn). Zn-bearing zeolite clinoptilolite, (Zn-ZCP), prepared by ion exchange methods at 80 ppm ofNPs improved growth and feed intake ratio of broilers consistent with a significant increase in fresh weights of tibia and pancreas. A key regulator, metallothionein, for zinc absorption and transportation was also used to improve the effectiveness of the zeolite (Tang et al., 2015).

Carbon nanoparticles, or more specifically carbon nanotubes, have been an increasing area of interest due to their high conductivity, high-strength and mechanical properties. They have been used as "conducting components in polymer composites" and are now expanding towards additional, diverse applications. Other uses of carbon nanotubes include electrodes in lithium batteries and improve of structural purpose in plastics components (Baughman, Zakhidov, & de Heer, 2002). Carbon nanotubes are synthesised by either laser ablation or electric arc methods and require materials to be thoroughly purified. Other methods such as Chemical Vapour Deposition (CVP), which uses catalyst or hydrocarbon as precursors, are currently being developed as there is a lack of scalable techniques in producing bulk amounts of nanopatticles of carbon (Ajayan & Zhou, 2001). Although carbon nanotubes have not yet been introduced as supplements in poultry diets, one study was able to use the reverse notion of recycling and reusing of chicken bits into producing NPs of carbon. An example involve the recycling of chicken feathers, usually regarded as waste, converted into N-doped carbon nanotubes to be used in the catalytic reduction of 4-nitrophenol (Gao et **al.**, 2014).

5.8. *Further directions*

Nanoparticles are now widely studied for use as new tools for targeted drug delivery activity and nutrition improvement. Their distinct size and properties can be both beneficial and disadvantageous; while their nano size enable drugs and other components to be delivered to desired target and rapidly produce an effect, nano particles can also easily pass through natural barriers of the body such as the blood-brain and mucosal barriers causing inflammation and toxicity. It is therefore vital to manipulate and produce desirable NP's size and concentration in order to achieve maximal benefits from NPs while avoiding toxicity.

NPs have been used in broiler studies effectively; improving growth and performance of chickens, the probable accumulation and toxicity of silver in the body cannot be disregarded and can greatly impact the immune response. Materials, such as silver, are naturally attacked by the immune system, generating an inflammation response due to its foreign nature in the body and as a result more attention is seen directed towards nutrients and minerals, an example is selenium where it is already required for proper cell function therefore causing no such stress to the body. Selenium is an essential mineral required in the body for the first cell line of defence and selenium nanoparticles have been observed to greatly improve immunity and overall performance of a broiler. The main focus surrounding broiler studies using nanotechnology includes improving growth performance and reducing disease and pathogen load. The biggest role is to grow poultry more efficiently, improved health and development, while reducing feed conversion ratio. A low FCR value contributes to a decrease in feed requirements to achieve market weight, and in cost of waste disposal. Other NPs studied for improving broiler performance include drugs encapsulated NPs such as Newcastle virus vaccine encapsulated in a polymer. Polymers are at present widely used for drug delivery in the body as they are biodegradable and increase retention time of the drug in the body. NPs may therefore be an advanced method of delivering nutrient requirements to chickens at very low dosage. More studies should be conducted on their influence on intestinal microbiota of the chickens to understand NPs' role on beneficial gut microbes and metabolites production. AJI current studies investigating effect of different NPs on microbiota, especially known pathogens are done using culturing methods. Recent advances in sequencing technology presented molecular microbiology options that allow screening of both culturable and not yet cultured microbiota at once. This methodology will provide complete overview of NPs interactions with intestinal bacteria. Studies should include more basic toxicology, histology and NP residue analysis in broilers of market weight or in egg before any wide adoption could take place.

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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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Chapter 2

The synthesis and characterisation of highly stable and reproducible selenium nanoparticles

Chapter 2 showcases a rapid bottom-up approach synthesis of Se NPs, and a full characterisation including various spectroscopy and microscopy techniques. The synthesis of the NPs was carefully optimised by controlling variables such as temperature, stirring rate and the concentration of reducing and stabilising agents. In this chapter, NPs are synthesised by reducing Se metal ions to Se metal atoms, with ascorbic acid. These atoms form “seeds” during a nucleation stage process, providing a platform for the particles to grow in a desirable size. Aggregation slowly occurs due to the surface energy of the particle, and a stabilising agent is added forming an electrical bilayer around the particle. This results in the repulsion of the particles, thus forming a homogenous colloid suspension of NPs of an average size of 55 nm. The characterisation of the NPs confirmed the presence of Se using spectroscopy, while instruments such as scanning and transmission electron microscopy were used to confirm shape, size and crystallinity of the NPs. This chapter has been published in Inorganic and nano-metal chemistry journal, and has been cited 5 times.

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The synthesis and characterisation of highly st able and reproducible se lenium nanoparticles

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ABSTRACT

This paper describes a simple and reproducible solution phase synthesis approach for selenium nanoparticles by reducing selenium tetrachlmide in the presence of ascorbic acid. An optimization study with poly (sodium 4-styrenesulfonate) produced stable and spherical narrowly size distributed nanoparticles (46 nm) which are considered highly monodisperse. The presence of seleniu m nanoparticles was confirmed by UV-visibl e spectroscopy for surface plasmon resonance (262 nm), elemental dispersive spectroscopy (11 KeV and 12.5 KeV) and size ranges characterized by dynamic light scattering (PDI = 0.04, size range of optimized nanoparticles = 35 nm to 75 nm), and visualized using scanning and transmission electron microscopy.

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Introduction

Developing u niform and monodisperse nanometre sized particles has been an exciting area of research for the last 10 years owing to the technological and fundamental science improvement associated with their uses. These nanoparticles often exhibit interesting electrical, optical, magnetic and above all, chemical properties, which cannot be achieved when compared to their bulk counterpart materials. The majority of nanomaterial research has focussed upon II-IV semiconductors and noble metals. Selenium is a metalloid possessing the characteristiGs of both a metal and a non-metal. Selenium is photoelectrically active and is a semiconductor where its uses include xerography, glass production and solar cell assemblyP ·21 The use of selenium in its nanoparticle form has recently attracted more attention due to the nano size range property enhancing the photoelectric and biological properties of selenium. Already we know that selenium is an essential dietary element required for the production of amino acids and enzymes, reducing cell and tissue damage caused by free radicals in the body[? 3 Ik benefit of selenium nanoparticles used in other applied sciences such as agriculture has attracted attention due to the nanopartide's ability to produce responses in cellular activity/ 41 Selenium nanopartides are being studied as potential supplements in agriculture and other health sciences to provide new and improved performance responses. Owing to the sensitive nature of the public perception of regulation towards these areas, nanopartides need to be reproducibly synthesised and studied intensely for their safety, stability and cellular responses.

Nanoparticle synthesis and their uses

There are various methods used to synthesise nanopartid es[by physical means including evaporation- condensation, [61 laser ablationY 1 biological approaches using plants,[BJ fungi,[gae 101 or bacteri a/ 111 chemical rnicro-em ulsion,l121 photo-induced reduction,l131 lN-initiated photo-redu ction,l141 electrochemical synthetic method,[Is] irradiation,[161 microwave-assisted,l171 using polymers and polysaccharides,l181 the Tollens process - the reduction of silver ammonium ion, Ag (Nl-hh (Tollens reagent), into silver nanopartides, where it has been previously prepared from a mixture of silver nitrate and ammonia and finally chemical reduction[20,211 Chemical reduction is the most widely used technique as a 'bottom-up' synthesis approach whereby nanopartides are formed through chemical reducing and protecting stages using chemicals such as: ethylene glycol used in the polyol method 221 and trisodium citrate used in the citrate method[23

N anopartide synthesis occurs through the reduction of metal ions to neutral metal atoms by the addition of a reducing agent. The first stage, nucleation, allow atoms to form small dusters, called "seeds", of a stable structure and defined crystallinity.[24·251 The next stage involves the "seeds" to form nanocrystals of different shapes and stru ctures.l251 As aggregation occurs, the surface energy of the metal also grows and smaller particles readily interact with each other to form larger particle sizes. A capping agent or stabilizing agent is used to prevent further aggregation by forming an electrical bilayer around fhe nanoparticle occurring from the adsorption of ions onto the surface of the nanoparticle. This process induces the repulsion

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force between th e nanoparticles and is significant in cont rolling particle size.[26 28]

Different reducing and protecting agents when used in combination with each other produce various shapes and sizes of nanoparticles.[29] For example, glucose can act as both a reducing and protecting agent for silver producing sphere-shaped nanoparticles with a smooth surface morphology and size range of 7 nm - 30 nm.[30] Here th e glucose acts as a reducing and a protecting agent which occurs by the formation of gluconic acid by releasing electrons. These electrons reduce the silver complex and encapsulate the silver nanoparticles formed. If nanoparticle synthesis protection is not adequately carried out, aggregation ensues and can be visualized by transmission electron microscopy (TEM) as aggregated clusters rather than well separated and defined mono-disperse garticles. The synthesis of silver nanoparticles is well repo rted. [30] In a synthesis using $NaBH_4$ as a reducing agent and polyvinylpyrrolidone (PVP) as a protecting agent produced size ranges of larger nanoparticles, 320 nm.[31] When other reducing agents are used, such as trisodium titl"\lte, coarse grain nanoparticles are produced with lower surface area to volume ratio. When ascorbic acid is used as the reducing agent with polyvinyl alcohol (PVA), this reaction produces nanoparticle sizes ranging from 55 nm to 68 nm compared to using PVP as a protecting agent resulted in larger nanoparticles from 101 nm to 227 nm, respectivelyP [91] The modification of concentration and ratio of solutions are equally important, as variations of these parameters can produce different shap es of nanoparticles, and high concentrations of metal salts, $AgNO_3$ with a low molar ratio of PVP results in silver nano-cubes while a lower concentration of $AgNO_3$ with an unchanged molar ratio of PVP and metal salt produces silver nanowires.[33] With respect to the relevance of this work using nanoselenium synthesis, a number of methods are available in the literature; (a) biosynthesis using raisin extract avoiding the need for organic solvents,[34] (b) green synthesis,[35] bacterial synthesis,[36] and finally hydrothermal synthesis based on liquid-solid-solution routes.[37]

The a im o f this study was to synthesize uniform and disperse nanoparticles using a fast and reproducible chemical reduction method using ascorbic acid as the reducing agent due to its biocompatibility characteristics and low toxicity when compared to other reducing agents.[31] Although not part of this paper, these particles are aimed for use in biological applied research to deliver novel effects in nutrient delivery in food. Characterization by lN- Vis spectroscopy, transm ission electron microscopy (TEM), dynam ic light scattering (DLS), scann ing electron microscopy (SEM) and energy dispersive X- ray (EDX) analysis was used to determine size, uniformity, shape and surface plasmon resonance, and crystallinity of the synthesized selenium nanoparticles. In this work a simple, reproducible benchtop method using aqueous chemistry, with ascorbic acid as a reducing agent and poly (sodium 4-styrene sulfonate) (PSSS) as a protecting agent is reported. The method demonstrates simplicity and reproducibility, where other meth- ods rely on multi-step processes that are often complex and require specialist equipment. Though multiple chemical techni- ques have been developed for the synth esis of nanoparticles, the requirement to exclude solvent contamination while pro- viding uniformity among the produced nanoparticles has been a challenge. This study aims at improving this knowledge while providing a robust method to eliminate these contaminants from the nanoparticles.

Materials and methods

Chemicals

The metal salt, selenium tetrachlor ide ($SeCl_4$), ascorbic acid ($C_6H_8O_6$), poly (sodium 4-styrenes ulfonate) and solvents (eth anol, m ethanol , propanol, dichloromethane, ethylene glycol and aceto ne) were purchased from Sigma Aldrich, Australia and stored at room temperatur e until use.

Preparat ion of nano-selenium

A modified chemical reduction method was used, where 5 mM of $SeCl_4$ solution was prepared. Ascorbic acid was used as a stock reducing agent at a concentration of 100 mM and the protecting agent, PSSS was made up at 0.1% (v/ v) co ncent t ation . All solvents were dissolved in Milli-Q water at room temperature, and used on the day of preparation .

Reactants were introduced in the following order: 2.5 mL of PSSS was added to 15 mL of selenium tetrachloride and followed by 2.5 mL of ascorbic acid. The solution was mixed under mild stirring rates at 160 °C for 1 - 2 min, until a deep red clear colour was achieved, this colour demonstrates reaction progression.

Precipitation and re-dispersion of Nano-Se (washing)

The first wash involved the solution of selenium nanopart id e of approximately 20 mL, to be diluted with 20 mL of Milli-Q water and centrifuged at 1008 x g. Following the wash, the precipitant was removed as it contained larger nanoparticles > 100 nm while the supern atant is re-suspended with Milli-Q water of approximately 40 mL. The second wash involves the new solution of selenium nanoparticle to be centrifuged at 8500 rpm for 25 mins and the supernatant is re-suspended clgain in Milli-Q water. The second wash is repeated twice more and lastly the supernatant containing any remaining surfactants and/or reactants, is removed while the precipitant containing selenium nanoparticles of sizes 50 nm and smaller, is collected for analysis.

Nanopartide characterization

UV- vis spectroscopy

UV-vis spectroscopy (Cary 50 Bio Spectrophotometer), was used for the determ ination of selenium nanoparticles in solution. A 2 mL aliquot of the selenium nanopartides (SeNP) was measured in a 1 cm path-length quartz cuvette and scanned at a medium scan rate (2 nm per second), 200 to 800 nm. The analysis was perform ed in triplicate for reproducibility.

Dynamic light scattering spectroscopy

The particle size and size distribution of the selenium nanoparticle sample was analysed by dynamic light scattering using the Malvern Zetasizer Nano ZS. A volume of 1 mL of the Se NP solution in 2 mL of Milli-Q water was placed in a polystyrene

cuvette and measured at 25 " C. The viscosity and refractive index were set to those specific to water and the quartz cuvette. Measurements of size and polydispersity Uidex (PD1) were obtained for both freshly syn thes ized e Ps and washed nano-patticles in triplicate. The particle size was given as mean and standard error.

Scanning electron micros copy

Scanning Electron Mic-roscopy (SEM) (JEOL JSM-6360LA) was used to determine the shape of the ,ian opartid e . *One* drop of the selenium nanopa.rticle solution was air dtied under cover on a carbon stub for l 0-15 min.

X-ra y diffraction study

The nanopar ticl e sample was dispersed on a low backgrnund noise sample hokl.er and analysed i'n a Bruker 08 Advance X-Ray diffractometer equipped with a LynxEYE detector. X-ray diffraction analysis was operated at a voltage of 40 kV, with current of 40 mA, with copper radiation of 1.54060 A. The scanning was per.formed in the *20* range of 10° to 40° at 0.02°/:min with ti.me constant of 1.2 s.

Transmission electron microscopy

A sample of 5 /.tL volume was placed onto a formvar-coated copper grid - grid bought from Prosc.itech and fonnvar-coated at University of Queensland - and air d ,ied. The morphology and size of selenium nanoparticles were characterized using transmission electron microscopy (TEM; Jeol 1011 JSM) operating at an accelerating voltage of 80 kV. Images were captured using an Olympus SIS Morada CCD camera with analySIS software.

EDS study

.Energy d.ispersi ve spectroscopy (EDS) was performed to confirm the conversion of selenium ions into elemental selenium (Se) using a FEI Ternai F20 TEM/STEM operated at 200 kV. A small aliquot of the sample was pipetted onto a carbon coated 200 mesh copper grid.

St atistical analysi s

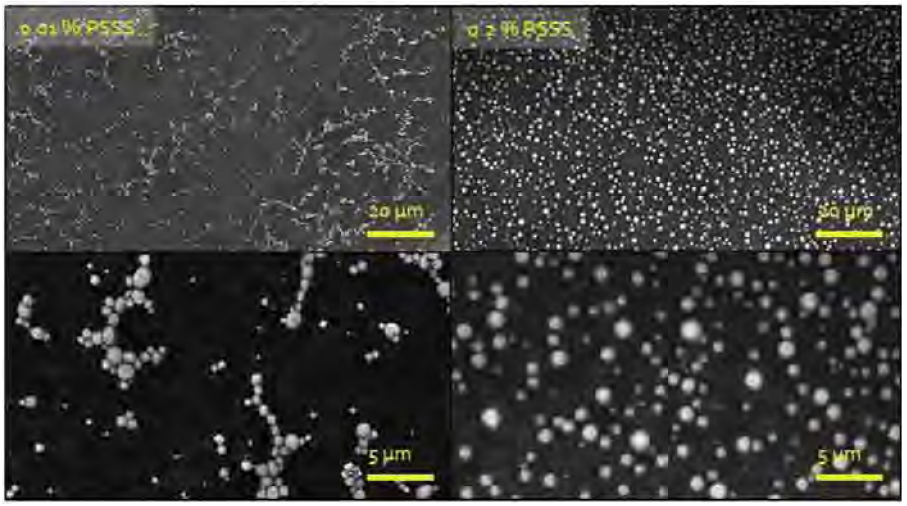
All stages of the synthesis and measurements including the UV-Vis and partide sizer analysis were cani.ed out in triplicate and expressed as a mean with a standard error using Sigma Plot. The synthesis was routinely replicated, n = 5, demonstrating the reproducibility of the method. Sl:'.M images were analysed using lrnageJ software, downloaded from TrnageJ - RSB Home Page, bttptd /imagej.nih.gov/ij/down lo ad.htrnl, with plugin 64-bit fava l. R. 0_77, where nanoparticle siz e was calcuJated.

Results and discussion

Nanoparticle synthesis and formation

Selt:nium nanoparticles were synthesised from the chemical reduction of selenous acid, obtained by adding selenium tetra.-chlotide in water, with the use of PSSS as a protecting agent and ascorbic acid as the reducing agt;o,t, as seen in figure l.

Ascorbic acid is often a preferred reduci.ng agent due to its biocompatibility and low toxicity in the body compared to other reducing agents[81] Jt was observed that higl1 concentrations of ascorbic acid produced a clear red solution containing smaller nanopatticles, < 70 nm while lower concentratioos of the reducing agent resulted i.n a cloudy, orange coloured solution ·with larger nanoparticles size ranges > 100 run. Different effects from different concentrations of reducing agents have been observed in other papers such as Henglein (1999) where high concentrations of a reducing agent can destabilize the na.nopaiticle causing the formation of irregular shapes whi.le low concentrations of the reducing agent caused aggregates of the nanoparticles, from large particle sizes, > JOO nm [91] The concentration of PS, S was important **i.n** this reaction as **it** has provided an electrical repulsion layer on the smface of the nanoparticle; a lower concentration, < 0.01% showed a tendency for the nanopartides to aggregate and form snowflake-like n.ao.o-structures. *In.* contrast, a higher conceotJ;ation (0.2% w/v) fonned uniform and widely distributed na.nopa.rtides (Figure I). If the protecting agent concentration is too high, a larger hydrodynamic diameter of a nanoparticles is usually experienced / [401] sU1face ch al:ge of nanopa.rticle is increased as



Figu re 1. Scanning electron micrographs to show the difference in (L) low and (R) high concentrations of protecting agent, PSSS. The 0.2% PSSS concentration has enable d Improved resistance to aggregat io n. /\ll experiments were repeated in triplicate.

more electrons are added onto the surface resulting in the nanoparticle ha,ving a larger n iameter. [1411] S:ome other authors have shown that higher concentrations of protecting agent produces a larger particles size range (>0.5 µm) along with various shapes: triangles, rods and cylinders rather than spherical-shaped nanoparticles (.100 - 300 nm in size).[1' 21]

The abilify to: invoke size changes of nanoparticles is important in order to explore their potential applications for cenu1ar upt ak:e,[4 3] catalysis, energy production, food science and many other areas of nanotechnology.[1221]

This work shows that the protecting agent was particularly important for the stabilisation process of the solution of the selenium nanopartldes preventing aggregation, thus improving the surface charge repulsion and zeta potential properties of 1he nanoparticles.[44] The crystal growth of metal nanoparticles is known to be influenced by the strength of adsorption of the capping agent and also the competition between the inter particle aggregation for the growth of the particles. For crystal growth to occur, descrrption of the capping agent must proceed, allowing the metal atom tu gain access to the prevailing nanoparticle surface.[145] Similarly, in this reaction, selenium metal nanoparticle synthesis has occurred when a change of colourless solution (seed) turned to a 'brick' red colour, indicating atoms had built onto the seeds in solution. The colour change was confirmed with the 260 nm plasmon resonance value. Controlling the shape of the nanopartide has been most successfully achieved using a see:d t emplate. For our reaction, a spherical seed template was used, this template provided a constrained environment durin g the nanopartid e growth phase and thus shapes were tuned according to the s,eed plat form _ The red colour associated with the nanoparticle solution occurred 1 min into the who.le reaction and intensified suggesting that the longer periods of reaction time increased th e concentration of mcmodisperse nanoparticles, as shown in[1461] and exhausted when all seed solution was used[2 4 6] . this seed and ripening method is known as the Ostwald ripening pro- cess. [1] AS the growth of nanoparticle crystals can be stopped by reducing th@ reaction kinetics: through tempernture and reaction t ime.[1 4 61] If all these parameters are optimised, such as the ones in this study, highly crystalline and well defined nanoparticles are ,;:reated. ff the values of the reaction parameters are too high or too low, irregular shaped nanopartlcles can result. Figure 2 shows the mechanism by which seienous acid was reduced to form the precise selenium nano-spheres.

Equations (1) and (2) show the main synthesis for this reaction including the formation of selenous acid from selenium tetrachloride and synthesis of selenium nanoparticle from reduction of ascorbic add:

$$SeCl_4 + 3H_2O \rightarrow H_2SeO_3 + 4HCl$$

The reaction of selenium tetrachloride with water produces selenous acid (lnd hydrochloric acid:

$$H_2Se0_3 + i\,Hd + CoHs0_6 \quad Se^{\circ} + Coli_i0 + H20 + d_{2(g}$$

(1)

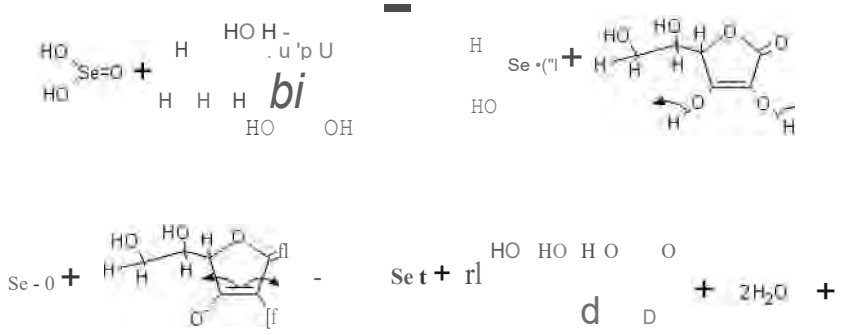
Selenous acid is reduced by ascorbic acid producing dehydroasrnrbic acid and water while the hydrochloric acid from the first equation (1) also result in water production and chlo- rine gas.[8]

UV-visible spectroscopy of the resulting nanoparticle solution displayed a surface plasmon resonance band at 262 nm confirmmg the presence of selemum nan0part1c1es[50 52] **m** Figure 3 below.

The noticeable broadness of the abso rption peak coincided with the increase **in** particle size at 1 h when compared to the initial reaction times of 1 and 10 mln, respectively, indicates the nanoparticle size profiles remained constant. The exact position of the surface plasmon band may shift depending on the individual particle properties including size, shape, solvent and capping agent.[1221] The initial I min absorption spectrum has another broad brand at 285 nm. This band was aligned with the seed solution. For the nanoparticles produced in fuis work, we have halted the aggregation process by optimising the capping agent concentration for the synthesis. An optimised, analytical approach produced an ideal optimal concentration "sweet-spot of 0.2% w./v PSSS. Temperature can also be used -to prevent side reactions if washing has not been carried out.[147] Refrigerating the selenium nanoparticle solution after syn1'hesis at 4 °C showed the ability of the nanoparticle solution to remain a dark red colour and clear with no change in wavelength absorption and particle size.[54]

Nanopartic:Je wa shlng, clean-up and aggregation studies

For the application of nanoparticles to be used in biological systems, it is necessary to clean the nanomaterial using a series of washing stages. Washing the nanoparticles was iJnportant **in** this study as it removed any un-reacted products left **in** the solution and also optimised the stability of the particles. In this study, selenium nanoparticles were washed and then re-suspended **in** MiUi-Q water. An observed change to the UV -vis



f igu re 2. An illu,tration of the proposed mechanism of selenium anopartide formation.

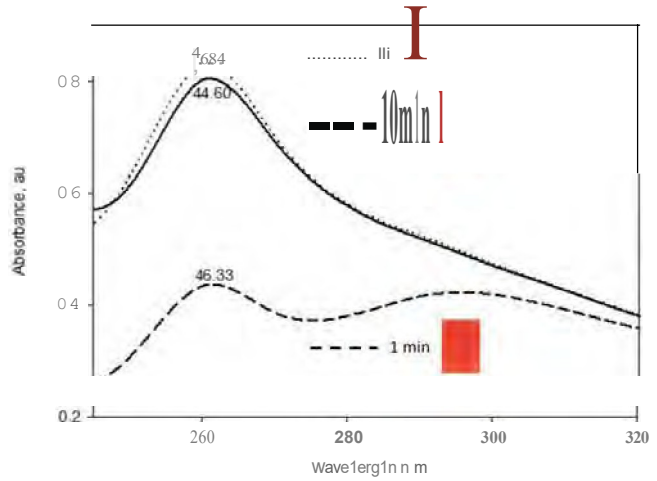


Figure 3. Top graph shows UV-vis absorption spectra of selenium nanoparticles at 1 min, 10 min and 1 h, photographs of the nanoparticle solutions during the synthesis stages are also overlaid.

spectra has shown that the seed solution was removed following the washing steps performed in the 1 h spectrum.

Few papers have used solvents for washing, clean-up and redispersing nanoparticles[17,18] showing that improved dispersity of the nanoparticle solution needs to be investigated. Potentially, a switch in dispersant may also provide new strategies to improve the end-application of the nanoparticles. This study found that when using ethanol, the nanoparticle solution became turbid and changed in colour from red to brown, where the aggregated particles precipitated from the solution. This result provided a rationale for the investigation for other solvents to be used without aggregating the nanoparticle solution. In this study, we have investigated ethanol (log K_{ow} = -0.31), methanol (log K_{ow} = -0.77), propanol (log K_{ow} = 0.25), dichloromethane (log K_{ow} = 1.25), ethylene glycol (log K_{ow} = 1.36) and acetone (log K_{ow} = -0.24) as a means to clean and re-disperse the nanoparticles, as shown in Figure 4. When polar solvents were used, nanoparticle destabilization is observed

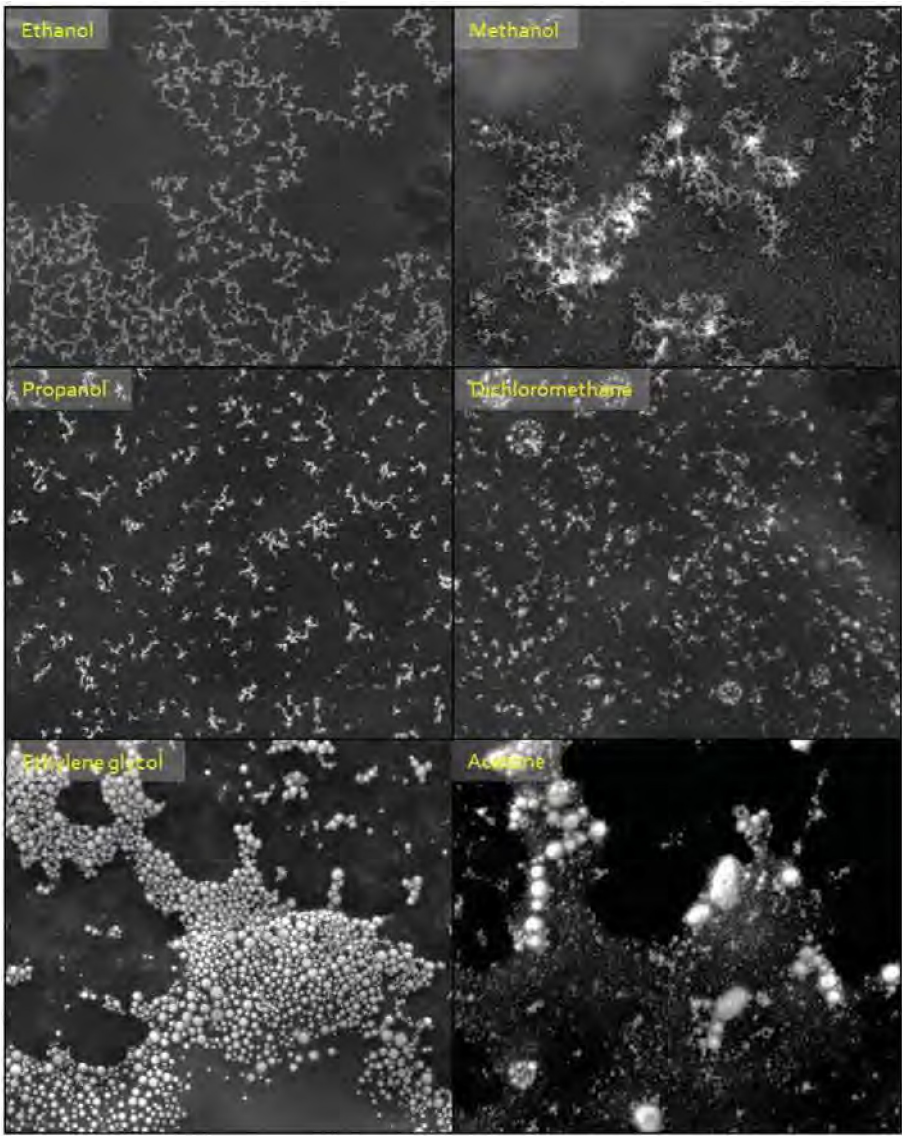


Figure 4. Scanning electron micrographs of selenium nanoparticle re-dispersed in different types of solvents and the effect the solvent has on the nanoparticle solution.

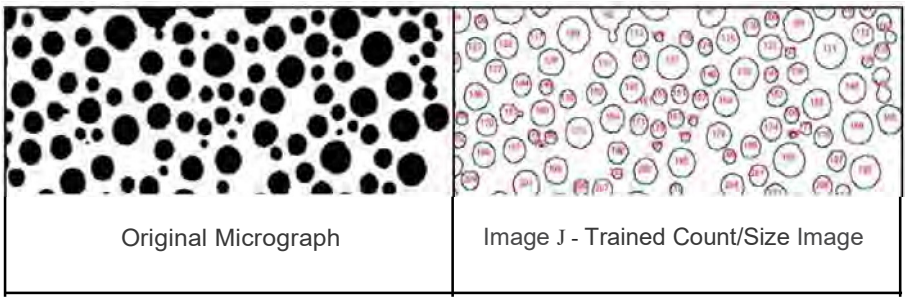


Figure 5. Orig inal transm issi on electron mlc:rogr aph (L) and (R) ove rall size and size distribution pixel plot of selenium nanoparticles produc:ed using ImageJ softw are .

where the concentration of the prott-cting agent was too low resulting in poor ieta potential of the nanoparticles[39 .41 causing aggregation. The zeta potential of the nanopartide solution in this study has sh,m, 'll low negative values bet\\l"een 10.11 and 10 .81 mV where preferred values are higher than 30 mV or lower than 30 m v [5] Ethylene glycol and dichloroi;netbane were the only solvents to disperse the nanoparticles successfully when used as pure solvents - the spherical particles displayed no sign of attraction or precipitation. This could be due to ethylene glycol and dichloromethane having higher octanol and water coefficient values, furthermore, their viscosity values are enabling the separation of the nanoparticles based on their di.fferi.ng viscosities - ethylene glycol, 13.5 t/ (m.Pa s) (20°C) while the other solvent have lower viscosity values of less than 2.[56 57] Washing illld re-dispersion with water affected neither colour nor appearance of selenium nanoparticle solutions where the electron micrographs demonstrated good visual data and si:Ze ranges of nanopartides, Figure 4. Other studies have attempted to re-disperse in water as the solvent and also show that no signs of aggregation occur, indicating good stabilization inducing repulsion between the particles and th surrounding solvent.[5 1

To support the size di1>tributi.on data from the dectron micrographs taken for each synthesis stage of this work, the selenium nanoparticle solutions were characterized with dynamic light scatteri.ng achieving sizes ranges 46 ± 0.17 nm with a polydispersity index intensity value of 0.04 ± 0.01 where nanopattides were of a spheriOl.1 shape. The small PD[wlue indicates the Sc-NP to be 1llon odis_persc.[J It is possible to improve the PDI value as shown in Malhotra (2014) by coating the nanomaterial with a substrate such as dext:rin, that can reduce the PDI value of nanoparticles_[53] Analysis of the SEM images with lmageJ and Sig maPlot further supports the DLS and SEM data. Figure 5., used lrnagef coupting functions to count na.noparticles and also their size dist1ihution. To the best of our knowledge, this is the firi;t time this bas been used to perform accurate measurement to support the nanoparti.cle si.z.e data.

The use of scanning electron microscopy , while excellent in providing routine infonnation on the overall size ranges of materials, resolution is not always retained. In order to fully cha ractetise the optimised solutions in this study, transmission electr on tnicroscopy ,vas used. TEM has confirmed the 1,1 orphology and structur e of selenium nanoparticles being sphe1ical. Figure 6 shows the sizes of Se NPs in solution and further corrobo rate Wit h the data obtained from DLS.

X-ray diffraction analysis was petfonned to show the crystalline nature of the selenium nanomatnials. Two diffraction peaks were observed around 16.4 and 32.8 degrees which were closely situated near 15.5 and 31.5 degrees conesponding to the p1·es.-cnce of selenium nanopa rticlcs. Other papers have shown the intensity of selenium occurring aro1111d 20 and 30 degrees[M.] furthermore, calculated lattice constants arc in agreement with tl1e values obtained in this study for spherical shaped selen i.utn . The peaks also exhibit low strength and thus potentialJy indicated that the Se• P may be a mix of amorphous and

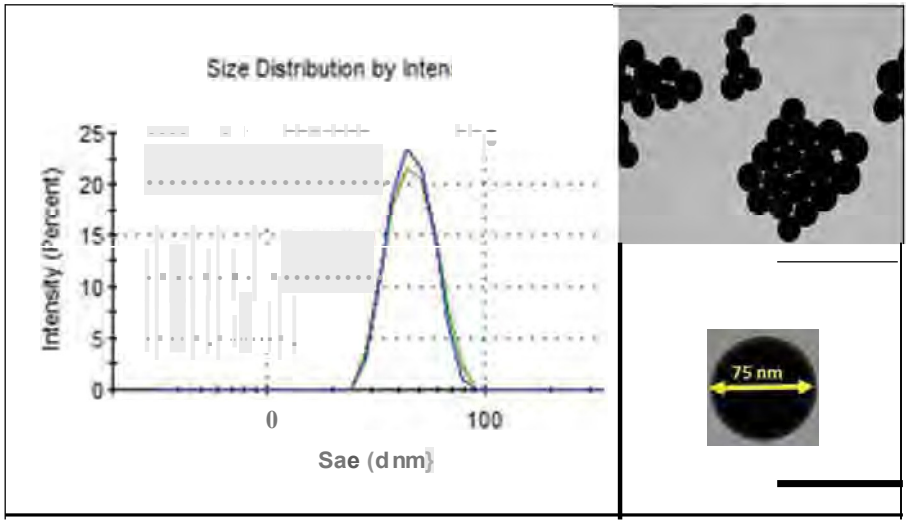


Figure 6, (L) Selenium si ze distributio n using ImageJ analysis of complex electron microgr.iphs and (R) T ransm ission electron micrograph show in g (a) a sing le isolated selen ium nanosp here of an average size of 55 nm.

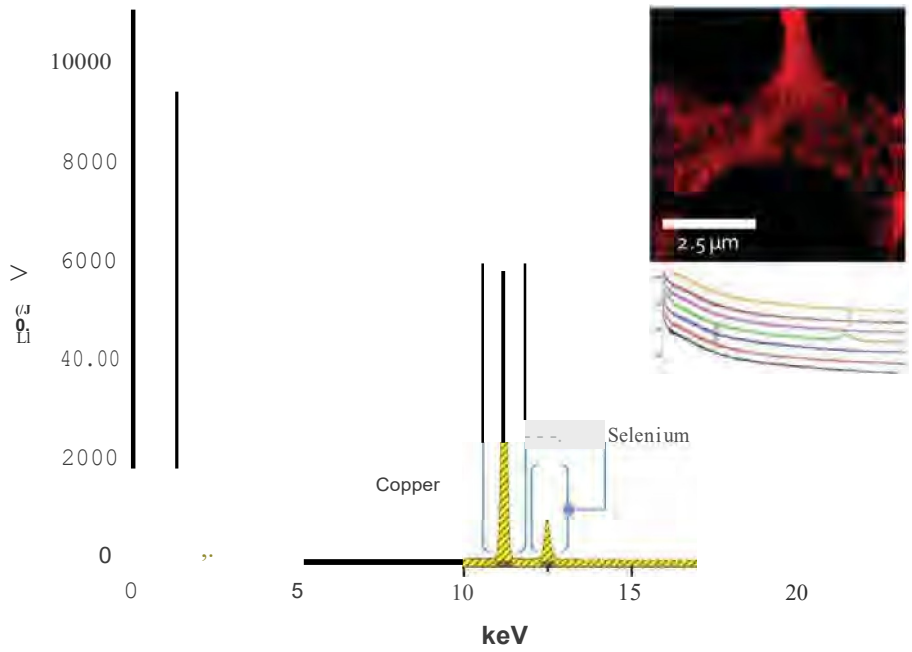


Figure 7. Energy dispersive spectrum of elemental composition of optimised selenium nanopartide solution, insert of transmissio n electron micrograph (top Insert), and X-ray diffractogram for multiple se-np analyses (bottom insert).

monoclinic selenium, mostly in an amorp hous form , while monodinic belongs to one of the seven cry stal sys tem s, an runor- phou i, i,tructur e refers to non-crystalline materia ls w hic h could signify the nanopa1tides to be non- c.rystaJline. [59]

l; he energy dispersive ,X ray spectroscopy analysis confirmed th e presence of elemental selenium show i ng absorption peaks of selenium at l.4 keV, 11 keV and 12.5 keV at Lal, K,BL a,nd K/32 peaks, respectively. All of these characteristic peaks refer to the X-ray emissions "mr se eruurn m of er Stu d' lcs.[31.sMb]

Conclusion

This study has demonstrated a fast and reproducible method to synthesise selenium nanopart:icles by reducing selenium tetrachlo1ide with asc01bic acid in a bottom-up design synthesis approac h. We have demonstra ted fu t low concentrations of reduci ng agent compared to tnetal salt produce larger nanoparticlcs > 100 om while high concentrations of reducing agent compared to metal salt produce mucb smaller sizes, < 70 nm of selenium nan oparti.cles. Aggregation of the nanoparticles ·was red uced using PSSS in an an alytica lly optimised method, resulti ng in an electrical layer producing repulsi on against other nan op.ut icles in the solution. The presence of selenium nanop ru·ticlcs \vas con.firmed through UV- vi15 b le spectrollCopy showing th e surface plasmon reso nan ce bands_ X-ray diffraction studies have shown that selenium nanoparticles are am or phou s, supp 01t- in g the dark red colo ur of the nan oparticle solut ion obtained. l'EM. S£M and DLS have shown nanoparticles to of spheri.cal shape and size ranging less than < 100 run.

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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

Chapter 3

Selenium nanoparticles in poultry feed modify gut microbiota and increase abundance of *Faecalibacterium prausnitzii*

Chapter 3 examines the supplementation of Se NPs in poultry feed at different concentrations and compare its effect on gut microbial ecology and SCFA production against two most commonly used Se supplementation in the poultry industry, sodium selenite (inorganic Se) and selenomethionine (organic Se). The results show the ability of Se NPs to improve gut microbiota by increasing beneficial bacteria such as *Lactobacillus* and *Faecalibacterium,* as well as increasing healthy gut metabolites, particularly butyric acid which acts as an energy source for colonic cells. Histopathological analysis was performed on duodenum, ileum and caecum tissue samples, and results shown there was no toxicity induced by the Se NPs. This chapter has been published in Applied Microbiology and Biotechnology journal, with an impact factor of 3.670, and has been cited 14 times.

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Selenium nanopartkles in poultry feed modify gut microbiota and increase abundance of *Faecalibacterium prausnitzii*

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Abstract
The poultcy industry aims to improve productivity while maintaining the health and welfare of flocks. Pathogen control has been achieved through biosecurity, vaccinations and the use of antibiotics. However, the emergence of antibiotic resistance, in animal and human pathogens, has prompted researchers and chicken growers alike to seek alternative approaches. The use of new and emerging approaches to combat pathogen activity inducting nanotechnology, in particular, silver nanoparticles (NPs), has been found to notonly eradicate pathogenic bacteria but also include issues of toxicity and bioaccumulation effects. Other novel metal nanoparticles could provide this pathogen reducing property with a more tailored and biocompatiblenanomaterial forthe model used, something our study represents. This study investigated the benefits of nanomaterial delivery mechanisms coupled with important health constituents using selenium as a biocompatible metal to minimise toxicity properties. Selenium NPs were compared to two common forms of bulk selenium macronutrients already used in the poultry industry. An intermediate concentration of selenium nanoparticles (0.9 mg/kg) demonstrated the best performance, improving the gut health by increasing the abunda;nce of beneficial bacl:eli a, such as *lactobacillus* and *Faecallbacterium,* and short-chain fatty acids (SCFAs), in particular butyric acid. SCF*As* are metabolites produced by the intestinal tract and are used as an energy source for colonic cells and other important bodily fimctions. Selenium nanoparticles had no significant effect on live weight gain or abundance of potentially pathogenic bae,'teria.

Keywords Intestinal microbiota • Selenium • Nanoparticles - *Faecalibacterium prausnitzii*

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Introduction

Nanoparticles (NPs) are particles with dimensions smaller than 100 nm (Chapman et al. 2010; Gangadoo et al. 2016). Owing to their size, metal NPs have been found to possess unique physical and chemical properties (Rai et al. 2009) such as erihanced biocompatibility and a greater surface area to volume ratio while frequentiy demonstrating reduced toxici!J compared to the corresponding bulk metals. The size of a nanoparticle offers a unique delivery platform to an organism including unintenupted passage through the endocytotic and lymphatic membranes for particles smaller than 5000 run (Chen and Langer 1998; Sanders and Ashworth 1961) and through cellular membranes for particles smaller than 500 mn (Florence et al. 1995). Because of their useful properties, NPs are used in a range of everyday products, from medical , building , optics and electronic industries to food and agricultural products (Peters et al. 2014).

A current issue in the poultry industry is that supplementation of chicken feed with antibiotics, still practised in many

countries to maintain health and productivity, has been linked to the emergence and spread of antibiotic resistance-encoding genes (Obeng et al. 2012; Vieira de Souza et al. 2012). An alternative to antibiotics must be found so that the use of antibiotics can be reduced without compromising the health, welfare and productivity of poultry. NPs have advantageous properties which may make them a worthwhile alternative to antibiotic usage.

Interest in the potential application of NPs in agriculture started with efforts exploring the antimicrobial action ofNPs, in particular, using nanosilver (nanoAg). Silver has been suggested as an alternative to antibiotics in poultry production (Zarei et al. 2014). NanoAg can also protect from the harmful effects of aflatoxins (Gholami-Ahangaran and Zia-Jahromi 2012), which can be an issue in overall poultry nutrition. A fundamental drawback ofnanoAg is now emerging, nan1ely, the toxicity of silver that accumulates in organs, especially the liver or muscle, and has halted the use of nanoAg in livestock products (Jennifer and Maciej 2013). Toxicity studies on nanoAg have shown that there are clear bioaccumulative effects owing to the fact that silver is not inherently required in most metabolic processes in living systems. Other metals and metal oxides when presented as NPs demonstrate improved biocompatibility and some researchers have been focusing on antibacterial activity against main poultry pathogens (Gangadoo et aL 2016).

Most o.f the NP products, being trialled in the poultry industry as pathogen controlling supplements include silver, gold and metal oxides (Gangadoo et al. 2016), are not required for cellulm· functions. We propose the use of an essential metal, in NP form, to deliver nut1ients necessary for optimal poultry health and, at the same time, produce positive effects by their influence on various bacterial genera. Thus, we have investigated the deliveiy of nutiients that represent a part of standard poultry supplemental vitamin/mineral mixes known to be beneficial and essential for anin1al growth m1d immunity. Here we hypothesised that this scientific approach will overcome the bioaccumulative and toxic effects previously observed using nanoAg and provide a faster and more efficient mean of essential metal delivery in addition to producing .antibacterial effects against a range of susceptible microorganisms.

Most vitamin and mineral supplement mixes contain a range of essential metals. The presence of trace amounts of these metals, including iron, zinc, copper, manganese, chromium, molybdenum and selenium, is vital for human and animal health, due to their role as enzyme cofactors (Maathuis 2009; Speich et al. 2001). Essential metal deficiencies are serious health probleins in humans as well as in the livestock industries. Selenium is routinely added to poultry feed in two primmy forms, inorganic and orgmlic, forming a supplementary macronutrient component in most feed. The inorganic selenium, typically reported as selenite when used in poultry feed is reported to be less efficient when compared to the organic form of selenium (Brandt-Kjelsen et al. 2014). Organic selenium sources are considered more suitable f01: feed, and a number of attempts to use selenium enriched yeast and wheat have been suggested and investigated (Brandt-Kjelsen et al. .2014). Ready uptake is a major advantage of NPs for transport to the desired cell and results from the particle size and shape (Pal et al. 2007). NPs are able to deliver the desired nutrient directly into the cell without involving complex uptake pathways and energy loss to the host.

Here we present a study to investigate the potential of selenium nanoparticles (nanoSe) to deliver selenium's essential metal properties to both the chicken host and the intestinal bacteria more efficient y than tlie inorganic and organic selenium sources in feed. The study will also demonstrate the potential modification of the gut microbiota in a positive direction to enhance the abundance of beneficial bacteria and 1imit the pwliferation of pathogenic bacteria. **This** study compared the .influence of both organic and inorganic Se supplementation that is commonly added to poultry feed in Australia against three different concentrations ofnanoSe: 0.3, 0.9 and 1.5 mg&g.

Materials and methods

Chemicals and reagents

Chemicals chloroform and methanol (HPLC grade) were purchased from Th.e rmo Fisher, Melbourne, Australia. Cellulose filters, pore size of 0.45 µm, were acquired from Sartorius Stedim Biotech Gottingen, Germany and gilS chromatography vials .from Restek, Sydney, Australia. Ultra-pure water (18 Mn) was used throughout the study. Standards of acetic acid, propionic acid, butyrate aoid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid and heptylic acid were purchased from Bio-Scientific P ty Ltd., Sydney, Australia

Nanoparticl preparation

NanoSe were synthesised using a simple chemical reduction with the following chemicals; selenium tetrachloride, ascorbic acid and polystyrene-4-sulfonate as the metal salt, reducing and protecting agents, respectively. The solution obtained was of a dark red colour indicating presence of amorphous selenium. Full characterisation of the NPs was done including, size, shape, morphology and crystallinity as well as composition of the solution as detailed in Gangadoo et al. (2017). The nanoSe which were dispersed in Milli-Q water (18 MD) wei·e mist sprayed onto poultry feed, rotating in an Ozito CMX-125 mixer (Ozito Industries Pty. Ltd., Bangholme, Australia) and then air dried for 24 h.

Animal trial

A total of 70 1-day-old Ross 308 broiler male chicks were purchased from Bonds hatchery (http://www.bondenterprises.com.a u/). The birds were weighed and randomly divided among five groups, each containing 14 chicks, in a light and temperature controlled room. Water and feed were supplied ad libitum. The room temperature was set to follow the guide in the Ross Broiler Handbook (Aviagen). The feed used in this trial was a chicken crumble formulation detailed in Supplementary Table Sl. Experimental diets included two controls, supplemented wi1h ei1her organic (Alkosel 3000 inactivated whole cell yeast containing elevated levels of organic selenium, min. 98%) or inorganic (sodium selenite) selenium, at concentration of 133.33 mg/kg, and three treatment groups supplemented wi1h different concentrations ofnanoSe: 0.3, 0.9 and 1.5 mg/kg.

Bird weights and feed eaten were recorded regularly at weekly intervals. Birds were eu1hanised at 29 days post-hatch by CO_2 asphyxiation. Faecal and caecal content samples were collected and immediately stored at - 80 °C Faecal samples were collected during necropsy by sampling directly from 1he colon in the section 2 cm adjacent to the cloaca. Caecal samples were collected by squeezing 1he content from both caeca into a sterile vial. TotalDNA from bothorigins was extracted wi1hin 1 week

Microscopy

For histological analysis, tissues were collected from the duodenum (mid-section), ileum (mid-section) and caecum (end points), washed in phosphate-buffered salineand fixed in I 0% buffered formaldehyde, and paraffin embedded and stained using haematoxylin and eosin (H&E) and Alcian blue stains. The histological images 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.

The morphometric indices, including crypt depth, villus height, villus area and villus height to crypt dep1h ratio, were evaluated for duodenum, ileum and caecum, using ImageJ software. Morphometric analyses were performed on 20 technical replicates (different histological sections) from 5 biological replicates (birds) per treatment group.

Short-chain fatty acid analysis

Short-chain fatty acid (SCFAs) metabolites were extracted from caecal samples (0.2 g) as described by Roume et al. (2013). To summaiise, an extraction solution of chloroform and me1hanol at a ratio of 2:1 was used in extractions and the final extract was filtered using a 0.45-µrn cellulose filter (ThermoFisher, Melbourne, Australia, cat. no. 54504-RC). Calibration curves were constructed using standard stock solutions and then stored as a method processing parameter in selective ion monitoring mo.de for the following SCFAs, acetic, n-butyri c, isobutyric , propionic , n-valeric and isovaleric acid

The GC-MS system consisted of a Shimadzu (Sydney, Austra li a) QP2010-Plus equipped with an AOC21 autosampler. The GC was fitted with a high-polarity column HP-Innowa:x (30 m x 0.25 mm x 0.25 µrn). The GC temperature programme started at 60 °C and held for I min, ramped at 15 °C per min to a temperature of 160 °C and then fmally ramped at 70 °C per min until a fmal temperature of 260 °C was reached and held for 0.90 min (a total of a 10-min programme). The GC oven temperature was set as presented in Supplementary Table S2. A 1-µL sample was injected at 250 °C using helium as a carrier gas (Coregas, Gladstone, Australia) at 2.0 mL per min in a split injection mode. Pressure was maintained at 122 kPa, with a total He flow of 15 *mUmin* and using a split ratio of5. Themass spectrometer was operated in the electron ionisation mode (EI) at 0.2 kV with a source temperature of 220 °C where both scan and -selective ion monitoring modes were used from 33 to 150 *m/z.* The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST) library, http://chemdata.nist.gov/.

DNA extractio , amplification and sequencing

Total DNA for rnicrobfota analysis was extracted usirtg Bioline .Isolate Faecal DNA kit (cat. no. BIO-52082, Bioline, Eveleigh, Australia). Primers were selected to amplify the V3-V4 regi0n of 16S rRNA geRes: forward ACTCCTACGGGAGG CAGCAG and reverse GGACTACHVGGGTWTCTAAT. The primers also contained barcodes, spacesr and Illumina sequencing linkers as previously described (Fadrosh et al. 2014). Sequencing was performed on 1he Illumina MiSeq platform using 2 x 300 bp paired-end sequenc ing.

Initial analysis of microbial communities was performed in QIIME v.1.9.1 (Caporaso et al. 2010). Paired end sequences were joined lli\ing the Fastq Join algorithm and no allowed mismatches within the region of overlap. Only sequences with Phred quality threshold higher than 20 were retained in the analysis. Operational taxonomic units (OTUs) were picked at .97% similarity using Uclust (Edgar 2010) and inspected for chimeric sequences using P intail (Ashelford et al. 2005). All taxonomic assignments were pelformed in QIIME against the GreenGenes database and QIIME default arguments (DeSantis et al. 2006). Further data exploring tools included Primer e v7, blastn (Altschul et al. 1997) and the NCB[l6S Microbial database and Calypso (Zakrzewski et al. 20.16).

OTUs wi1h the relative abundance ofless 1han 0.01% were filtered out. The data analysis was performed on a rarefied table, and square root transformation was used in all statistical

analyses. 1"he figures comparing relative abundance show untransformed data. ANOYAwas used to detect tbc si 6JJificaoce of the differences between the groups. Analysis of simi\ruities (ANOSIM) and two-way pcrmuwtional multivariate analysis ofvariancc (PE R MANO VA) analysis were perfmmed in Primer • v7 on weighted and unweighted Unir rnc distaoce matrices calculated in Q flME. each with *99,999* pernmtatioos. Ca lyp o was used to imp l e me nt the upc rvised multivariate rcdtmdancy analysis (R DA) using 999 pe1mutatiori .

T he complete a:nno tatcd sequence dataset is publicly available on tbe MG-RAST database under library 10 mg1620750.

Results

Animal health and performance

AU birds appeared b.e althy, and m01iality in the trial was restricted to early. post-transport days. Total mortality was 4%. The bird weights for each treatment (Fig. L) were not significantly different *(P* < 0.05) altllOugb the 0.3 mg/kg naooSe group was the numericaUy lowest weight for the dmation of the tt-ial. Individual *l.* test comparisons. performed for each day separately, show that tbe only compariso ns witb. significant differences in weight we.re those comparing organic or inorganic Se groups with slowest growi ng 0.3 mg/kg nan oSe, wbere compari ons for eve1y weight measu1ing time poin t after week 1 were signi ficant ly differentia l with *P*< 0.005 for organic and *P* - 0.006-0.024 for inorganic Se control vs 0.3 nanoS e. This i11d ia1 tes that 0.3 nanoSe did indeed bow reduced growth rntc compar ed to the con trols. S imi la rly. high 1 . 5 nanoSe was in - s ig nific ant ly reduced. Regiuding weight gain, the intermediat . 0.9 mg/kg nanoSe . concentrat io n was not distinguishable from org,mic and i norganic control. (Fig . I a, b).

Overall miaobiota composition

O verall, the caecaJ samp les bad higher richness (P - 0.002) and evenn ess (P = 9.34E ^{4})than the faecal samp le s. ln both weighted and un weigh ted UniJ-"rac ana lyses. effects of both diet treatments and samp ling origin were highly sig nifican t (all *P*< L-0 ^{5} with *99,999* pe rm,ut1tio ns) while the interaction was more sign ificant u ing wei.g bted *(P*= 0.028) tban unwe ig)1ted (P = 0.048) Unifrac-bascd ana lysis. s ho wing that sample origin (faecal or caecal) affected the re ponse of mi.crobiota in the five diet treatm ent . To fiirther inve tigate this effect, ANOSlM rnu ltiva ria te analysis of group similatities was perfonm:d and is presented in an ANOSCM ca lculated s ignific ance derived pairwi e grcnlp-to-group similarity matix: as mMDS p lots (Fig. '.2a, b). In both weighted and unweighted metrics. am.pies from the 0.9 mg/kg nanoSe and 1.5 mg/kg nanoSe groups were clustered by feed treatment with tJ1eir respective faecal and caecaJ samp l.es close tog e t h er and separate from other treatments, wb.ile ofb.er samp les were separated by faecal m caecal origin. thus reducing the difference between tbe faecal and caecal conununitie in the moderate and higb concentrated nan.oSe groups. Tb.e significance oftb.e two-way PERt\rlANOVA (diet vs sampling origin) interactions indicated tbat different response to treatment were observed in the faecal and caeca l s amp les from the same birds. This stage of the ana ly sis indicated that the microb iot a i11 faecal and caecal , amp le s sh ould be se parately analysed and inte rpreted.

GIT microbiota differences between Se supplement treatments in faecal samples

Tbere were no sign ificant differences between the Se supplementation treatments in Li.chness or evenness **of** the communities detected in faeces. Multivariate RDA ana lysis indicated significant difference between the treatments at both OTU *(P*= 0.002) and genus level *(P* .:c 0.001) (Fig. 3a, b) witb the 1.5 mg/kg nanoSc- upplemented group, sepam tin g fu1thermost, followed by moderate separati on of the 0,9 mg/kg nanoSe treatment group, while the lowest concentration of 0.3 mg/kg nanoS e overlapped with organic and i norganic Se supplementation groups. At the OTU level. 31% of al'l OTU were sigojficantly *(P* < 0.05) altered in re lative ab unda nce between the treatments. This high le vel of response is pmt ly cx1llained by sign i ficant changes induced by the l.ig hest

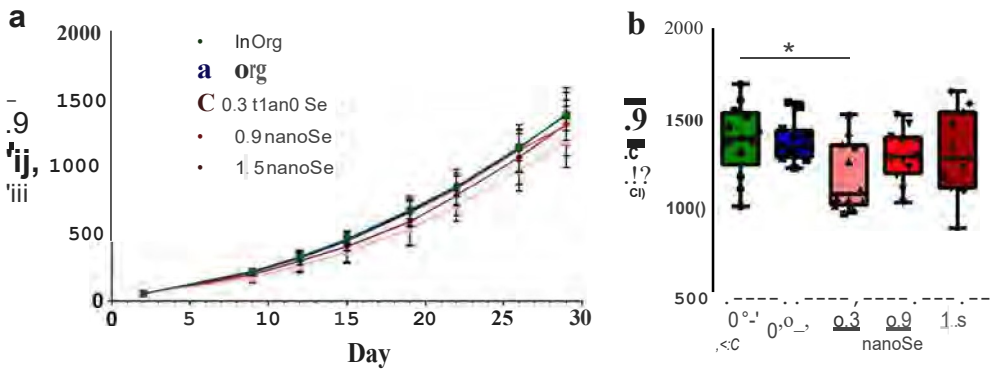

Fig. 1 Bird weigh ts (mean and *SD)* oE elenium eXperimenlal groups. The legend sho ws abbreviated group labelJin,g [nDr g for inorgani c Sllpp le mented Se. Org for organic -and three concentratioru; of nano e supplomcntation (0.3. 0.9 and 1.5 mg/kg nanoSe). **a** Growth over time. b Final weig)it distribution at 29 days of age

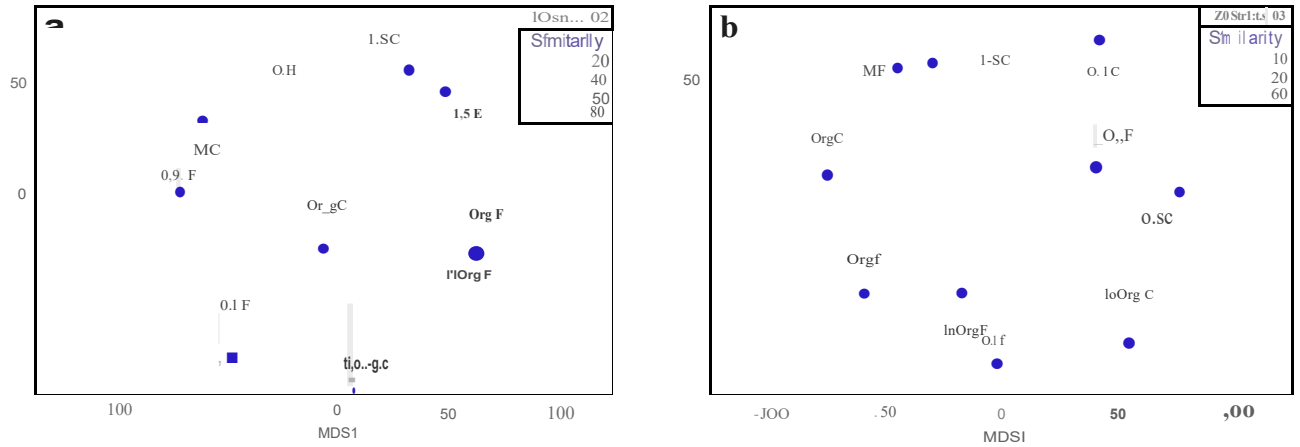

Fig. 2 Multivariate ANOSJM calculated. s.ignincance deiived. group to-group imiJmi ly vi, ualised as MDS plot was based on we.ighted *(a)* and unweighted (bJ Unffrac distance

nanoSe concentration (l .5 mg/kg). Blc ven of the top 50 most abundant OTUs stroogly *(F* < 0.01 re ponded to tbe. treatment differeoces (Fig. 3c).

Genera were also b.igbJy influenced by tbe trea tmcot , with 48% of genera significantly *(P* < 0.05) different in relative abundance between the feed treatmcot . The most effected genera *(P* < 0.0 l) included *Jeotgalicoc*, *Brevibacteriwn, Paecalibacleriwn. Parabac reroides. Sta phy lo L·oc ·us, Brachyba cterium. PhascolarL'!obacterium. Turicibactl?.r* and *Lactobacil/ us* (Fig. 3d). The genera that were comparable in faecal samples, m most cases, responded to the different selenium treatments similar ly in caecal samples Fig. 4).

and *Rum in.o co r;c u.* species were inerca cd i n the nanoSc group , while *l acwhac;dlus* was jn greater relative abundance in the groups treated with low and intermediate concentrations of naooS e, but suppressed by 1.5 mg/kg of uanoSe, indicating tbc possibility of a thrcsltold concentration. after which the beneficial effects regress. Tbe analyses of faeca l and caecal rnicrobiota indicated that the effects of low nanoSe conccntra" tiolJ (0.3 m_wkg) remained comparable w'itb. organic and inorganic controls wbile high concentrnti.olls produced microbiota changes that may prove unhealthy for the host gastrointestinal system. as shown by reduced abundance *ofLac:tobacillus* and an increase in *Turfctbacler* and *Staphylococcus.*

GIT microbiota differences between Se supplement treatments in the caecum

1n the c aecu,m smaller responses to treatments were observed than in the faecal community; with 18 % ofOTUs and 36% of genera significan tly altered by treatments (P < 0.05). RDA analysis indicated that treatment groups bad different microbiota structures at both OTU (P < 0.001; Fig, Sa) and a genus level *(P<* 0.001; Fig. Sb). As in faecal samples. the most abundant OTUs and genera were influenced (Fig. Sc. d) as well a not-readi ly culturable microb io ta.

f aecalibacterium and *Jeotgalicoccus* were strongly c01-related with th concentration ofoanoSein tb.e feed *(P*= 2-E ^{1} , *r*= 0.63 and *f*>= 1.SE ^{8} *r* = 0.49. respectively). Figure 4 shows that *Faecalibaclerium* responded to nanoSe in such a robust way that nanoSe could potentially be used to emich the *Faecalibacterium* relative abundance in mixed cultures. *Jeotgahcncc11s* was similarly hi gh ly COITelated witl1 rn.i.n0Se level but remained at low abund ance .in the high concentration Se- P treatment.

Neither faecal or caecaJ data aruilyses indicated that any potential pathogens, suc ll as *1l.1ricibac1er* and *Staphylococcus,* were reduced in abundance by addition of nanoSe. On the other band, some known beneficial bacteria like *Faecalibaderiwn*

Microscopic evaluation of nanoSe influence on the gut

Desquamative processes are processes where cells 'shed' from the skin layer. The study demonstrates that desquamative processes involving tbe apical and middle paits oftbe intestinal villi were obse1ved io the duodenum in all experimental groups. However, these desquarnative prncesses were observed at a higher degree in control birds supplemented witheither inorganic (Supplementary Fig. S I A) or organic selenium (Supplcmentaiy F'ig. S1B). In contrast in the groups treated with 0.3 m g/ kg (Supplementary Fig. S1 C), 0.9 mg /k g (Supplementary Fig.SID) and 1.5 m_wkg (Suppkmentaiy Fig. Sl 13) nanoSe, desqurunative processes were cononcd to 1he tipsurface of the villi without affecting the middle and lower vil li sections Ti,0r the deeper layers of the intestinal mucosa In the intestinal lumen of aU g row s, fulle n eprtheLial cell oftbc intq;;-tinaJ epithelium were detected; this was al o more pronounced in organic or .inorganic selenium supplemented birds.

In nanoSe-supplemented birds (0.3. 0.9 and 1.5 mg/kg). slight to moderate b ypera entia of s1tbmucosal blood vessels and m.inor subserosal oedema (Supplementary Fig . SlC-E) was observed. Changes of this natnre were not detec ted in

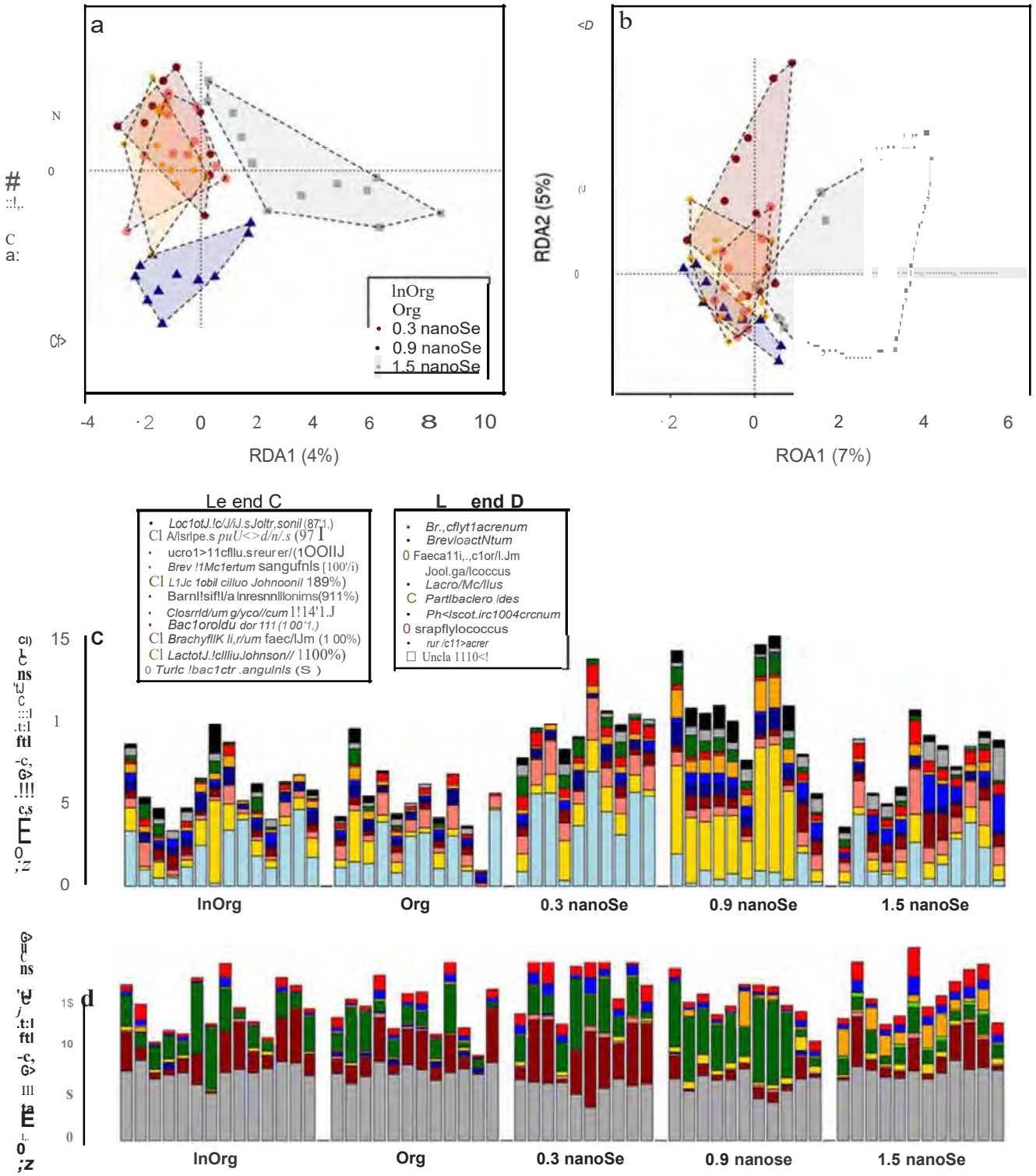

Fig. 3 The treatm en t differences in faecal m icrobiota comp osition. RDA plot at an OTU **(a)** and a genll! (h J leve l separate 1.5 nano Se treatmen t group as mo ! distinct from othe r n"eatmcn ts. T here were 11 OTUs among the lop 50 most abundant that we re ig nificantly *(P* < 0. 01) allered between tl1c treatments (c). d The genera *(P* < 0.01) influenced by treatn1ents

inorganic or organic sele nium control groups (Sup plementary Fig. SJ A, B). Ln the duodenal mucosa of all groups, positive PAS-Alcian blue reaction was obseived, proving the presence of acidic and neutral mucins (Supplementary Fig. SlF-1).

The morphomettic analysis showed no significant differences between treatments in caeca, slightly significant differences in. viUus height of ileum, with organic control and 0.9 nanoSe having reduced villus height compared to the other

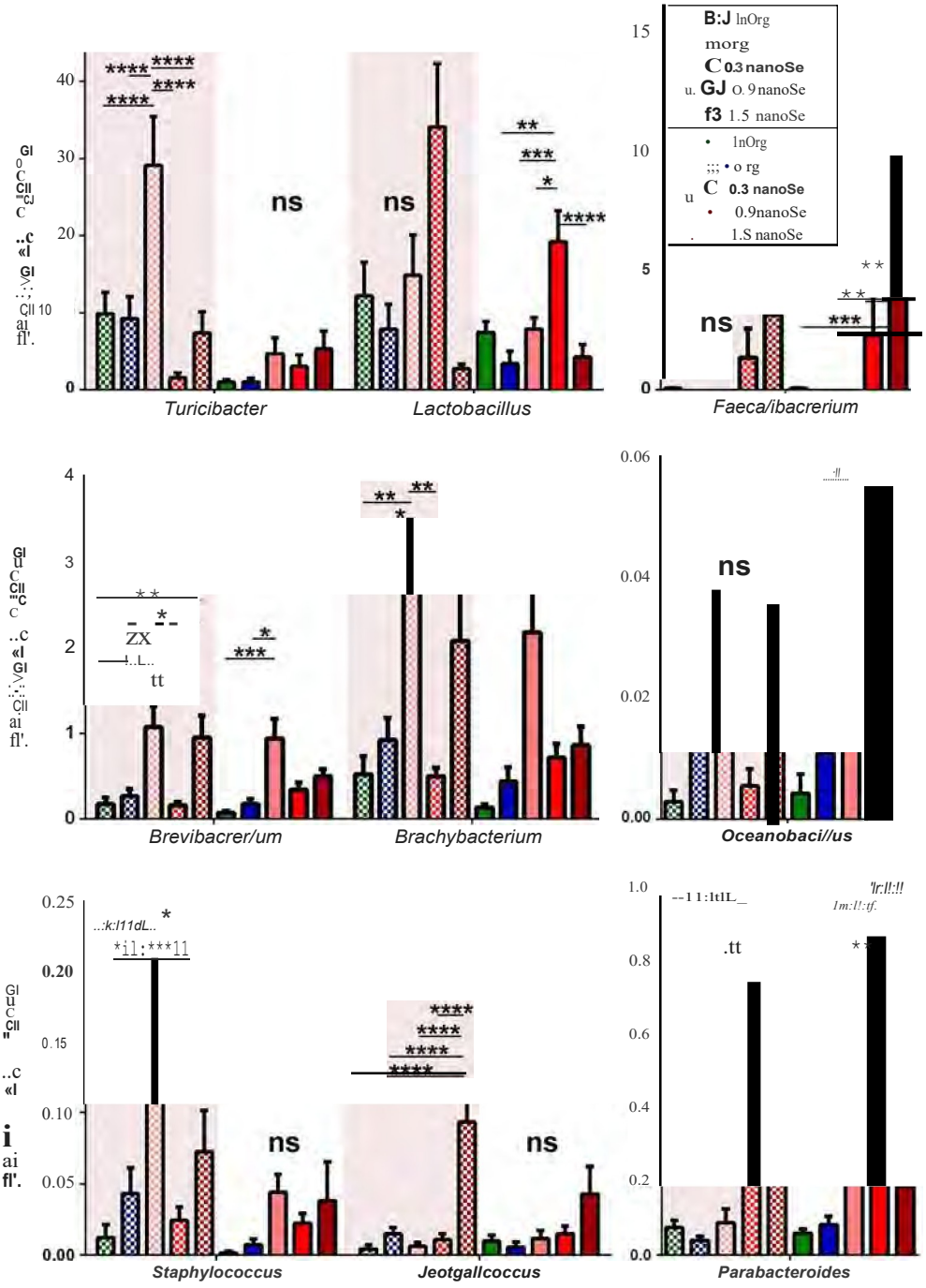

Fig. 4 Comparison or response in faecal and in caecaJ microbiota lo different Se-supplemented treatments. The same colour pallem was us ed for treatments with faecal amples displayed with whi le pattern bars and caecal with full colour bars

groups (Fig. 6a-c). The differences in the duodenum (Fig. 6d-t) demonstrated that the villus to ctypt ratio was significantly higher in l.5 mg/kg nanoSe group than in all otb r group (Fig. 6-f).

NanoSe influence on short-chain fatty acid concentrations

The analysis of SCFAs included acetic. isobutyric, isovaleric, propionic, n-butyric and n-vaJeric acid. We perfonned two-way ANOVA with two factors: SCFA and different seleoium treatment. There was no significant int ractioo between dif ferent SCFA and Se treatments *(/'* = 0.9993), indicating that tbere was no major diff rence in *SCFA* profile between the treatments. There was significant difference between individual SCFAs *(P* = 0.0004) and in SCFA profiles between the treatments *(P* = 0.0014). The 0.9 nanoSe bad significantly higher overall SCFA production than inorganic *(P* = 0.0055). organic *(P* = 0.0274) and 1.5 nanoSe (P = 0.0202). The differences in individual SCFAs are shown

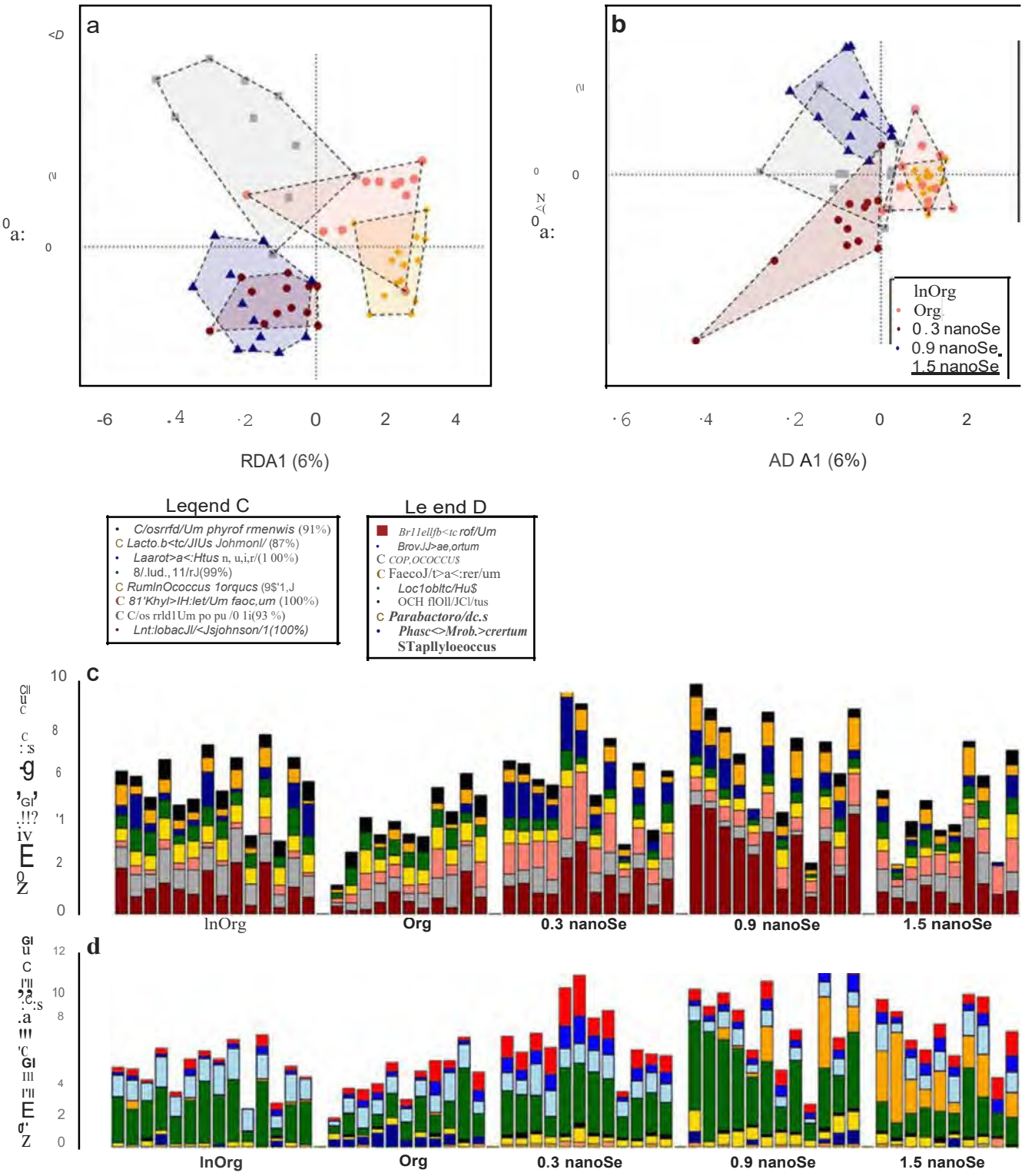

Fig. S Visualisation. of the differences in caecal microbio1a compo ition aero s tTealmeu! groups. RDA plot a! OTU **(a)** ,md genu (b) level. The OTUs among the top 50 most abundant that were significantly *(P*< 0.01) altered between the b-ealrnents (c). d The genera (P< 0.01) influenced by tTeatTnents

in Fig. 6g). The only sigrufican t differences between SCFA concentrations were significantly higher acetic acid compared to isobutyric acid *(P* = 0.0311) and iso val er i c acid *(P*= 0.0457).

Discussion

Results showed that nanoSe supplementation in feed did not sigru.ficantly affect bird weight but indicated the presence of

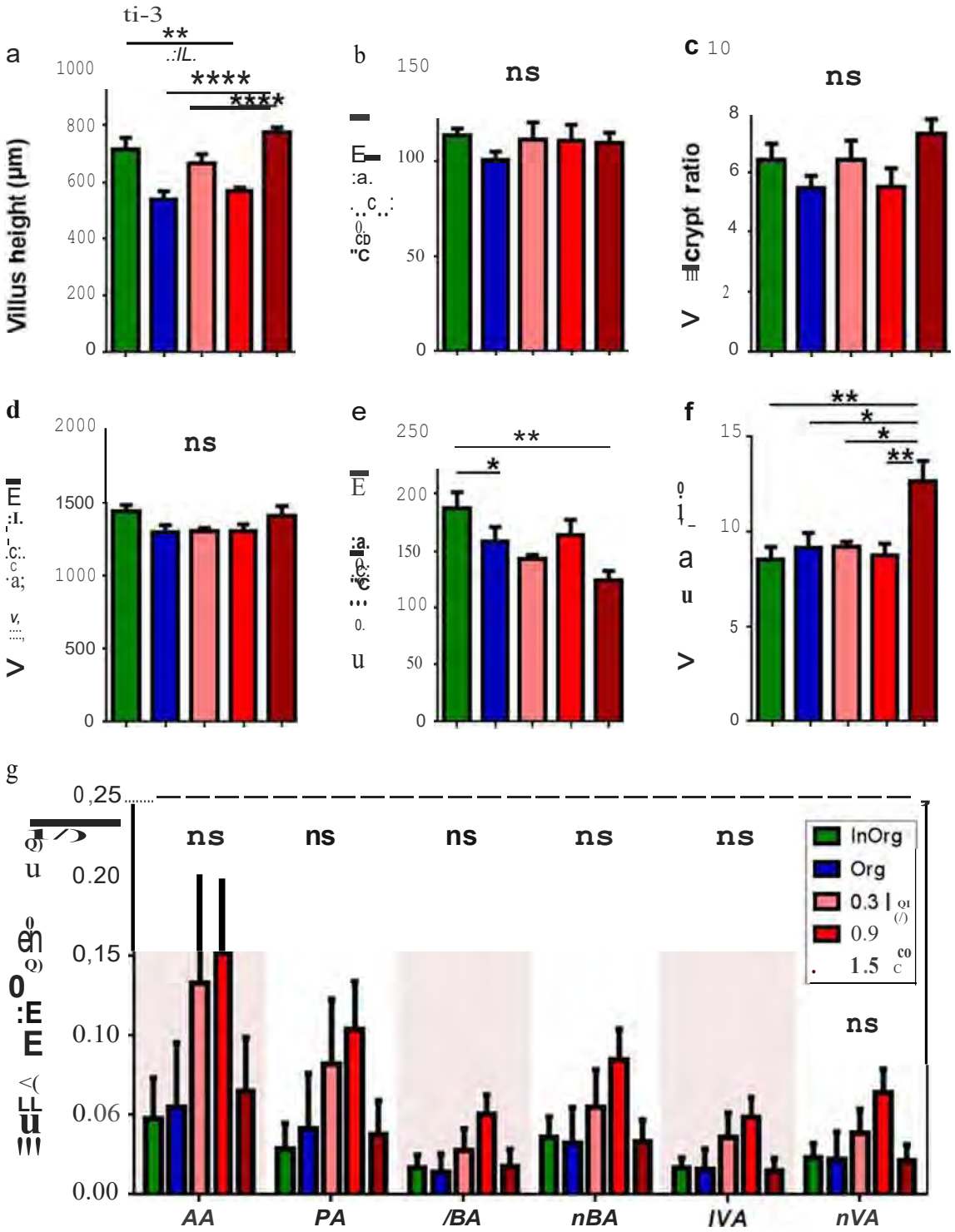

Fig. 6 Basic h1stological measurememːs (a- f) from illeal (a) and duodenal (d - f) his tological ections and caecal SCFA profile (g); AA acetic acid. fBI\ isobuty1ic acid, JVA isovaleric acid. PA pmpionic acid, nBA n-buty,ic acid and nVA n-valeric acid

an optimised thresbold for naooSe concentration effects concerning bird weight gain. The weight gain was reduced at tb.e lower and higher nanoSe concentrations but was comparable with the two controls using the industry standard organic and inorganic selenium at the intetmediate concentration of0.9 mg/kg. anoSe altered the microbial community of the birds. introducing a sigruficant increase of opportnnistic pathogens , *Tztricibacter* and *Staphylo occus,* as shown in Fig. 4. Although *1i1n'cibacter* is commonly found in animal intestine , a member oftb.is genus was onlyJecently.reported as an opporn.mistic pathogen (Bosshard et al. 2002). On the other hand, *Staphylocoe(;us* are known to cause a range of diseases

in humans and animals through both proliferation and toxin production combined with the increase in antibiotic resistant strains, with an extreme example being meyhicillin-resi1,tant *Staphylococcus aureus* (MR.SA) (Emaneini et al. 2017). The nanoSe-induced proliferation of genus *Staphylococcus* can have serious clinical consequences for both chickens and humans alike. Additionally, this may upset the balance between the pathogens and beneficial strains in the gut causmg dysbiosis that is implicated in a wide range of medical conditions (Underwood 2014). The nanoSe concentration, at 0.9 mgikg, is recommended as an optimised feed concentration for future exploration.

As reviewed m Gangadoo et al. (2016); strong antibacterial effects were reported for a number of nanoparticles trialled for poultry pathogen control and in vitro studies, showmg areduction *in* pathogenic bacteria, such as *Escherichia coli* and *Staphylococcus.* The results for this trial indicated different results: pathogenic bacteria, *Turicibacter* and *Staphylococcus*, were increased in mmoSe supplementation as compared to con-n:ols but additionally increased health-promoting bacteria such as *Lactobacillus* and *Faecalibacterium.* **The** sampling origin also proved to be a major influence on microbiota composition and abundance between faecal samples and caecal samples caecal samples are typically richer due to the caecum actmg as the primary site of fe1mentation in the gastrointestinal system (Stanley et al. 2015) while faecal bacterial community was highly variable due to constant emptying of different gut sections, therefore varying significantly between time points (Stanley et al. 2015). While faecal sampling can be used to detect major occurrences in the gut profile, in birds, caecal sampling by necropsy is considered as better, more reproducible, representation of the composition and abundance of the microbial environment and provides a more accurate observation into the complex environment of the gut. NanoSe supplementation significantly affected the microbial profiles and made faecal and caecal samples in medium and high nanoSe groups more similar to one another than commonly seen in chickens and other treatment groups.

The capacity for absorption **off** and nutrients from the GIT fully depends on the condition of the intestinal mucosa and intestinal morphology such as villus height. The feed and intestinal microbiotas have profound influence on intestinal epithelial morphology (Sittiya et al. 2016) with improvements repmted -with delive.ty or increase in probiotic species in the intestine. The largest duodenal villus/crypt ratio was observed in the highest (1.5 mg/kg) nanoSe concentration treatment used; this concentration was also found to induce the strongest changes in intestinal microbiota composition. The villus/crypt ratio is an indicator of tl1e digestive capability of the small intestine. Reductions in this ratio can indicate compromised intestinal function while an increase in this ratio may relate to an increase in digestion and absorption capability of the gut (Lim et al. 2015; Mohiuddin 1964). The increase in duodenal villus/crypt ratio coincides w.ith the increase of *Faecalibacterium prausnitzii,* a known prompter of epithelial health owing at least partially to its strong metabolite production, especially butyrate (Miquel et al. 2013).

Poultry feed is predominantly grain based and is fibrous. Thus, production of SCFA.s in the chicken gut can be indicative of bacterial groups that are beneficial to health and a contributor to improved growth performance in chicken (Rehman et al. 2007). The role of SCFAs and the interplay between diet, gut microbiota and host energy metabolism are routinely described (den Besten et al. 2013) and support the data obtained in this study. In all 6 SCFAs measured, the 0.9 nanoSe treatment group showed high SCFA concentrations compared to all other groups including 0.3 and 1.5 nanoSe. foterestingly, the group with highest SCFA production, 0.9 nanoSe, was the best growing of the three selenillill supplemepted groups; however, this much higher SCFA concentration did not induce better growth in 0.9 nanoSe than in controls that both had significantly lower SCFA and almost identical growth rates. This would however result in a number of non-weight gain-related healtli benefits.

SCFAs are produced in the colon via bacterial fermentation of non-digestible complex polysaccharides. Acetic, butyric and propionic acids are the most abundant representing more than 90% of colonic SCFA.s (Rios-Covian et al. 2016), With improved knowledge on the roles of gut microb'iota, the new knowledge on protective and health beneficial roles ofSCFAs are continually emerging. Previous studies involvmg mostly human and mice subjects show that SCFAs stimulate the immune system (Goverse et al. 2017); strengthen epithelial tight junctions and promote gut integrity (Asarat et al. 2015 Kelly et al. 2015; Park et al. 2016) reduce incidence of inflanima-to1y bowel disease (Huda-Faujan et al. 2010), colitis (Macia et aL 2015), asthma (Thorburn et al. 2015) and diabetes (Marino et al. 2017); control bacterial pathogenesis (Sun and O'Riordan 2013); improve colonic mucosal functions; inhibit inflammation and carcinogenesis and decreasing oxidative stress; and act as a major energy source for epithelial cells of the colon (Barner et al. 2008; Rios-Covian et al. 2016). It is possible that an increase in SCFAs due to nanoSe treatment could have produced benefits that we did not investigate in this study.

The differences between low, moderate and high nanoSe concentrations were profound, and higher nanoSe groups behaved oppositely, most evident from Fig. 4. Our data indicate that, as in Cai et al. (2012.), nanoSe did not improve bird performance as previously reported by others under different conditions (Zhou and Wang 2011) and that moderate concentrations of nanoSe resulted in similar growth performance as commonly used organic and inorganic selenium supplementation, however they showed the benefit of high SCFA production and an increase *inF prausnitzii* abillldance. Currently,

major effort is under way to produce *F prausnitzii* probiotics for treatment of colitisand other intestinal medical conditions in humans. The level of increase in *F prausnitzii* achieved in our birds fed nanoSe-supplemented feed exceeds levels of expected enrichment in the gut via orally delivered probiotic. This warrants further investigation in use of nanoSe to enrich *F prausnitzii* for improvement of both chicken and human intestinal conditions using higher sample size and multiple trials. Our results also encourage further investigation into optimal nanoSe concentrations for harvesting other benefits that may have resulted from increase in SCFAs, such as reduced intestinal permeability and integrity.

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Compliance with ethical standards

Conflict of interest Th e authors declare that they have no cbnflict of interest.

Ethical approval Animal ethics approvals were obtained from the Animal Etbics Committee at Central Queensland University with the approval number Al 5/07-333.

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Nature of Candidate's Contribution, including percentage of total

SG was involved in the data collection, statistical analysis, and writing of this scientific article (65%).

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All co-authors assisted with the conception and revision of the article. ID (5%); NW (5%); RJM (5%); JC (10%); OS (10%).

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

Chapter 4

Toxicity and bioaccumulation of selenium nanoparticle feed additives in chicken

Chapter 4 investigates the toxicity of Se NPs in poultry, following the animal trial performed in Chapter 3, by analysing the concentration of Se in various tissues of the bird, as well as conducting a thorough histological examination of the tissues mentioned. The tissues were collected following euthanisation of birds at 29 days post-hatch, and underwent digestion using concentrated acids, and a microwave digester at high temperature and pressures. The tissue digests were analysed using Inductively-Coupled Plasma Mass Spectroscopy and results observed an increased bioavailability of Se NPs and reduced accumulation in detoxifying organs, such as liver, as compared to inorganic Se additive used in the poultry industry. The tissues were also subjected to a detailed histopathological analysis, using haematoxylin and eosin dyes, and confirmed no toxicity were caused by the addition of Se NPs in poultry diets. The chapter has been submitted for publication in 'Environmental Science and Pollution Research' on 27th of August 2019.

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RESEARCH ARTICLE

Nanoparticles of selenium as high bioavailable and non-toxic supplement alternatives for broiler chickens

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Abstract
Selenium is commonly used in the poultry industry as an additive in broiler feed to improve immunity and overall health. The selenium comes in different forms, inorganic and organic selenium, as sodium selenite and selenomethionine, respectively. This study proposes the use of nanoparticles of selenium (nanoSe) for improved delivery and absorption of the trace element while causing no toxicity. Previous studies have shown the success in utilizing nanoSe in broiler feed, with increased absorption and diffusion of material into organs and tissues, and increased antioxidant capacity. However, the.mechanismof nanoSe conversion remains illlknown, and the gut microbiota is believed to play a significant role in the process. The use of inorganic selenium in poultry feed demonstrated a lower bioavailability in breast (P < 0.01) and duodenum tissue (P < 0.05), and increased accumulation in organs involved in detoxification processes as compared to organic selenium and selenium nanoparticle supplementation. Histopathological analysis showed that nanoSe did not cause any damaging effects to the tissaes analysed, revealing intact epithelial cells in the digestive system and neuronal bodies in brain tissue. The results indicate that nanoparticles of selenilllll operate a similar way to organic selenium and could potentially be used in poultiy feed as a trace element additive.

Keywords Selenium · Nanoparticle · Poultry· Additives · Histology · Toxicity

Introduction

The presentation of compounds as nano-sized particles can result .in new properties, change the interactions with o1he r materials, and alter uptake and retention in biological systems, compared with presentation of compounds as larger particles or in solution. The novel propeities of nanomateiials have been exploited for a range of applications, particularly in.food (Gangadoo et al. 2016), sensing (Power et al. 2018), the environment (Rajapaksha et al. 201S), materials (Chapman and Regan 2012) and health (Gangadoo and Chapman 2015). Trace elements have also been delivered for nutiitional supplemen1ation using a nanomaterial (< 100 nm), representing a novel and effective method to deliver essential me1als to support immunity and health (Gangadoo et al. 2016).

Nano-sized particles have already demonstrated a number of benefits such as enhanced absorption, bioavailability, antimicrobial activity (Chapman et al. 2013; Chapman and Regan .2012; Chapman et al. 2010; Regan et a.1. 2 012; Sullivan et al. 2012), and excretion of the nano-mateiials (Pan et al 2002; Schiifer-Korting et al. 2007; Shaikh et al. 2009). Nanoparticle delivery of minerals and vitamins has essentially been shown to be effective in improving feed conversion ratio, promote growth and development of muscle cells, improve the gut microbial environment, treat common parasitic disease such as coccidiosis and reduce mortality in poultiy (Gangadoo et al. 2018; Gangadoo et al. 2016).

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Selenium (Se) is an essential micronutrient that is routinely added to the feed of production animals to promote the optimal functioning of the immune system {Surai 2002b). It is currently delivered in two distinct forms, either inorganic (selenite) and organic (selenomethionine) (Surai 2002a). There has also been a recent focus in micronutrient uptake efficiency using nanomaterials (Gangadoo et al. 2016), wh ich has also shown to modulate gut microbiota (Gangadoo et al. 2018), improve immune and musculoskeletal function, and growth performance (Beski et al. 2015; Lin et al. 2015; Wang et al. 2011). The delivery of Se in the form of a nanoparticle is appealing as it does not need to be metabolized before being incorporated into selenoproteins and can be taken up by the body at a much faster rate than inorganic Se (Gangadoo et al. 2016; Suzuki and Ogra 2002).

Se is added in poultzy feed as inorganic Se (0.5 ppm) or organic Se (0.3 ppm), the latter demonstrating improved bioavailability, with multiple studies focusing on using selenium-enriched yeast and wheat as feed additives (Fisinin et al. 2008; Sumi and Fisinin 2014; Utterback et al. 2005). Previous studies showed that nanoSe increased daily weight gain of broilers, and resulted in improvement in antioxidant functions (Cai et al. 2012; Fwtiang et al. 2008). Despite the promising results, some concerns have also been raised, with a few recent reports of unexpected nanoparticle toxicity (Ahmadi and Branch 2012; Ahmadi and Kurdestany 2010; Pinget et al. 2019), some specific to the gut (Ruiz et al. 2017). For example, the use of silver nanoparticles (nanoAg), while effective against aflatoxins in poultry nutrition (Gholami-Ahangaran and Zia-Jahromi 2014), was found to accumulate in the liver and muscle tissues of livestock and therefore, discontinued (Jennifer and Maciej 2013). Histopathology studies have shown that nanoAg can cause lesions in hepatocytes contributing to liver inflammation and necrosis (Loghman et al. 2012). In a previous study, we investigated the ability of nanoSe to control poultry pathogens (Gangadoo et al. 2018). Here we extended the study to investigate nanoparticle tissue bioaccumulation and histopathological toxicology, producing findings that are relevant to both chicken and human consumer health.

The aim of the current study was to examine the tissue distribution of Se supplemented to broiler chicken in the form of inorganic, organic and three different concentrations of nanopmt icles . Trace elements from biological tissues were extracted using a simple, standard, acid digestion at high temperatures and pressures using a microwave oven. The digests were analysed on an ICP-MS instrument, and concentrations as low as parts per billion were determined (Liang et al. 2000). Additional histopathological analysis was carried out to corroborate and to assess the integrity of tissues exposed to the different sources of Se.

Materials and methods

Reagents

Nitric acid (concentrated HNO_3) and hydrogen peroxide (30% (w/W) H_2O_2) were purchased from Sigma, Aldrich, Australia and used without further purification. A high purity standard of selenium (Se), 1000 µg/mL in 2% HNO_3 was purchased from Choice Analytical Pty Ltd., NSW, Australia. A Dogfish Liver Certified Reference Material for Trace Metals and other Constituents (DOLT-5) was purchased from NRC-CNRC. Milli-Q water was used to perform dilutions throughout the digestion protocol.

NanoSe synthesis and characterization

NanoSe was prepared as previously described by Gangadoo et al. (2017); briefly, metal salt, selenium tetrachloride ($SeCl_4$), was chemically reduced with ascorbic acid, and a stabilizing agent, poly (sodium 4-styrenesulfonate), was added to obtain stabilized and monodispersed nanoparticles. NanoSe parameters, -size, shape, crystallinity and presence of elemental selenium, were characterized using UV-vis spectroscopy, dynamic liglit scattering **(DLS),** scanning electron microscopy (SEM), transmission electron microscopy (TEM), x-ray diffraction and energy dispersive spectroscopy (EDS).

Animal trial

The animal trial was conducted as described in Gangadoo et al. (2018). Briefly, 70 one-day-old Ross 308 broiler male chicks (Bond Enterprises, Toowoomba Qld, Australia), were randomly divided among five treatment groups; two control groups: organic Se (Alkosel 3000 inactivated whole cell yeast, selenomethionine, containing elevated levels of organic selenium, min. 98%), inorganic Se (sodium selenite), and nanoSe supplementation given at three different concentrations, 0.3, 0.9 and 1.5 ppm. For the nanoparticle manipulated feed, the nanoSe was homogenized in a poultry remix (Rabar, PTY, LTD) which had no selenium concentrations. The poultry remix had been manufactured to possess no basal selenium content, as determined by the manufacturer (Nestel and Nalubola 2002). To be able to experimentally compare the standard poultry remix containing the standard selenium forms: organic selenium and inorganic selenium (sodium selenite) with concentrations of 133 ppm, versus the synthesized nanoparticle selenium feed system, feeds were homogenized in an Ozito CMX-125 mixer (Ozito Industries Pty. Ltd., Bangholme, Australia) at a revolution of 200 rpm and air-dried for 24 h. This homogenisation method is a standard method for commercial poultry remix formulation where many commercial

manufacturers mix vitamins, trace minerals, medicaments , feed supplements, and diluents (Armstrong and Behnke 1996, Monsalve 2006, Nestel and Nah.tbola 2002). The birds were fed twice daily in a light- and temperature-controlled room and were euthanised at 29 days post-hatch by CO_2 asphyxiation. Tissue samples were taken from the following organs: breast, liver, spleen, duodenum, ileum and brain, and were stored at - 80 °C until analysed.

Tissue preparation

Tissue samples (n =5 per treatment group) were thawed in a fridge overnight and dried at 105 °C for 24 h. The percentage moistme was calculated using the following formula: [1 - ((dry sample weight/wet sample weight) x 100)]. Dried samples of 0.5 g were digested using a TANK PRO Microwave Digester with 6 mL of concentrated HNO_3 and 2 mL of concentrated H_2O_2 using the method shown in Table I. The sample was reconstituted in Milli-Q water prior to analysis on the ICP-MS.

Preparation of standards and quality control

External calibration standards were prepared using the Se standard with a stock solution of 100 ppm, and standards were prepared at the start of each ICP-MS run, at concentrations of 0.05, 0.1, 0.5, 1, 2, 5 and 10 ppm with M illi-Q water. The limit of detection (LOD) and limit of quantitation (LOQ) were evaluated using digestion blanks (n. = 10) consisting of 6 mL of cone. HNO_3, 2 mL of cone. H_2O_2 and 2 mL ofMilli-Q water. Reference materials and blanks were prepared, digested and analysed, along with the diied tissue samples, for quality control. The dogfish liver certified reference material (DOLT-5 CRM) is a tissue standard with known elements and concentrations, provided by the National Research Council Canada, and was used to calculate recoveries of Se from the digestion procedure.

ICP-MS instrument settings

Sample analysis was performed on a Shimadzu ICPMS-2030 instrument, and the method parameters used by the instrument are as described in Table 2.

Table 2 Instrument settings for ICPMS-2030 and measurement par;uneters

Parameter	Setting
Radio frequency power	1200W
Plasma gas	8 L/min
Auxiliary gas	1.l L/min
Carrier gas	0.7 L/min
Nebuliser	Coaxial
Sampling depth	5mm
Spray chamber temperanrre	5 °C
Collision cell gas flow (He)	6 mL/min
Internal standard tube	Mini torch

LOD and LOQ values were calculated as 3 and IO times the standard deviation, respectively, using ten measurements of acidic blank solutions ($HN0_3$ and H_20_2) divided by calibration cmve

Tissue sample histology

For histological analysis, tissue samples (n = 5) of approximately 100--500 mg were collected from organs: breast, liver, .spleen, brain, duodenum and ileum, and washed using phosphate-buffered saline. The tissues were cut at 4 μm and fixed in l0% buffered formalin and stained with haematoxylin and eosin (H&E) dyes, which allows for the differentiation of the nucleus and cytoplasmic components respectively (Cardiff et al. 2014; Wu and Zhou 2013). The histological images were .scanned at the Translational Research Institute Microscopy Core Facility (Bris b an e, Australia) using a .N'ikon Brightfield, Olympus VS120 slide scanner and analysed using Olympus :microscopy software, Olivia.

Statistical analysis

All statistical. tests were conducted using the SPSS Statistics package and the results are reported as mean valu s ± standard error of mean (SEM). Normality tests, Kolmogorov-Smirnova and Shapiro-Wilk, and Levene's Test of Equality of Error Variances were conducted to confirm equal variances and normal distribution across datasets of treatment groups ($P > 0.05$). The differences between the Se supplementation groups were analysed by a one-way analysis of variance (ANOVA), followed by a post hoc Tukey multiple comparison test when a statistically significant ($P < 0.05$) result was observed among the different treatment groups.

Table 1 Digestion protocol settings using the TANK PRO Microwave digester

Step (N)	Temperature (T)/°C	Pressure (psi)	Heating up. time (t)/min	Keep tlrne (t)/mln
1	150	400	8	3
2	180	400	3	l:0

Animal ethics statement

Animal ethic approvals were obtained from th e Animal Btbics Co mm ittee at Cen tral Quecns h'md Un iversity witb the approvaJ numb er A JS/07-333.

Results

NanoSe synthesis & characterization

T he synthesized nanoSe we.re: an average of 45 ::r: 0.17 om and demonstrated a polydis j,e csjty index value of 0.04= 0.01 nm, indi cating the uanopartic les were well dj persed and djd not induc e aggregation . As sbowo in Fig. I , the E 'DS analy is confirmed the presence ofseleoium, while TEM & SEM con-filmed the sbape and size ofoaoopruticles to be spherical and less tbao I 00 run respectively. The size di h'ibutioo of the nanoSe was farther supp01ted from ilddit iortal aoaly i with DLS, showing the size ofnanoSe to be elow 100 nm

Se concentration varied among treatment groups and different types of tissues

A linear calibration curve (R^2 = 0.9999) was obtained from the extenJal Sc calibr(ltion standards Se, with a LOD of269 ppb and a LOQ of7.81 ppb. The DOLT-5CRM Se concentration obtain-ed from tbe digestion procedttrc was 8.49 ± 0.38 ppm, showing a l02% Se recove.iy . 11 Je concentration of Se, fl-om the different supple.meutatioosources varied, as did the coo centrntioo of Se in the di.ffc1 nt tissue types, as sbown by Fig. 2.

The highest average concentration of Se within all treatment group was fo1md in the spleen. followed by duodenum, brain. ilc11m, liv e r a nd breast (sp lcen>dtmden nm>brai n>ileum > livcr>breast).[oorgarucSe supplemeotatioo exhibitedfhe highest Se concentrations in the sp leen, liver and brain. aod the lowest concentration in duodmrnm, ileum and brea t (plceo> live r>br ain>duod ermrn>ik:nm>brc ast). Orgaruc Se demonstrat-ed bigher Se couoentratioos in sp lee o. duodenum and ileum, showing higher Se absorptioo in the gastrointestinal tract while lower Se concentration were folllld in the bra.in, liver and brea.5t (spleen>ileuro.>du odcnum>brain> ljver>brea t). NanoSe tipplc-mentation sbowed compamblc results witb orgaoic Se, whb 0.9 ppm u,anoSe treatment producing the best result, with bjgber coocentrations in tbe sp.le en, duodenum and ileum , and lower concentrations in brnm. liver and breast tissues. Additiona lly. 0.9 ppm nanoSe showed improved absorption in the duodenum, and lower retention in the brain tissue , tban the low and high nanoSe concentration, confirming that there was no indicati on of a dose-responseeffect in the tissue concentrations produced by the different supplementation levels of nanoSe.

The difference in the Se concentration were -not significant. among treatrnent group s in the brain *(P* = 0.05 l). ljver *(P* = 0.182), pleen (t'=0.449) and ile um *(P=0.092)* but differed iguificanlty in breast (P = 0.01) and duodenum (P=0.013), with high er concentrations observed in birds supple men ted with uauoSe. A simila r trend was obse1ve d in tbe ilea! ti sue. witb a higher Sc content observed with nanoSethan inorganic Se. The re was an opposing trend observed with the liver, spleen and brain tissues with inorganic Se displaying the highest S content

Histopathological effect of Se sources

A histological exa mination was pe rfonned oo the tis ucs ob-tajned from the fiv e treatment groups. **AU** Se treatmen t groups showed normal his tologica l tructure of breas t ti ss ue .

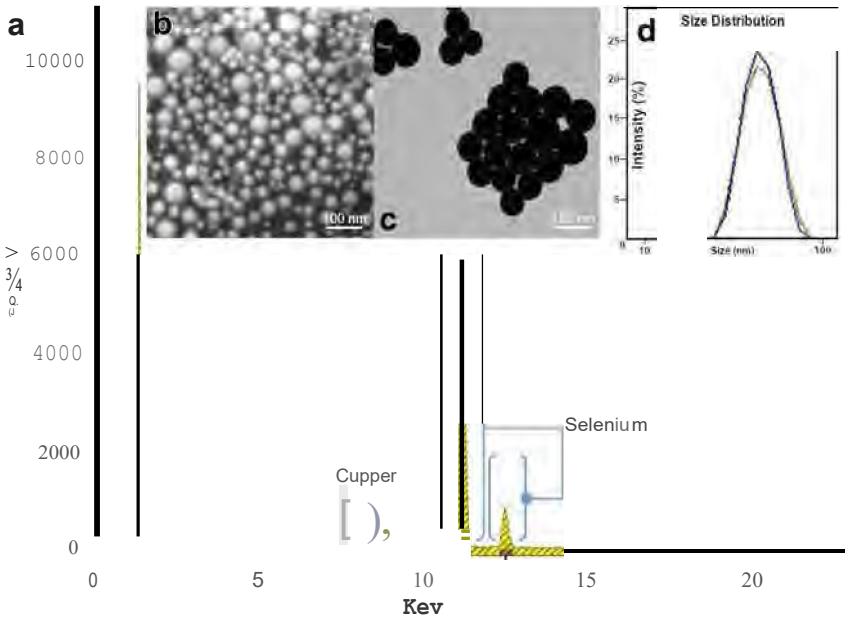
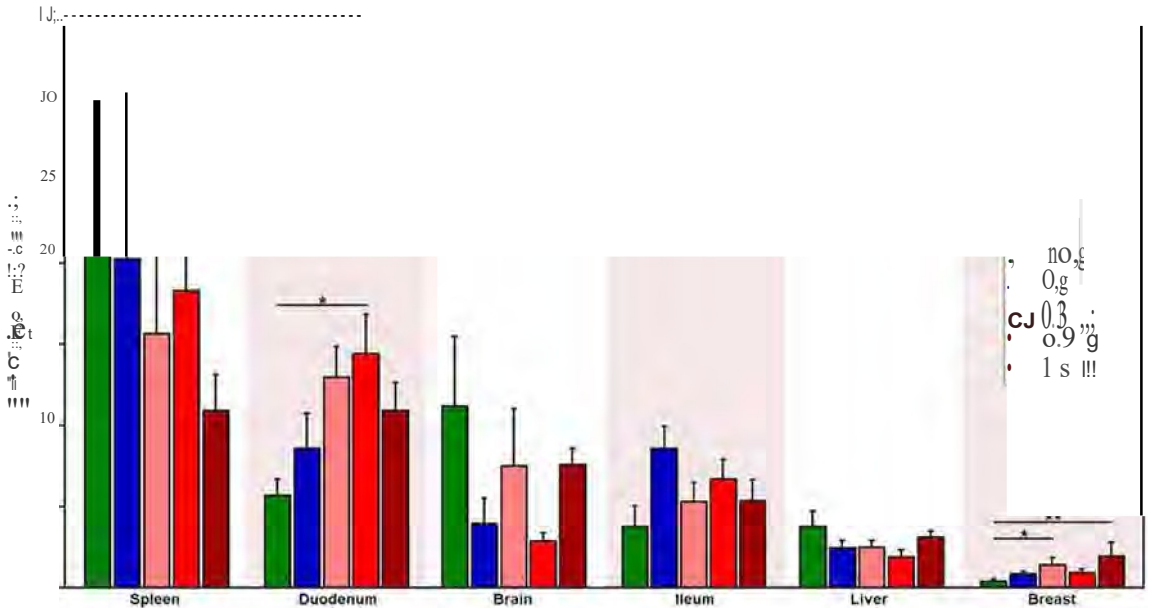


Fig. 1 NanoSe charncteriw tion obtained from ;:i: EDS; b: SEM; **c:** 'l'P.M; **d:** OLS



f ig. 2 Se concerilrnti\>n among treillmenL groups in difterent tissue types

d moustrate d by the relatively tmifo1m diameter oftbe cross-cut myofib1il la r bundles and intcrmyofibrillar paces of th muscle fibres. The integ1ity of the muscle fibres was demo n-strated by the well-defined cross-striation, as shown in Fig. 3(a) . and preserved nuclei of the myofibtil (b). The cross-stliation of myofibrils was less expressed in inorganic Se than the other Se groups. There was no pathological dam- age observed in brain samples with neurnnal bodies between the new-oglia and blood vessels of brain substance shown to be normally dishibuted in all Se treatment groups. Figure "" c) shows basopbilically stained Nissl bodies /tigroid clustered mainly in the ,u-ea around the oucleus/per ikaryon and io the periphery of the cell. An i nitia l to moderate degree of fat infiln·ation in liver epithelial cell was ob crvcd in liver tiss ue.

1--fopatocyte aod Kupffor cell picttJJ"ed in Fig. 4 (a) and (b). respectivel y, showed no damag in any of tbe Si;: h·catrncnt group . Spleen samples across all Sc treatment groups sbowed normal structural characteristics, with minor to mod crate amounts of e1y throblasts 0b$ervcd io the spleen pulp as pic-tmcd in f'ig . 4 (c).

The re wru; no histopathological damage observed m either the duodenal or ileal tissue across any of the five Se treat-ments. The in testina l villi of the mucosa in the duodeoum were covered by a monolayer prismatic epithelium. Single goblet cells (Fig. 5(b)) were located between the no1mal in-testinal cover ceUs. The epithelial cells covering the c typts showed preserved microvilli (Fig. 5(a)). **The** intestinal C1ypts of the ileum are shown in fig. 5(c), with the villi evenly covered by a monolayer prismatic epithelium and a common density of goblet cells observed. The ileum tissue also showed dense ly located, mucous -assoc iated, accumu lations of lym-phoid cells forming Peyer' s patches, pi·tured in rig. S(d).

Discussion

Selenium is an important micronut1ient.aud is u constituent of the immlmC . ystem (Rayman 2000). Selenium nanopart icles were synthesi7,ed u ing a typica I b o ttom -u p approach, allowing the conh·ol and fonnation of stable and defined c1ys - tals, and delivered to a chicken model to improve the uptake and absorption rate ofth trace clement. The model used was a broiler chicken model with a rationale to investigate whether nanoSe supplementation would induce toxicity and/or alter bioavailability. compared to the two commonly used Se sup-plementation products by the poultty industry (organic and ioorganic selenium). Previous studies utilizing nanoSe for im-provement of poultry's health and performance only investi-gated cooceott·ation levels of the oanoparticle rather than its parameters such as size. surface charge and crystallinity. The oanoparticle size was an average of80 nm and studies showed levels exceeding 2.0 ppm resulted in a decline in the immune system (Gangadoo et al. 2016). This ttial included the com-mon concentration ofScused in poultty iodll5tiy, 0.3 ppm as a minimum le vel and a maximum concentration level of J .5 ppm. However, there was no con-ela tion observed witb tbe concent:1·ation of uanoSe and effect presented in tlli study, indicating fi.rrfl1er studies with a wider range of concen-tration le vels to b beneficia l.

The bistopatbological aoalysi of the ampled tissues indi-cated that there were no damaging cftects from any of t he Se sources, however, significant differences in Se concentration betv,een the nanoSe and inorganic Se treated birds were ob-served. The tissues from birds with nanoSe supplementation displayed h.igher Se concentration in breast and intes tina l tis-sues than the birds treated with ino:rganic Se , indicating its

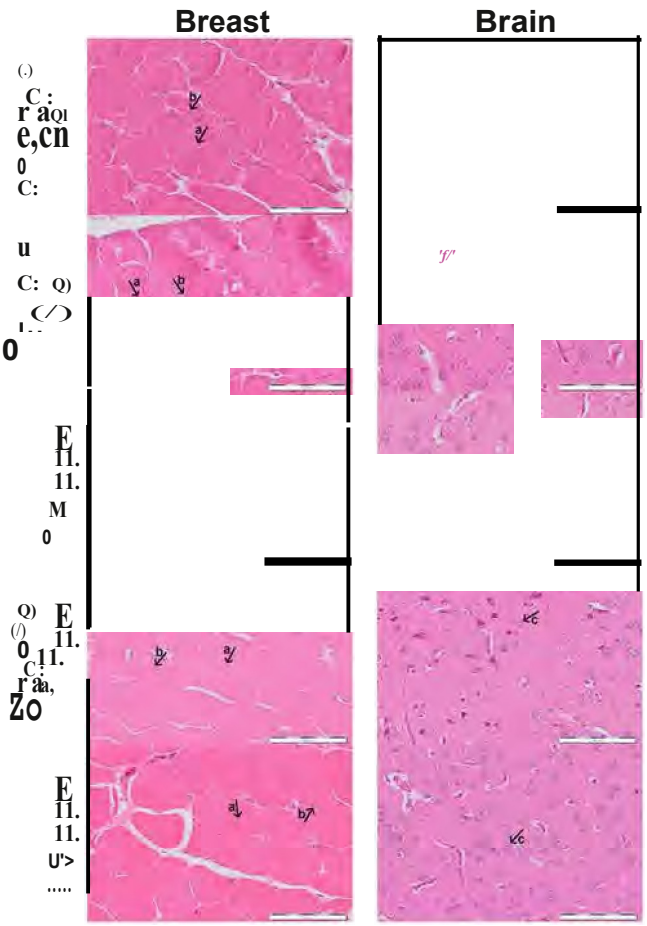


Fig. 3 Hi to logic al a ess ruen t of 55 Se treatmen t on breast ti sue showing myofib rils **(a)** and cell nuclei **(b);** and b1ain with Nissl bodi s (c)

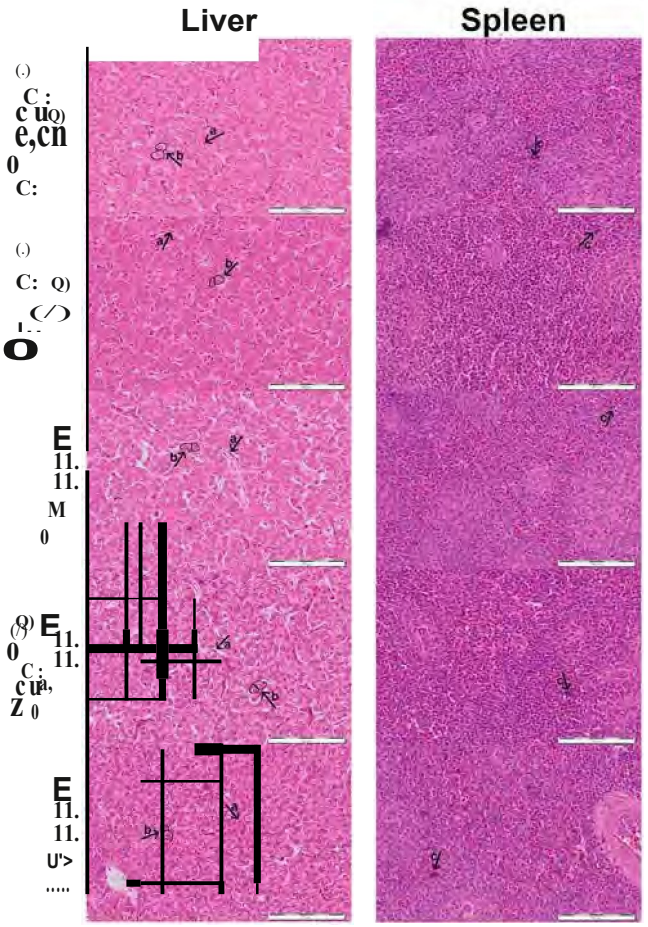


Fig. 4 Histologica l asses ment of Se treatment on liver tissue showing hep atocytes (a) an.d Kupffer cells **(b);** . pleen tissue with niinor to modemte amounls of erylhroblasts **(c)**

supe1ior bioavailabiLity and suggesting an active transpo1t mechanism similar to organic Se (Shi et al. 2011). Se species differ within the treatment groups, affecting its bioavailability and absorption. Inorganic Se combines with other food components during digestion and form insoluble complexes. reducing its abso1ption. while the Se form in organic Se undergoes amino-acid uptake mechanisms in the intestine, increasing its transportation across the intestinal wall (Con taotinescu-An1x andei et al. 2018; Mab.ima et al. 2012), and this bas been observed from greater bioavailability of organic Se in broi le 1 and egg-laying hens from organic Se than inorganic Se (Dobrzanski et al. 200 3 ; Jiakui and Xiaolong 2004; Payne and Southern 2005). NanoSe is consequently elemental S and its transportation aero s tbe biological body is determined by its physicochemical prop rtie , including size and shape (Cons tantine scu -A ruxandci et al. 2018). This study demonstrated the higher bioavailability of nanoSe obtained through a spherical and non-c1ystalline morphology. with an average size of46 run.

An opposing trend was observed in li ver and spleen tissue. with the birds from the inorganic Se treatment group showing higher Se concentrations. wh1le nanoSe and organic Se showed comparable results. The liver *is* a major organ of Se accumulation (Smai 2002a), where selenides, from inorganic Se sources, are incorporated into seleno-proteins before being distributed throughout the body (Pilarczyk et al. 2011· Suzuki 2005). The spleen is the largest lymphoid tissue and is important in regulating immune functions around the body (Attia et al. 2010; Chen et al. 2014). This suggests that inorganic Se contributes to higher Se retention and accumulation in organ involved witb detoxificatjon proce se .

Al.though our data indicates tbat tbere is no Se toxicity occuning in the tissues and no diJ"ect damaging effects on tbe intcstinal morphology, more tmcicity stu dies s ucb as irnmunogen.icity, cytotoxicity, NP accum ula tion and excretion kinetics, would be required p1-ior to engaging in nanoSe supplementation to li vestock ou an industrial scale. The determination of selenium concentrations in tissue samp les and histological analysis of multiple broiler tissues showed nanoparticles of selenium to be non-toxic, while exhibiting higher absorption in intestines and a lower retention in tissues involved in detoxification as compared to selenium additives

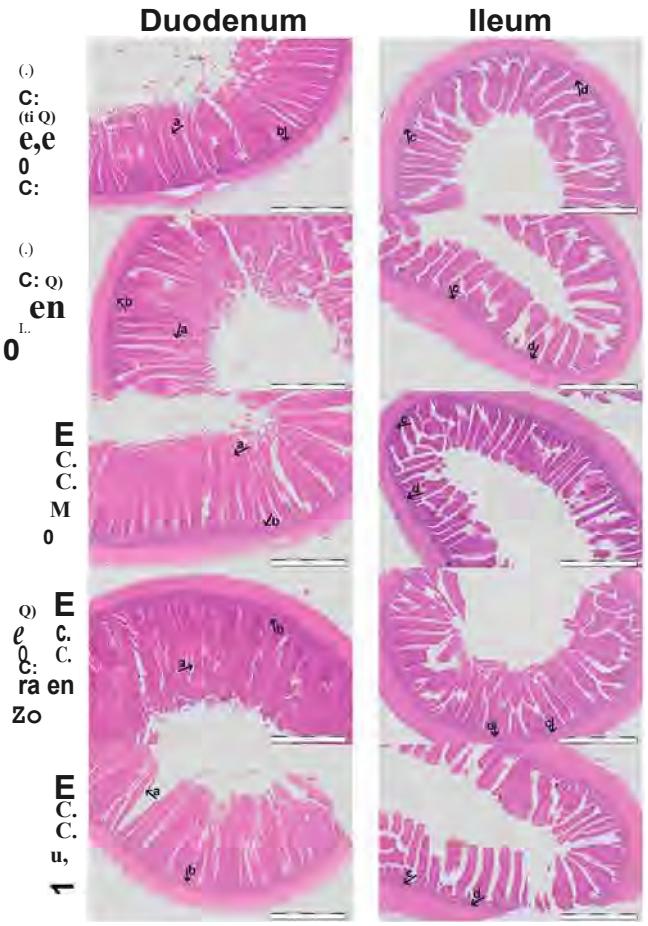


Fig. S Hi tological assessment of Se trea tment on duodenum with microvi ll i **(a)** and goblet celJs **(b);** and ileum snowing inrestioal crypts /c) a.nd acl,1.unulation of lymp hoid ce lJs forn1ing Peye r's patches (dJ

c ommonly used in the pouJtty industry. such as sodium sele - nite and selenomet rnone (sele nium yeast).

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Compliance with ethical standards

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

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Nature of Candidate's Contribution, including percentage of total

SG was involved in the inception of the paper, data collection, statistical analysis, and writing of this scientific article (70%).

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

All co-authors assisted with the conception and revision of the article. BBW (5%); SYB (5%); TTHV (5%); RJM (5%); OS (10%).

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

Chapter 5

In vitro growth of gut microbiota with selenium nanoparticles

Chapter 5 investigates the influence of Se NPs on caecum microbiota in an anaerobic environment, following the animal trial conducted in Chapter 2. Intestinal samples were approved for collection from backyard growers and subjected to *in vitro* growth in an anaerobic environment. Following the extraction of DNA and analysis of SCFAs, using 16s rRNA gene sequencing and GC-MS respectively, Results show Se NPs had moderate effect on the gut microbiota and SCFAs, with the ability to significantly ($P < 0.05$) reduce *Enterococcus cecorum*, an emerging poultry pathogen. This chapter has been published in Animal Nutrition, with an impact factor of 1.368.

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Original Research Article

In vitro growth of gut microbiota with selenium nanoparticles

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ABSTRACT

The application of nanoparticles rose steeply in the last decade, where they have become a common ingredient used in processed human food, improving food properties such as shelf life and appearance. Nanoparticles have also attracted considerable jnterest to the livestock industry, due to their efficacy in intestinal pathogen contro l, with the regulatory and consumer driven push for the removal of antibiotic growth promoters. The influence of selenium (Se) nanoparticles was investigated on a diverse and mature broiler caecal microbiota using *in vitro* culturing and 16S rRNA gene sequencing methods for microbiota characterisation. Caecal microbiota was collected from 4 traditionally grown heritage roosters and grown for 48 h, in the presence and absence of Se nanoparticles, with 2 technical replicates each. The effect of rooster as a biological variable strongly overpowered the effects of nano-Se in the media, resulting . in moderate effects on the structure and diversity of the caecal microbial community. However the nanoparticles showed a significant reduction ($P < 0.05$) in the abundance of an emerging poultry pathogen, *Enterococcus cecorom* identical operational taxonomic units (OTU), which could be of notable interest in poultry production for targeted *E. cecorom* control without significant disturbance ro the total microbial community.

1. Introduction

The application of nano-scaled materials, 1 to 100 nm, has rapidly expanded across various disciplines, including but not limited to, electronics, technology, consumer goods, biomedical science, agriculture and microbiology. They display unique physicochemical properties due to high surface energy and increased surface area to volume ratio (Regan et a l., 2012). Nanoparticles are used in everyday applications such as self-cleaning surfaces (Mue lle r and Nowack, 2008), topical products (Goyal et al , 2016) and food preservation (Es pitia et al., 2012). They have excellent antimicrobial properties through the disruption of microbial cell membranes (Hajipo ur et al., 2012; Thill et al., 200 6) and oxidation (Le Ouay and Stellacci, 2015). Culture studies have been used to examine their biocidal interaction (J ia et al., 2017; Teodoro et al., 2011) towards both prokaryotic, bacterial pathogens and eukaryotic cells such as tumour and stem cells (Arora et al., 2008; Greulich et al., 2 009; Kaul and Amiji, 2005). They have also been intensively investigated for their use in joint and bone reconstruction therapies (Gangadoo et al., 2015), agricultural products (Gangadoo et al., 2016) and delivery of drugs and other substances to the body (G upt a and Curtis, 2004), as they exhibit high biocompatibility (Lu et al., 2010; Naahidi et al., 2013) and biodegradability (Mahapat ra and Singh , 2011; Panyam and Lab hasetwar, 2003).

Nanoparticles can be used as vehicles to transport substances to the body effectively and fast, by avoiding complex pathways and defence mechanisms as compared to their bulk counterparts (Desai e t al., 19 96; Mohanraj and Chen, 2006). Many studies have shown

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the positive effect of NP formulations delivered to the gut microbiota, focussing primarily on reducing the pathogenic load with an antibiotic based approach, by inhibiting the growth of harmful microbes (Karavolo s and ll olban, 2016). Since various materials have successfully targ eted detrimental microbes through NP delivery, it was proposed that a NP-based system, using metal salts and complex, could also be used to enhance beneficial bacteria by delivering essential nutrients to the gut microbiota. Nanoparticles have been used to increase the abundance of beneficial species such as *Lactobacillus* and *Bifidobacteria* and reduce pathogenic bacteria and coliform counts (Gangadoo et al., 2018 ; Han et al., 2 010 ; Yausheva t al., 2o-t8). The gut ecology is a complex community and it is necessary to consider the complex interactions of multiple bacterial species, the chemistry of their growth environment and the metabolites produced.

Selenium (Se) is an important trace element required by the body for the proper functioning and development of the immune system, and it is routinely supplemented to poultry rations to prevent detrimental effects of Se deficiency in birds (Gangado o et al., 2 0 16). About one quarter of the gut microbiome has the ability to express selenoproteins and Se availability in microbiological media affects their expression (Kasaikina et al., 2011). These proteins play an important role in both bacteria and mammalian host where they are essential in numerous bodily functions (la blɪnskyy et al'., 20 14).We have previously investigated the ability of seleniumnanoparticles (nanoSe) to improve the delivery of Se to birds and have characterised the resulting modifications of the intestinal micrnbiota (Gangadoo et al., **2018).** We found increased abundance of some beneficial bacteria, **for** example *Lactobaclllus* sp. and *Faecalibacterium prausnitzii,* however, without significant pathogen reduction. The quantity of butyric acid in different gut sections was increased. Butyric acid is a primary energy source for intestinal colonocytes and can promote good gut health (Va.n lm merseel et al., 2017).

Traditionally raised free range chickens generally show higher diversity of intestinal microbiota compared to intensively reared birds (Chen et al , 2 008 ; Cui et al , 2ffl 7). Their microbiota may contain bacterial species that are not commonly encountered in the microbiota of birds raised in modern high-density production systems. We are interested to study such novel microbiotas and determine the effects of feed additives; with the expectation that some novel members of the microbiota may have application in modern chicken production (e.g. novel probiotics). However, it is difficult to obtain sufficient numbers of such traditionally raised birds to carry out statistically powerful *in* vivo studies. Here we present an in vitro study that has investigated the effect of nanoSe on in *vitro* cultured chicken, caecal microbial communities. We used a growth medium specifically developed to support a range of unknown and uncultured species as well as more routinely cultured bacteria, to culture caecal material from ·tr aditionally raised free range chickens.

2. Materials and methods

2J. *Animal ethics*

Collection of chicken caecal and intestinal material from backyard growers was approved by the Animal Ethics Committee at Central Queensland University with the approval number A1409-318. All animal ethics procedures were in agreement with the Australian Animal Welfare Standards and Guidelines.

2.2. Media preparation

LYHBHI medium (Brain-heart infusion medium supplemented with yeast extract [5 g/L, Alfa Aesar], cellobiose [1 g/L, BD], hemin [5 mg/L, BD], cysteine [0.5 g/L, Alfa Aesar]) and resazurin (0.5 mg/L, Alfa Aesar)] (Fenn et al., 20 17; Zl1an g et al., 20 14) was enriched with a multivitamin mix (1 mL), trace element mix (1 mL), feed extract (100 g/L) and bacterial ferment (100 mL). The multivitamin mix was prepared with 5 capsules of SO + MULTI Vitamins & Minerals (CENOVIS) and 5 capsules of vitamin K (Caruso's Natural Health, Queensland, Australia), dissolved in SO mL of Milli-Qwater, and the resulting mixture was filtered twice through a 0.45-µm and a 0.2-µm syringe filter. Appen dix Table 1 shows the resulting vitamin concentrations in the medium. The trace element mix was obtained from Youngevity (California, USA) and contains plant-derived minerals. The feed extract was prepared by mixing 100 g of poultry feed (Red Hen Chick premium micro starter, Lauke Mills, Daveystone SA, Australia) to 1 L of Milli-Q water using a 1,500 W blender (Nutri Ninja Duo Auto-iQ) and left to soak overnight. The mixture was then autoclaved, centrifuged and 100 mL of the supernatant extract was added to the medium. The bacterial ferment was prepared by aerobically growing cultures of *Lactobadllus plantarum* (ATCC BAA-793) and *Lactobadllus Thamnosus* {ATCC 53103) to mid-stationary phase in LYHBHI media. The supernatant of the bacterial ferments were then mixed at a 1:1 ratio with a final volume of SO ml, filter sterilised and added to the media. The volume of water was adjusted to ensure that the original LYHBHI medium was not diluted. The enriched LYHBHI was purged for 30 min prior to inoculation of caecal content with anaerobic gas mix (80%N_2 /10%$C0_2$/10%H_2 BOC, Queensland, Australia).

NanoSe was prepared as previously described (angadoo et al., 2017). Briefly, selenium tetrachloride was reduced with ascorbic acid to Se atoms , to which a protecting agent, polystyrene-4-sulfonate, was added to allow the formation of nanoparticle clusters. The synthesised, dark red solution was washed by multiple centrifugation with Milli-Q water and a full characterisation, including size, shape, morphology and crystallinity, was conducted. The nanoSe was then diluted with Milli-Q water to 0.9 mg/kg.

2.3. Cecum starter cultures

Caeca, from 4 roosters, were donated by a local heritage breeder. The roosters were raised with organic feed without antibiotics and had exclusive outdoor access, including overnight outdoor roosting, which provided intensive contact with wild flora and fauna. The whole intestine of each rooster was removed and placed immediately into an anaerobic gas pack (Cat. #260683, BD GasPak EZ Pouch Systems) and stored at - 20 °C. The caecal samples were slowly allowed to defrost at 4 °C for 30 min. The contents of the whole ceca, for each rooster separately, was squeezed out and diluted in SO mL of enriched LYHBHI media with 15% glycerol **in** an anaerobic **work** station '(A35, Whitley, Shipley, UL). The caecal starter cultures for each rooster's caecal content were then aliquoted as 50 x 1 mL stock and stored at -80 °C until the start of the experiment. This would eliminate cold sensitive species and allow t,he reproducible use of each 1 mL stock for the future in vitro experiments.

2.4. In vitro *growth cultures*

On the day of the experiment, a single glycerol stock for each one of the 4 roosters was thawed and inoculated into SO mL of enriched LYHBHI media to grow parent cultures for the experimental inoculation. The experimental cultures were prepared in 20 mL of media in SO mL Erlenmeyer flask with a cotton stopper, allowing for gas exchange, and incubated at 37 °C on a digital orbital shaker (Heathrow Scientific), shaking at a speed of0.21 xg in an anaerobic hood (Whitley A35 Anaerobic Workstation, UK)

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running on a nitrogen rich gas mix (80 % N2/10% C0 2/10% H2). Four cultures were prepared from each rooster's caecal content. 2 as control and 2 with 0.9 mg/kg of nanoSe, by inoculating late exponential growing parent,11 culture to achieve a starting culture 00620 of O.l. Thus. the final experiment was performed on 16 cultu res; 11 - 8 for contr-ol and the nanoSe treatment each, on 4 biological re plicates (rooster's caecal content) and 2 technic,11 replicates each, as shown in Pig. 1. Sampling of the cultures was done at 24 and 48 h and the samples were centrifuged at 18.500 *xg* at 4 QC for 10 min . The pellets and the supemata □ts were used for microbial and metabolite analysis, respectively.

2.5. DNA extraction

DNA was extracted from the centrifuged pellets of the mic robial cultures. The lysis step was based on the method suggested by Yu and Morris on (2004), fo\lowed by a DNA purification step . The lysis buffer (0.5 m,L) and 0.1 g of sterile zirconia beads (Cat. #11079101, BioSpec Products) were added prior to bead -beati ng (Mi□i- beadbeater , 13io5pec Products) for 5 min . Following a 15 - mi□ incubation at 85 •c, the samples were centrifuged for 5 min , and binding buffer (0.8 mL) was added to the supernatant and placed through DNA Silica Memb rane Mini Spin Column (Cat. # 1920 -250, Epoch Life Science, I nc.), followed by a tw o-ste p washing with wash buffer (0.7 mL). The washed and dried column was then eluted with 50 fLL of elution buffer. The composition of the buffers is included in Appendix Tab lr **2.**

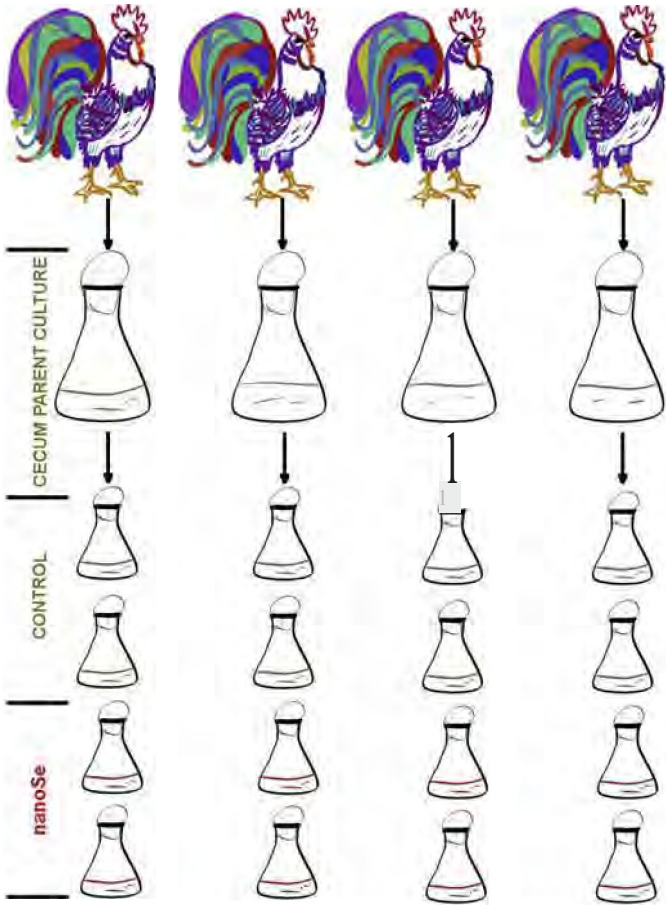

Fig. 1. A sche matic or Lhe in viiro ex perirnell1 perfom ed to examine llle effect of nanoSe on growlh cul tures or rooster caeca sam ples.

2.6. DNA ampli ficatiori and sequencing

Sequencing of 165 rRNA gene DNA am plicons was performed on the lllumina MiSeq platform using 2 *x* 300 bp paired-end sequencing. Prime rs were selected to amplify the V3 - **V4** region of 16S rRNA genes: forward S'- ACTCCTACGGGAGGCAGCAG-3' and revers e 5'-GGACTACHVGGGTWTCTAAT-3'. The primers contained barcodes, spaces and lllumina sequencing linkers as previously described (ad rosh e t al., 20 14). Two samples, one from the rooster 2 and one from the rooster 4, failed the sequencing process , and were thus excluded from the analysis.

2.7, Statistical ana lysi s

The analysis of microbial communities was performed in QII ME v.1.9.1 (Capo ras o ct al., 2010). Paired end seque nces were joined using the Fast q-Jo i n algorithm and no allowed mis match es using only seque nces with Phred quality threshold higher than 20. Operational taxonomic units were picked at 97% similarity using lfclust (J:d gar, 20 10) and ins pected for chimer ic sequences using Pintail (As he lford el JL, 2005). Taxonomic assignments were performed against the GreenGenes (De$ an tis et **al.**. 2006) database and QJI ME default arg um en ts . Further data exploration was done using Calypso (Zakrzews ki et a l., 2016). Tota l sum normalisation and a square root transformation was performed prior to statistical analysls. Student's t-test was used to detec t th e significance of the differences between the groups. An alysis o f similarities (ANOSIM) was pe rfom1 ed using Calypso on we ig hed and unweighed UniFrac distance matrices calc u lated i n QIIME, e.ich with 99.999 permutations. Calypso was also used to implement the supervised multivariate Redundancy Analysis (RDA) using 999 permutations and linear regression a nalys is us - ing Pearson correlation.

The complete annotated sequence dataset is publicly availab le on the MG - RASTdatabase under library ID (Id pend.i ng).

2.8. Short - chain fatty acid ana ly s i s

The supematants from the caecal cultures were diluted **with** 70% et hano l, filtered through a 0.45 pm syr inge filt er (Cat. #5 4504-RC, ThermoF lshe r) and analysed on the Gas Ch romatography - Mass Spectrometry (GCM S) syste m. A st anda rd stock solution (100 mg/kg) was used to construct cal.ibration curves and stored as a method processing paramet er in scan mode for the following short -cha in fatty acids (SCFA). acetic, n- buty ric , isobu tyric, propionic and n-va l eric acid .

The GCMS system used for metabolite analysis was a Shimadzu QP2010-Plus. fitted with a high-polarity column SH- Rxi- SSil- MS (3 0 m x 0.25 mm *x* 0.25 f1m, Restek) and equipped with an AO C- 20i - s autosampler. The GC temperature programme sta r- ted at 100 "C and was held for l min, increased to 12 °C per min to a temperature of 170 °C. and ramp ed at 100 *qc* per min, until a final temperature of 260 °C was reached and held for 1 min (a total of an 8.73 - min progra mme). The GC oven temperature was set as prese nted in Appe ndix 'fa ble 3. A sample of 1 tlL was i njected at 250 °C usi ng he li um (5.0 , Coregas, Austra l ia) as a carri er gas at 1.97 ml/ min in a split injection mode . The press ur e was held at 143.3 kPa, with a total He flow of 1.03.4 ml / mi n and using a split ratio of 5. The mass spectrometer was operated ln th e electron ionisation. mode at 0.2 kV wit h a source temperature of 220 °C where scan mod e was used from 33 to 150 m/z. The peaks were ident ified by mat ching the mass spectra with th e Nat1ona l I nstitut e of Standards and Techno logy (NIST) library, htt p:// ch emd, lt ;i. mst.gov /.

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3. Results

3.1. Sample origin influences overall microbiota composidon and abundance

The origin of caeca greatly in flue nced the microbiota comm unity of **the** samples, showing great biological variation. The abundance of phyla *Finnia1tes, Bacteroidet es, Proteobacteria* and *Actinobacteria* differed significantly between the 4 roosters (ANOVA, $P < 0.001$). *Lactobacil/us* (>20%) was the dominant genus in all roosters combined, followed by *Streptococcus* (> 15%), Enrerococcus and *Clostridium* (>5%) (A ppend ix Table 4). Principal coordinates analysis (PCoA). as shown in Fig . 2A, was performed on both unweighed and weighed U niFrac matrices and shows similarities between roosters 2 and 3, while roosters one and 4 were very distinctive (Fig. 2B). The miuobiota of roosters 2 and 3 included multiple genera not found in roosters one or 4, such as *ColliJ1Sella. Coprobacillus. Slackia* , ,1nd unclassified families ofBurkholderiales and Ruminococcaceae. Rooster 4 had the most distinctive microbiota; dominated by *C/ ostridium,* with lo wer abundan ce of *Lactobadllus* compared to the other roosters, and higher amounts of *Trichococcus* , Proteus and unclassified families comprising of Clostridiales and Burkholde 1i ales .

32 . NanoSe irifluence 011 microbial composition and metabolite production

The enriched LYHBHI s upport ed tl1e growth of a diverse range of genera comprising of multiple previously uncultured species as shown in A ppe ndix Table 3. NanoSe supplementation significantly- ($P < 0.05$) increased 20 operational taxonomic units (OTU). as shown in Fig. 3 A and reduced 8 OTU ($P < 0.05$), one of which was identified as 100% identical across the amplified region to *Enrero-* coccus *cecorum.* followed by 2 other *Enterotoccus* OTU significantly red uced by nanoSe (Fig. 3 B). *Enteracoccus* OTU, including pathogenic£. *ceconim,* were exclusively reduced by nanoSe while genera *Lactobadl/us* and *Streptococcus* were rea1Tanged with some OTU significantly reduced and other significantly inc reased. Although nanoSe treatment was correlate d with changes in abundance of some specific OTU an ANOSIM mul t ivariate analysis of group similarities showed that the overall gut microbial composition was not affected by nanoSe (P - 0.991) or an add iti onal 24 h of incubation

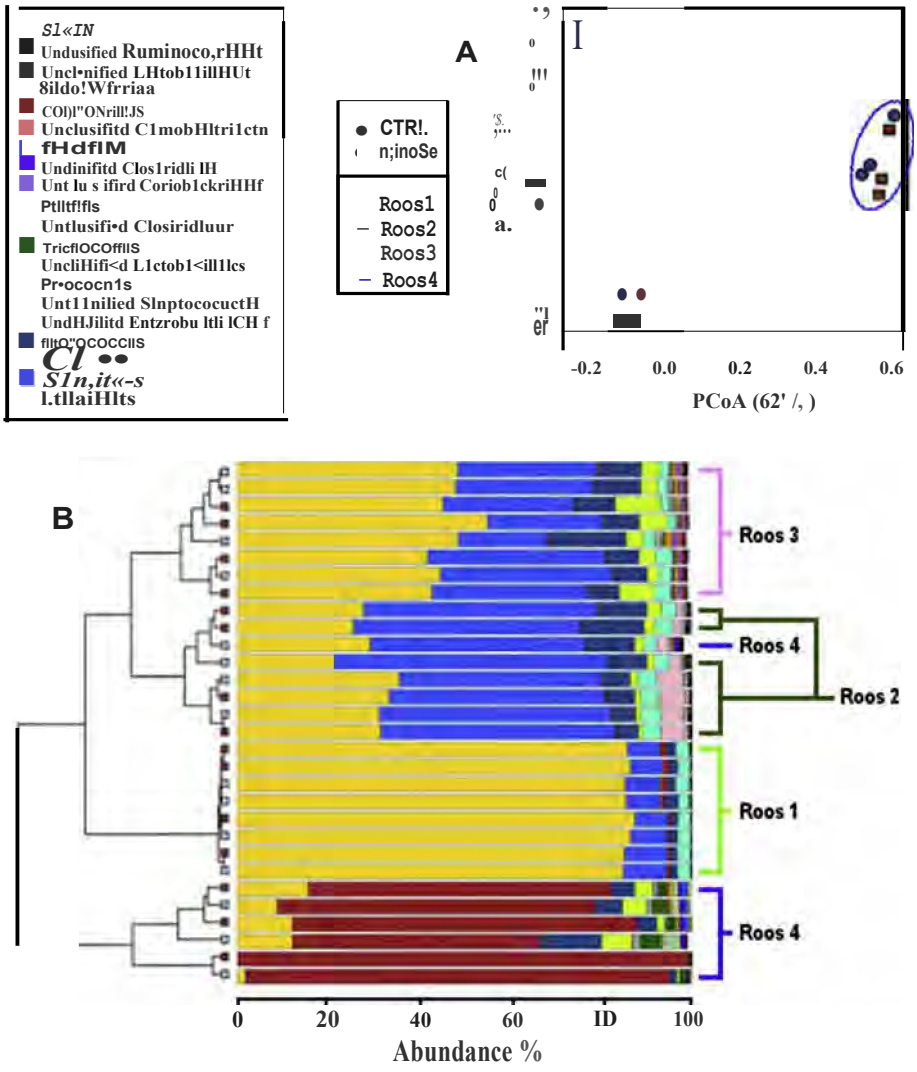

Fig. 2. Gut microbiota profile was clustered based on the bird sample.(A) PCoA analysis performed on weighed Un1Frac matrices shows gut community profiles clustered by bird origin. (B) The multiple genera present in the samples confirm imilarities and differences between rooster's guL communities. PCoA= Prind pal coordinatesanalysis.

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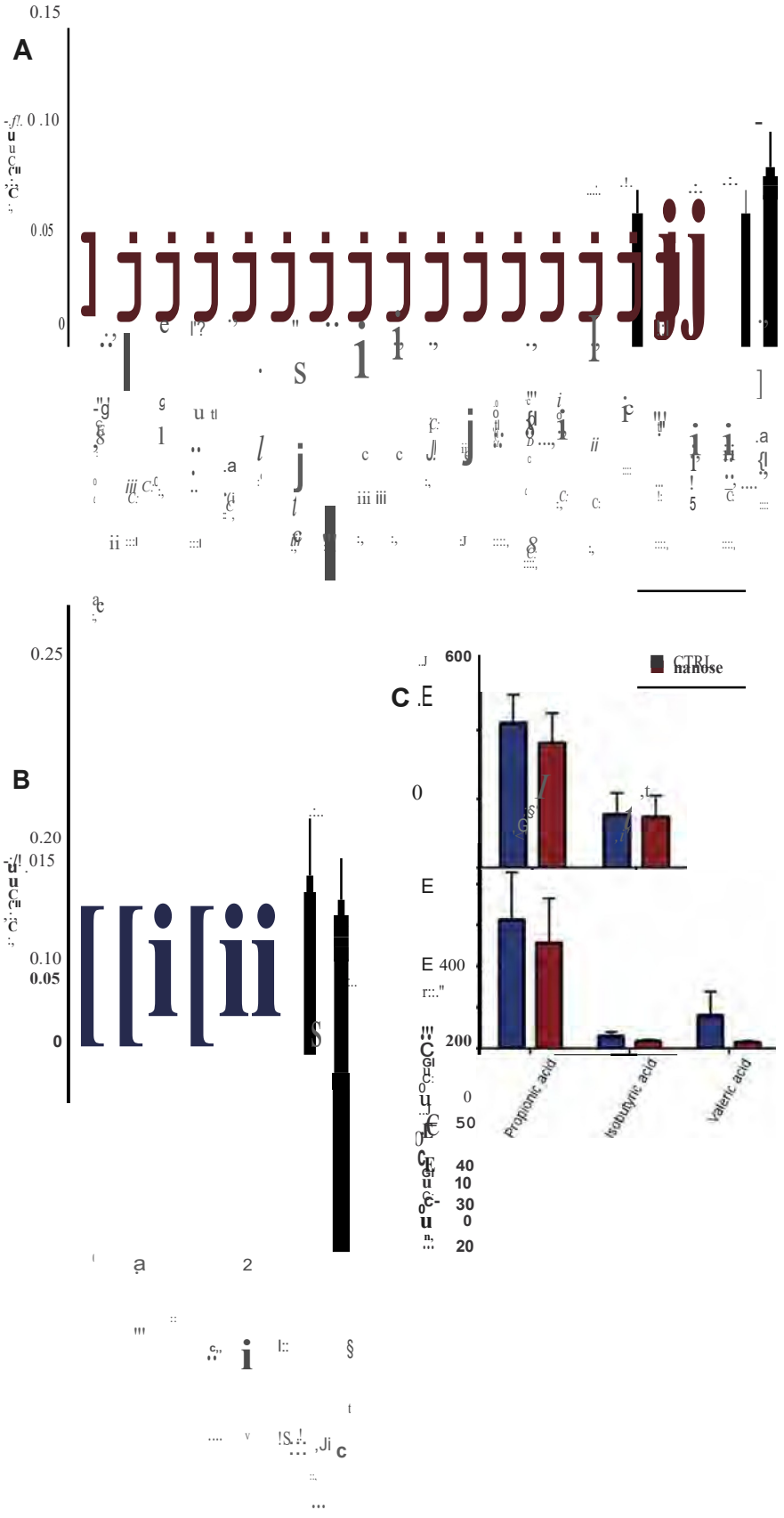
A
0.15
0.10
0.05
0
B
0.25
0.20
0.15
0.10
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0
C
600
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0
CTRL
nanose
Propionic acid
Isobutyric acid
Valeric acid
50
40
10
30
0
20

C: e

c ::,

Fig, J. NanoSe in Ouence on the gut m1crobiora of roosters at **OrU** level and the suhs equcn t effe ct on SCFI\ production.(/\) Increased and **(JI)** decreased OTU from seve rdi unclassified

genera and fam flie s. (C) SCFI\ production was not significantly affected by the addition of nanoSc in in vitro cul ture s. OUT = operational taxonomic uni ts: SCFA = shon-,h in fany

a.dcls : Un. = unclass ifie d.

(P - 0.55). furthermore, alpha diversity indices (Shannon's index. ridmess and evenness) were also not affected by nanoSe or time of

incubation ($P > 0.05$). The supplementation of nanoSe had no effect (using r-test) on SCFA production, as shown in 1-ig. 3 C

PERMANOVA showed that SCFA production was significantly

3 -3. Interaction *between gut* community and *sha rt-ch ain fatty add*

related to the microbiota composition ($P = 0.00067$) and RDA demonstrated that the microbial composition was significantly

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related to the SCFA ($P < 0.01$).The 5 SCFA. acetic acid, butyricacid.

isobutyric acid (IBA). propanoic acid and valeric acid correlated

with the abundance of a number of taxa (FJg , 4 A). Valerie acid and IBA strongly correlated ($P < 0.001$; $R > 0.85$) with the same

genera, including *Adlercreutzia, Desulfavibrio, Microbacterium,* unclassffied Bam es ie llaceae , unclassified Helicobacteraceae and

unclassified WPS2, (Fig. 48). Butyric acid and acetic acid shared one genus. an unclassified Clostridiales, exhibiting a. strong cor-

positive correlation ($P < 0.001$; $R = 0.86$) with *Clostridi'um* and an relation ($P < 0.001$: $R > 0.85$). Butyric acid additionally had a

inverse correlation ($P < 0.001$: R - - 0.9 0) with an unclassified Streptococcaceae.

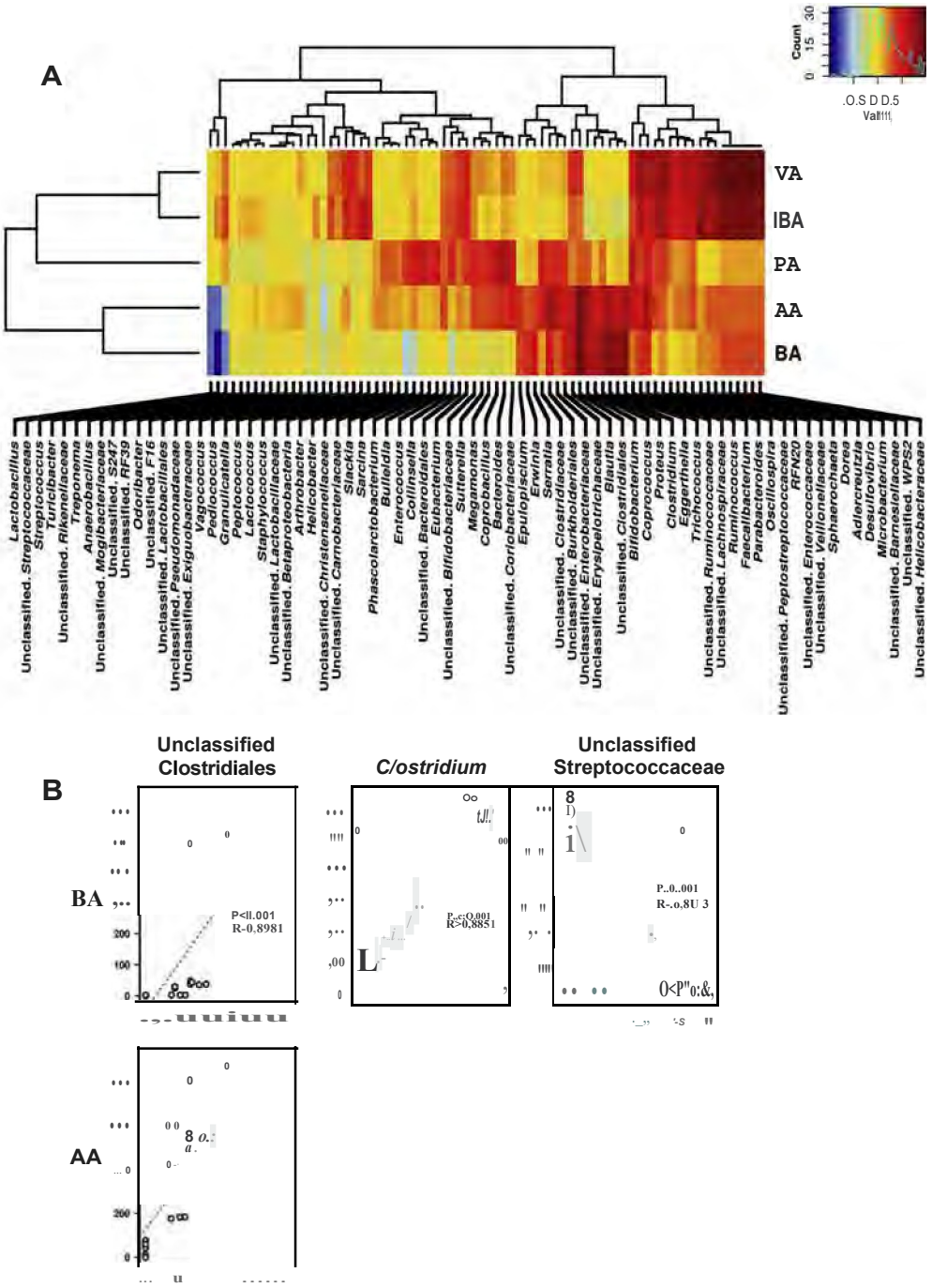

Fig. 4. Short-chain fatty acid1 (SC F/1) interaction effect with the microbial community. (/1) overall interaction effect of SCF/1 Wit h genera yielded by enriched I.YHBH l media (Brain-heart infusion medium supplemented With yeast extract [5 g/J /IJfa Aesar l, ce l\ob iose [1 g/L SDI. he min [5 mg/I, BDJ. cy steine 1 0.S g, fl Alfa Aes arl) and resa7.Urin (0.5 mgfl, /1\fa /lesar)I. (BJ Highest-con·e\ation (R > 0.85) of SCF/1 wtth generated IJac teii a. M = .icetic acid; BA = butyric acid ; PA = propanoic acid; !BA = isobutyric acid; VA = vale ric acid.

4. Discussion

The human and animal microbiota is continuously altered with different lifestyles and environ.mental changes, and has undergone major rearrangements since the introduction of industrialised, large-scale food production in the last few ce ntu ries (l' land roy et al., 2018). This change in eating habits and the subsequent changes in gut microbiota has led to the modern age being described as an age of "microbiota genocide" (Sonnenburg et al.. 2016). The lifestyle and eating habits of hunter-gatherer societies are. very different to that of modern western societies and the difference drive profound changes when comparing ancient and modern human microbiotas (Dave n port et JI.. 2017: !<umar s1nd Forster, 2017: Warinner et al., 2015). This effect spills over to livestock and birds with characteristics microbiota changes occurring because of altered husbandry and feeding practices. Industrial scale grown birds experience very different growth conditions compared to their ancestors: the eggs are hatched under rughly clean cond iti ons, removing the influence of parental microbiota passage to the next generation (Don aldson et al.. 2017). This results in aberrant microbiotas and high microbiota variation from one batch of hatchlings to another (Stan le y et al.. 2013). Microbiota analyses of chicken caeca across various

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projects have displayed an enormous discrepancy between bacterial species present in industrial birds and those present in birds grown in traditional low density open housing ways such as that found with "village chickens" or "backyard chickens" as called in Australia. Here we used the caeca of backyard chickens to investigate the effects of Se nanoparticles (nanoSe) on gut microbiota. The gut microbiota of an industrially grown domestic chicken, *Gallus gal/us domesticus,* is typically comprised of 4 main phyla; Firmicutes, Bacteroidetes, Proteobacteria and a low amount of Actinobacteria (Oakley et al., 2014 ; Wai te and Taylor, 2014; Wei et aJ,, 2013). The high number of unclassified genera, presented in this study, is possibly indicative of the influence of non-industrialised housing and other environmental conditions (Kers et aL, 2018), such as access to pasture, live plant and insect food content, full free range, and exposure to wild birds and animals.

Culturable genera, *Lactobad l/us, Streptococcus, Clostridium* and *Enterococcus* strongly dominated (>60%) the rooster's caecal community, while numerous uncultured genera remained in low abundance. The 80% to 90% sequence similarities render it impossible to infer function, pathogenicity or probiotic potential of these unidentified species to known cultured bacteria. The unknown and uncultured species often require metabolic feedback from other bacteria and can be cultured only in a complex community rather than as a single cult ure. The caecal microbiota communities were more diverse and different to the ones previously investigated with live birds treated with different concentrations of nanoSe (C-angado od al, 2018), and consequently the *in vitro* response of cultured caecal microbiota to nanoSe proved dissimilar to that seen in the microbiota of treated birds, including the lack of SCFA and *Lactobadllus* genus stimulation. It is not clear whether the different test systems or the different starting microbiotas have more pronounced influence in producing the different outcomes.

In contrast, the reduction of an emerging avian pathogen, *E. cecorum,* and 2 unknown enterococcus species, was observed with nanoSe at a concentration as low as 1 mg/kg. *E. cecorum* has been linked to enterococcal spondylitis and femoral head necrosis, resulting in symptoms such as hind limb weakness (Bo rst et .ii., 2017; Do lka et al., 2016) and lameness in poultry (McNamee and Smy th, 2000). Other symptoms observed include arthritis and spinal lesions (Da lka et al. , 20 17), with *E. cecorum* infection leading to a marked increase in flock mortality among all poultry types. Additionally, the ability to carry and spread antimicrobial resistance among other *Enterococcus* spp. has been observed from an analysis of retail meat samples (J un g e t al., 2 0'18). It can be deduced from this current study that nanoSe may exert targeted antimic ro bial activity against pathogenic bacteria such as £. cecorum within the complex environment of caecal microbiota without causing significant alteration to the rest of the community. Further investigations should focus on the mechanisms by which the nanoparticles may inhibit the growth of various pathogens.

5. Conclusion

The data presented in this study suggests an immense untapped potential for microbiota manipulation in unconventionally grown birds and could reveal useful information for future attempts in standardising the microbiome of industrial poultry. The application of nanoparticles, with careful optimisation, could help uncover a range of unknown bacterial species and their role in the expression of beneficial microbial products. Nanoparticles have rapidly emerged in the food and agricultural industry, and it is of vital importance to understand their gut microbiome interaction, while modifying their properties to our best advantage.

Conflict of interest

We declare. that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix. Supplementary data

Supplementary data to this article can be found online at https://d oi.org/10.10 16 /j.aninu2 019.06.004.

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Chapter 6
Future Work & Directions

In this final chapter, a quick summary of the Chapters 1 to 5 will be provided alongside a discussion for future work and directions, focusing on methodology improvement and certain limitations encountered during the animal trial study, *in vivo* experiment and techniques and instrumentation involved in conducting Se NP toxicity in a biological body.

6.1 General Summary, Strengths & Discussion

This thesis investigated the use of Se NPs as poultry additive to modify the gastrointestinal microbial ecology in order to improve the health and performance of broiler chickens. The NPs were synthesised using a simple chemical reduction method, using protecting and reducing agents to achieve maximal colloid stability [1, 2], and underwent a full characterisation process, identifying its physicochemical features. The Se NPs were compared against two most commonly used Se additives in the poultry industry. The main results revealed the ability of NP to rearrange the entire gut microbiota and influence the production of metabolites, while showing no comparable toxicity. Se NPs were further investigated *in vitro* in caecum samples, and results showed its ability in reducing the abundance of common poultry pathogen. The following sections summarise the findings of this thesis and discuss the implication and limitation of the findings.

6.1.1 Chapter 1

The first chapter reviewed the current use of NP supplementation and its main limitations within the fast-growing poultry agricultural sector, with most studies focusing on NP's direct effect on broiler's health, immune and performance status. Performance status of birds are typically assessed through various means, including feed conversion ratio (FCR), body weight gain (BWG), antioxidant and immunocompetence activities, and the reduction of poultry and zoonotic human pathogens [3, 4]. The review discussed the rise of antimicrobial resistance of poultry pathogens from the heavy use of antibiotics in feed supplementation, and the crucial need for alternatives to reduce harmful bacteria, leading to the conception to modulate the gut microbial ecology to improve the health and performance status of broiler chickens [5]. The gastrointestinal tract of broilers houses a complex microbial ecology, one which greatly contributes to its overall health and immunity, as well as its ability to absorb energy from food and resist pathogen invasion [6]. The bulk Se currently used in the agricultural industry, while efficient at improving the immune system of broilers, encounters problems in digestibility and absorption through the rapid breakdown of the component [7, 8]. NP generates multiple benefits, operating differently from its bulk counterpart, such as higher surface area to volume ratio and adaptable surface affinity. The transportation and permeability of NP across mucosal layers and other defensive

barriers in the body can be governed by modifying the surface charge and particle size, affecting its absorption and retention across the body [9, 10]. The main objective of this chapter was to explore the lack of research area within the poultry industry in regards to gut modulation and performance via nanoparticle feed formulations, and the literature review has comprehensively summarised the findings of the multitude forms of nanoparticle used in the poultry industry to enhance performance and growth. Furthermore, the review not only assessed the need for advanced molecular techniques to be utilised in this research area, but additionally underline the importance of using an essential metal and optimising the nanoparticle metal to be stable, non-toxic, the ability to permeate and transport into cells and finally the capacity to increase the abundance of beneficial gut-modulating microbes, improving overall performance and growth development of host.

6.1.2 Chapter 2

Chapter 2 explored the synthesis of Se NP using a simple bottom-up chemical reduction over a top-down approach which employs the physical breakdown of larger particles into smaller particles, and a bottom-up chemical approach demonstrates a simplicity in controlling the growth of nanostructures and the ability to produce NP with fewer surface defects [11]. A precursor, Se tetrachloride, is reduced and underwent the Ostwald ripening process resulting in a seed template, enabling the homogeneous growth of Se crystals [12, 13]. The use of a protecting agent and the control of reaction kinetics, such as temperature, were successfully employed in limiting particle size to remain under 100 nm and to induce colloidal stability [14]. The synthesis and washing processes were also optimised, producing monodisperse NPs, with a full characterisation conducted using various spectroscopy and microscopy techniques. Dynamic light scattering (DLS), along with scanning electron microscopy (SEM) and transmission electron microscopy confirmed an average size of 55 nm, with NP adopting a spherical shape. The combination of UV-vis and elemental dispersive X-ray spectroscopy confirmed the presence of elemental Se, with X-ray diffraction used to authenticate the amorphous crystallinity of the NP. The synthesis process involved an easy and fast method in producing stable and spherical Se NPs of an average of 46 nm and were further used in an animal trial, as discussed in the next section.

6.1.3 Chapter 3

Se NP effect on broiler chickens was investigated against sodium selenite and selenomethionine, standard Se additives used in the poultry industry. The results in chapter 3 showcased some interesting findings: whilst there was no significant effect observed with overall weight gain of broiler, NP inclusion remodelled the entire gut microbiota composition. An optimised threshold was observed with NP concentration, where higher concentrations exhibited negative effects such as reduced weight gain, introduction of detrimental microbes and reduction of SCFA production. NP also promoted the abundance of health-promoting bacteria, such as *Faecalibacterium prausnitzii,* where a strong correlation was observed with its abundance and concentration of Se NP. *Faecalibacterium* is highly sought after in gut microbiology research as it is linked to promoting high colonic health and production of healthy metabolites [15]. A largest duodenal villus/crypt ratio coincided with highest abundance of *Faecalibacterium*, indicating NP in promoting high digestive capability of small intestine [15, 16]. The overall concentration of SCFA was increased with an intermediate concentration, 0.9 ppm, of Se NPs, showing the concentration to be optimal for the immune system, the maintenance of tight injections and promotion of gut integrity. This study clearly demonstrates the superior efficacy of NP to transport and deliver substances fast whilst avoiding complex pathways and degradability. This is shown by the high promotion and abundance of healthy gut bacteria along positive modulation of the intestine's absorption and digestive capability, as compared to their bulk counterpart in the poultry industry [17]. The results clearly demonstrate the use of an essential metal NP as a solution to the bioaccumulative and toxic effects observed from silver (Ag) NP used in livestock feed and previous studies [18].

6.1.4 Chapter 4

The main objective of chapter 4 was to investigate the toxicity of Se NP in various tissues of broiler chickens. Se concentration was analysed using a closed-vessel digestion method, followed by quantitative analysis using inductively coupled plasma mass spectrometry. The ICP-MS is a strong analytical technique capable in detecting multiple elements at low concentration [19]. Results showed Se NP increased bioavailability of Se in breast and duodenum tissue while reducing Se retention in detoxifying organs such as liver and spleen compared to sodium selenite.

The histopathological analysis showed no damaging effects from NP, demonstrating its safe use in the biological body at low concentrations. Again, the use of Se NP and other essential metal NP provides a solution against toxicity observed from Ag NP in previous studies showing the presence of lesion formation, tissue inflammation and necrosis [20].

6.1.5 Chapter 5

Chapter 5 investigated the ability of Se NP to influence a diverse and mature broiler caecal microbiota *in vitro,* with the intention to act as a replacement for antibiotic growth promoters. The results from Chapter 3 showed the ability of Se NP to increase the abundance of beneficial bacterial species such as *Lactobacillus* sp. and *F. prausnitzii*. Studies show the diversity of intestinal microbiota to be higher in traditionally raised free range chickens than in industrialised birds, hence the *in vitro* trial used Se NP to cultivate and enrich novel species within the caecal microbial communities of the free range birds [21, 22]. The study showed variable results with biological difference of roosters overpowering the effects of Se NP. While the overall microbiota and SCFA production was not affected, Se NP significantly reduced an emerging poultry pathogen, *Enterococcus cecorum.* Results show Se NP enriched a high number of uncultured species, demonstrating the possibility to use nanoparticle as formulation for complex media cultures in isolating and identifying unknown intestinal species. This study, and overall, the thesis, explored the use of advanced molecular techniques in determining the superior advantages of NP formulation over its bulk counterpart to effectively deliver substances and modulate the gut microbiome improving intestinal performance.

6.2 Limitations & Future Directions

6.2.1 Chapter 2

This study mainly investigated the size control and aggregation properties of NP using a bottom-up approach. Future synthesis work should include thorough optimisation of surface charge, as the latter is an equally important parameter that can influence the transportation and uptake of NP in the biological body. As mentioned in chapter 1, the net surface charge can influence its permeability and thus absorption

and retention throughout the body. The use of various precursors, reduction and protective agents should additionally be considered to explore the different transportation and uptake effect of various NP shapes and uniformity, as well as sizes and crystallinity.

6.2.2 Chapter 3

Chapter 3 included two controls of most commonly used Se supplementation in the poultry industry, to compare their effect on gut modulation to those of NP. Further animal studies, including a control with no Se, should be conducted to distinctively isolate the effect of Se NP on the gut composition. Additionally, the feed conversion rate should be compared among broilers fed conventional poultry diets and broilers fed nanoparticle supplementation, to establish whether nanoparticle can improve feed uptake and overall improve performance.

6.2.3 Chapter 4

In Chapter 4, a closed-vessel digestion method was used to digest tissues prior to being analysed on the ICP-MS. This technique reported high volatilisation of Se observed from the slight charring of biological tissue during the drying process. A prolonged time and lower temperature have been previously observed in avoiding this mineralisation process. The rapid volatilisation of Se indicates its fast conversion from one species to another, resulting in differing stabilities and further affecting its biological activity. Further work should include a Se speciation step prior to ICP-MS analysis, and the coupling of methods with hydride generation and laser ablation to enable the separation of interferences from other materials, such as arsenic and chloride. The addition of coupling methods and derivatisation steps will ensure the reduction of instrumental and matrix interferences in biological extracts and may result in the better qualification and quantification of analysed materials. The examination of histopathological toxicity of tissues primarily involved haemotoxylin and eosin dyes. Further studies with other dyes, such as alcian blue and nissl stains, should be included for a more thorough examination of cell features, goblet cells and mucin production, and nervous tissue respectively.

6.2.4 Chapter 5

The high variability in microbial composition of the different roosters is a significant limitation of this study as the effect of NP was not properly grasped. Additional work should involve a higher number of biological replicates, focussing on one type of rooster at a time. This chapter additionally showed the introduction of uncultured bacteria by specialised media and the significant effect of NP on those bacteria. Further metabolomics and genomics studies should be conducted with various other media constituents to identify the relationship between those uncultured bacteria and Se NP supplementation.

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Appendices

Appendix A

Chapter 3: Selenium NPs in poultry feed modify gut microbiota and increase abundance of *Faecalibacterium prausnitzii*

Supplementary material

Applied Microbiology and Biotechnology

Selenium nanoparticles in poultry feed modify gut microbiota and increase abundance of *Faecalibacterium prausnitzii*

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Supplementary Material

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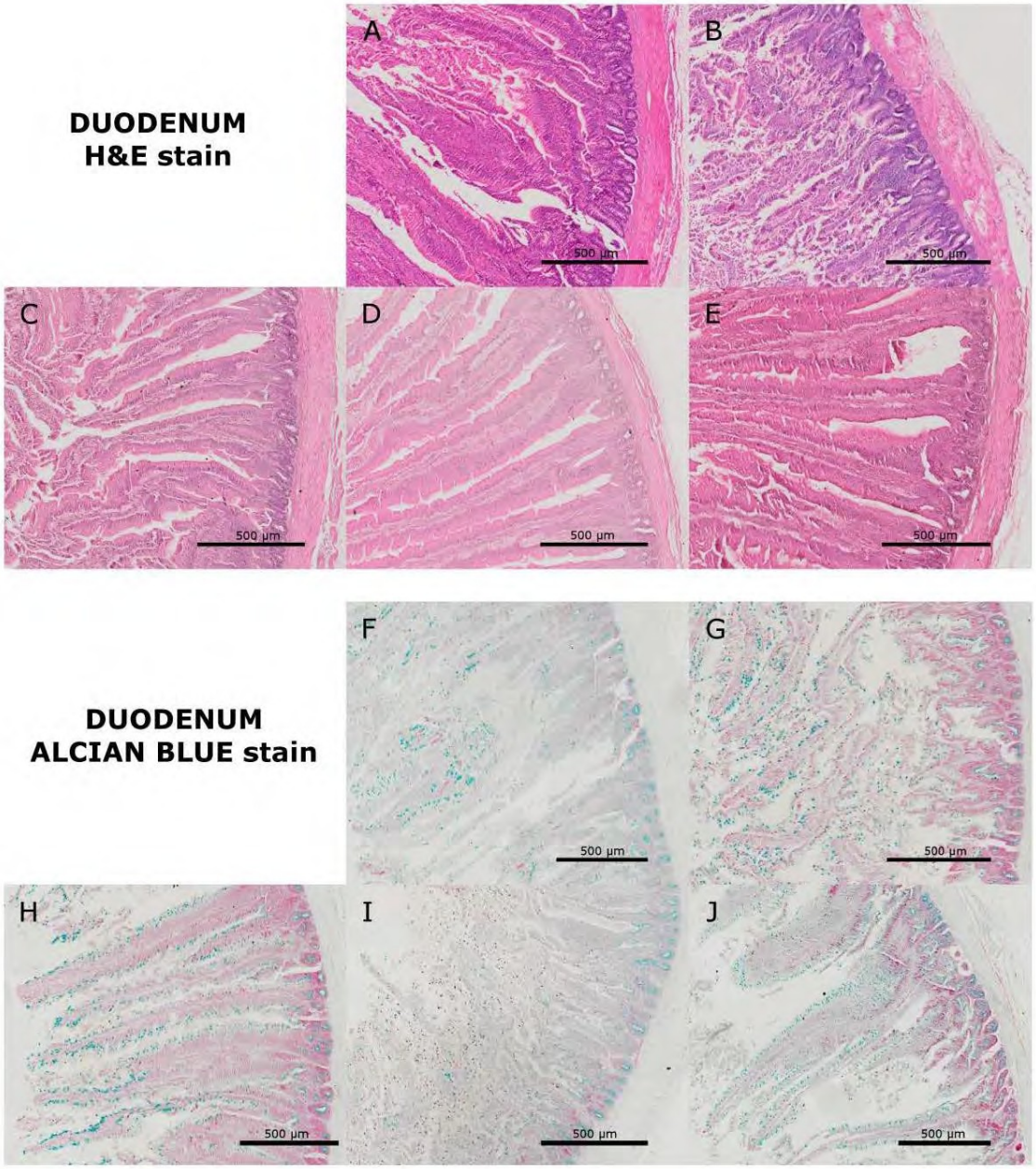

Fig. S1 Duodenum histology of the 5 treatments: **A-E** show haematoxylin & eosin staining of Inorganic Se (**A**), Organic Se (**B**), 0.3 ppm (**C**), 0.9 ppm (**D**) and 1.5 ppm nanoSe (**E**) and **F-J** show alcian blue staining of groups Inorganic Se (**F**), Organic Se (**G**), 0.3 ppm (**H**), 0.9 ppm (**I**) and 1.5 ppm nanoSe (**J**)

Table S1 Feed composition and poultry premix

The chicken starter crumble manufactured by Allora Grain Milling:

Ingredients available	**KG/Tonne**	**% Inclusion**
Corn/Sorghum meal	325.50	32.55%
Wheat ½	279.70	27.97%
Millrun	60.00	6.00%
Cotton seed meal	75.00	7.50%
He soybean meal	175.00	17.50%
Lysine	1.50	0.15%
Ag lime	20.00	2.00%
Salt flossy fine	3.00	0.30%
Bentonite	30.00	3.00%
Dicalcium phosphate	5.00	0.50%
Poultry premix	2.00	0.20%
Agrimol runny molasses	20.00	2.00%
Aniseed flavouring	0.30	0.03%
Clean feed (mould inhibitor)	2.00	0.20%
Dl methionine	1.00	0.10%

The poultry premix manufactured by Rabar PTY LTD consisted of the following nutrients:

FORMULA		**RABAR POULTRY PREMIX**	**CHICK STARTER**
		Inclusion rate kg/tonne	**2.0**
Nutrient	**Level**	**Ingredient to provide nutrient**	**Active level/Inclusion**
Vitamin A	MIU	Vitamin A 1000	13.333
Vitamin D	MIU	Vitamin D3 500 A	3.333
Vitamin E	g	Vitamin E-50 Adsorbate	40.000
Vitamin K	g	Vitamin K3 43.7% Menodione 31.2% Niacinamide	2.667
Nicotinic Acid B3	g	Niacin (B3) 99.5%	40.000
Pantho Acid (B5)	g	D-calpan 98% (B5)	13.333
Folic Acid	g	Vitamin B9 folic acid 100 (97%)	1.600
Riboflavin B2	g	Vitamin B2 80 SD B	5.333
Cyanoc. B12	g	Vitamin B12 10000 (1%)	0.020
Biotin	g	Vit H-2 (Biotin 2%)	0.133
Pyridoxine (B6)	g	Vitamin B6 (Pyridoxine HCl) 99%	5.333
Thiamine B1	g	Vitamin B1 thiamine mono 98%	1.600
Copper	g	Copper sulphate penta	13.333
Cobalt	g	Cobalt sulphate 21%	0.267
Molybdenum	g	Sodium molybdate	0.667
Iodine	g	Potassium iodide 68%	1.333
Iron	g	Iron sulphate powder	40.000
Zinc	g	Zinc sulphate 35%	93.333
Antioxidant	g	Oxicap E2	0.5

Table S2 GC oven temperature for SCFA analysis using a SIM method

Rate	Final Temperature (°C)	Hold Time (min)
-	60.0	1.00
15.00	160.0	0.00
70.00	260.0	0.90

Appendix B

Chapter 5: *In vitro* growth of gut microbiota with selenium nanoparticles

Supplementary material

1 Appendix

2 Appendix Table 1. The resulting concentrations of the vitamin mix supplemented to the
3 enriched LYHBHI media.

Vitamins	Concentration，μg/mL
Calcium (carbonate)	21
Riboflavin (vitamin B_2)	0.4
Thiamine nitrate (vitamin B_1)	0.4
Cyanocobalamin (vitamin B_{12})	0.005
Pyridoxine hydrochloride (vitamin B_6)	0.8
Nicotinamide (vitamin B_3)	4
Calcium pantothenate (vitamin B_5)	2.2
Zinc (oxide)	1.5
Ascorbic acid	4.5
Cod-liver soil	17.5
Magnesium (oxide heavy)	1.5
Total vitamin A	0.0593
dl-alpha-tocopherol (vitamin E 20 IU)	1.82
Iron (ferrous fumarate)	0.5
Folic acid	0.03
Betacarotene	0.12
Cholecalciferol (vitamin D_3 154 IU)	0.000385
Citrous bioflavonoids extract	0.2
Biotin (vitamin H)	0.015
Phytonadione (vitamin K_1)	0.0015
Iodine (potassium iodide)	0.015
Copper (cupric sulfate anhydrous)	0.1
Chromium (picolinate)	0.0025
Manganese (sulfate monohydrate)	0.1
Selenium (selenomethionine)	0.0025
Boron (boric acid)	0.3
Menaquinone-7 (MK7)	9

Appendix Table 2. Preparation of buffer reagents as used in the DNA extraction protocol of culture samples.

Buffers	Ingredients
Lysis	500 mmol/L NaCl, 50 mmol/L EDTA (Alfa Aesar), 50 mmol/L tris-HCl (pH = 8) (G-Biosciences), 4% SDS
Binding	5 mol/L Gu-HCl (Astral Scientific), 30% isopropanol
Wash	10 mmol/L tris-HCl, 80% ethanol (pH=7.5)
Elution	10 mmol/L tris-HCl

EDTA = ethylenediaminetetraacetic acid.

Appendix Table 3. Gas chromatography oven temperature.

Rate	Temperature, °C	Hold time, min
–	100.0	1.0
12.0	170.0	0.0
100.0	260.0	1.0

10 Appendix Table 4. Culturable bacterial genera grown with enriched LYHBHI medium[1].

Classified genus			
Adlercreutzia	<1%	*Megamonas*	<1%
Anaerobacillus	<1%	*Microbacterium*	<1%
Arthrobacter	<1%	*Odoribacter*	<1%
Bacteroides	<1%	*Oscillospira*	<1%
Bifidobacterium	>1%	*Parabacteroides*	<1%
Blautia	<1%	*Pediococcus*	>1%
Bulleidia	<1%	*Peptococcus*	<1%
Clostridium	>5%	*Phascolarctobacterium*	<1%
Collinsella	<1%	*Proteus*	>1%
Coprobacillus	<1%	*RFN20*	<1%
Coprococcus	<1%	*Ruminococcus*	<1%
Desulfovibrio	<1%	*Sarcina*	<1%
Dorea	<1%	*Serratia*	<1%
Eggerthella	<1%	*Slackia*	<1%
Enterococcus	>5%	*Sphaerochaeta*	<1%
Epulopiscium	<1%	*Staphylococcus*	<1%
Erwinia	<1%	*Streptococcus*	>15%
Eubacterium	<1%	*Sutterella*	<1%
Faecalibacterium	<1%	*Treponema*	<1%
Granulicatella	<1%	*Trichococcus*	>1%
Helicobacter	<1%	*Turicibacter*	<1%
Lactobacillus	>20%	*Vagococcus*	<1%
Lactococcus	<1%		

11 [1] LYHBHI medium, Brain-heart infusion medium supplemented with yeast extract (5 g/L,
12 Alfa Aesar), cellobiose (1 g/L, BD), hemin (5 mg/L, BD), cysteine (0.5 g/L, Alfa Aesar).