



Alternatives to antibiotics in the layer industry

Final Project Report | May 2023

A report for Australian Eggs Limited

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ISBN: 978-1-920835-73-6

Project Title: Alternatives to antibiotics in the layer industry

Australian Eggs Limited Project Number: 1I001

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Published in May 2023

Foreword

This project was undertaken to review the literature with respect to alternatives to antibiotic use in the layer industry to allow an assessment of the ability of such alternatives to replace antibiotics.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Acknowledgments

Australian Eggs Limited provided the funds that supported this project.

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Abbreviations

3HP	3-Hydroxypropionic Acid
ACTH	Adrenocorticotropin
ACTN1	Alpha Actinin-1
AECL	Australian Egg Corporation Limited
AGPs	Antimicrobial Growth Promoters
AMPs	Antimicrobial Peptides
AMR	Antimicrobial Resistance
APEC	Avian Pathogenic <i>Escherichia. coli</i>
APVMA	Australian Pesticides and Veterinary Medicines Authority
ATPV1A	V-type proton ATPase Catalytic Subunit A
AXOS	Arabinoxylan-Oligosaccharides
BW.	Bodyweight
CADN	Cecropin A (1-11)-D(12-37)-Asn
CAMA	Cecropin A (1-8)-Magainin 2(1-12)
CYP	Cytochrome P450
EOs	Essential Oils
EU	European Union
FCR	Feed Conversion Ratio
FOS	Fructo-Oligosaccharide
GIT	Gastrointestinal Tract
H ₂ S	Hydrogen Sulfide
IBD	Infectious Bursal Disease
IFN	Interferon
IOS	Isomalto-Oligosaccharide
MAPK	Mitogen-Activated Protein Kinase
MBC	Minimum Bactericidal Concentration
MCFA	Medium Chain Fatty acids
MOS	Mannan-Oligosaccharide
NF	Nuclear Factor
NRC	National Research Council
NSP	Non-Starch Polysaccharides
OM	Outer Membrane
ORT	<i>Ornithobacterium rhinotracheale</i>
P4HB	Protein Disulphide Isomerase
PFU	Plate Forming Units
PTS	Propyl Thiosulphinate
PTSO	Propyl Thiosulphinate Oxide
SCFA	Short Chain Fatty Acids
SE	<i>Salmonella</i> Enteritidis
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SLD	Spotty Liver Disease
SRBC	Sheep Red Blood Cells
tBHP	tert-Butyl Hydroperoxide
TLR	Toll-Like Receptor
USDA	United States Department of Agriculture
VCL	Vinculin
VIM	Vimentin
WDR1	WD Repeat-Containing Protein-1
XOS	Xylo-Oligosaccharide Soya

Executive Summary

As part of the Australian Egg Industries antimicrobial stewardship, Scolexia was tasked with undertaking a review of the literature regarding potential alternatives to antibiotic treatment in laying hens and pullets. The literature was searched using bacterial disease terms, laying hens, pullets and 'alternatives to antibiotics'. For the majority of diseases there were only a small number of papers in this class. There were many more papers discussing these diseases in broilers and in particular the replacement of so-called antimicrobial growth promoters. This literature was also consulted. Most classes of alternatives examined have a place in reducing the need for antibiotic treatment but few are suitable in currently available forms as actual replacements for therapeutic antibiotics in the face of a systemic disease outbreak.

Organic acids can have a positive role in prevention and treatment of bacterial diseases. For example a citric acid/dextrose blend was able to ameliorate the negative impact of a challenge with Avian Pathogenic *Escherichia coli* (APEC). Probiotics have shown more variable results but have been shown to decrease the level of pathogenic bacteria in the GIT of naturally challenged hens, as well as lead to increases in production and egg quality. Similarly, prebiotics appear to have more of a role in improving immune response and productivity (for example, a *Saccharomyces cerevisiae* culture increased egg production and improved humoral immune responses during heat stress in Hy-Line White hens) than in actual treatment in the face of a bacterial disease. A combination of prebiotic and probiotic was used in a study where the hens were challenged with *Salmonella* Enteritidis (SE), however, the SE infection of ovarian tissue was higher in the treated hens than the untreated. Other workers did show a reduction in the level of SE in the caeca of hens treated with a combination of probiotic organisms and prebiotic substances.

Of the nutraceutical (phytogenic) compounds, essential oils are of particular interest due to the antibacterial properties shown by some *in vivo* and in practical settings. Some have been shown to decrease overall faecal coliform and faecal *E. coli* counts in laying hens, and treatment of heat stressed hens with essential oils has decreased mortality rates. Male Hy-Line Brown birds were protected from the impact of challenge with APEC by the addition of essential oils to their diets. Other phytogenics such as mushrooms have been fed as part of the diet of laying hens and a modest reduction in *Salmonella* levels was observed.

The use of exogenous enzymes in layer diets had become more widespread and some workers have shown an increase in the proportion of beneficial bacteria such as lactic acid bacteria (LAB) following the feeding of xylanase, although there was no decrease in Enterobacteriaceae observed. The use of phytase has been associated with an increase in fertility in aged broiler breeders. Other workers have reported an increase in the abundance of *Bifidobacterium* and *Lactobacillus* following the use of added exogenous enzymes. As stress hormones have been shown to be growth factors for certain bacteria, the reduction of stress could be considered to be something that may help avoid the need for antibiotic treatment. Resveratrol is a compound that was shown to reduce the negative effects of artificially induced stress in laying birds (e.g. lower production and egg quality) but has yet to be studied in experimentally infected birds.

Bacteriophages have shown promise in infection control for almost a century, and in laying birds have been shown to decrease APEC related disease and production loss in layers. Antimicrobial peptides (AMPs) have been shown to significantly decrease the mortality due to artificial SE challenge in Hy-Line Brown chicks. Bacteriocins have been shown to inhibit the growth and survival of *Brachyspira* in laying hens.

However, overall the responses to alternatives to antibiotics have not been consistent and there are issues with availability in terms of consistency of product, price and availability and regulatory requirements before any of the classes reviewed will be readily available as a replacement for therapeutic antibiotics.

Overall Conclusions

The classes of alternatives to antibiotics that are commercially available in Australia are being largely utilised in the Australian layer industry at the moment, and they primarily act in the area of disease prevention or of allowing the birds to respond better to infection, either by immunomodulation or improved gut and general health. Some classes of phytobiotics such as saponins and essential oils have reported direct antibacterial or anticoccidial effects, and with further research and development may be developed in a form that would allow their use therapeutically in the face of disease outbreaks, thus replacing therapeutic antibiotics. The other classes that could potentially replace therapeutic antibiotics include bacteriophages, antimicrobial peptides including aptamers and bacteriocins. However, there are development and registration challenges that need to be overcome to allow the practical use of these products. The Australian egg industry will need to retain the use of the currently registered antibiotics with nil-egg withholding period for some time into the foreseeable future to treat the increasing level of bacterial diseases associated with free range farming.

1 Introduction

Two important industry issues are to be addressed by this project; firstly the real need for responsible antibiotic use by the industry to help minimise the problem of antimicrobial resistance (AMR). The importance of this issue was summarised by the USDA Agricultural Research Service (Allen 2017) as follows:

The antibiotic resistance problem is caused by the selection and transfer of genes that confer resistance to medically important antibiotics into human pathogens. The acquisition of such resistance genes by pathogens complicates disease treatment, increases health care costs, and increases morbidity and mortality in humans and animals. As antibiotic resistance continues to develop, antibiotics of so-called last resort become even more precious. Reducing or preventing the dissemination of antibiotic resistance genes into human pathogens is currently of high international importance.

Given limited registrations of antibiotics in laying hens in Australia and the overall responsible use by the industry, the Australian egg industry has one of the best antimicrobial resistance profiles of any egg producing country in the world (Veltman et al. 2021; Wilson et al. 2020). There is therefore only a limited scope for improvement in this area, however, this imperative needs to be maintained for the industry to have effective antibiotics available for the health and welfare of poultry when needed and as a responsible approach to preserving the use of highly important antibiotics for use in human health.

The second issue is the perception regarding antibiotic use, and marketing issues introduced by supermarkets etc. Some supermarkets and regulators (Wong 2019) do not understand the distinction between various antibiotics, such as the narrow spectrum gram-positive antibiotics like bacitracin (which were erroneously referred to as 'growth promoters' and may actually decrease the transfer of resistant organisms through the food chain), as well as the ionophore anticoccidial products, which are not used as antibiotics in human medicine and probably do not impact the overall level of antimicrobial resistance (Mathers et al. 2004), and the highly important classes of antibiotics that are used therapeutically in human medicine. As a result the assumption by the majority of consumers is that all antibiotic use in food producing animals will lead to antimicrobial resistance in human pathogens and must be stopped.

The production of antibiotics by organisms in the environment has led to the development of resistance genes, and when bacteria carrying those genes interact with human or livestock bacteria, they are able to transfer those resistance genes. This can occur via various mechanisms and it should be understood that as a generalisation the use of antibiotics will select for those bacteria that are more resistant to the antibiotic. It can be surmised that the major reason for the existence of resistant bacteria in the human population is the use of antibiotics in human medicine. Nevertheless, potential pathways for the transfer of resistance genes from production animals via direct contact or via food exist.

The increase in the proportion of free range hens farmed in Australia has been associated with an increase in various bacterial diseases such as Spotty Liver disease (SLD), Fowl Cholera, Erysipelas, *Ornithobacterium rhinotracheale* (ORT), mycoplasma and other opportunistic upper respiratory pathogens, and thus an increase in the need for therapeutic antibiotic use. (Grimes & Reece 2011; Groves & Underwood 2021; Kurian et al. 2012; Omaleki et al. 2022; Singh et al. 2013; Singh et al. 2014). While by international standards Australian use of antibiotics in commercial layers is low, the movement of larger numbers of birds into free range production systems has been associated with a significant increase in the use of antibiotics. In pullets, *Eimeria* spp. vaccines are used increasingly to control coccidiosis and so the need for ionophore anti-coccidials has been reduced. The authors are currently successfully using a saponin-based product in the place of ionophores in pullet and cockerel

rearing. Vaccination against other major diseases including *Mycoplasma* spp. has reduced the need for prophylactic treatments.

The aim of this review is to examine and summarise the literature on all available 'alternatives' to antibiotic treatment and if possible, to assess the degree to which each treatment is effective and a real potential alternative. Whilst 'treatment' is a term primarily utilised with reference to a response to a bacterial disease event that has occurred already, many of the alternatives discussed will be of more use in prevention or assisting in the control of the severity of the disease. Prophylaxis is defined as the prevention of a specific disease by one or more interventions. Many of the alternatives discussed or listed in this review will fall into this category.

This review will better equip the industry to maintain the availability of therapeutic antibiotics where needed for good animal welfare and demonstrate excellent antimicrobial stewardship. The term antibiotic specifically refers to those substances or their derivatives that are produced by microorganisms and at relatively low concentrations are able to either inhibit the growth of or kill bacteria. Such substances are still known as antibiotics when they are produced synthetically. Antibiotics are part of the broader class of antimicrobials that can inhibit the growth of or kill microorganisms but may be entirely synthetic. The term antibacterial simply refers to a substance that inhibits the growth of or kills bacteria. So antibiotics and some antimicrobials are antibacterial.

2 Materials and methods

2.1 Search results and general observations

A literature search specifically including the terms ‘laying hens’ or pullets and ‘alternatives to antibiotic treatment’ and bacterial or protozoal disease was undertaken. Literature varied in availability depending on the particular disease. For *E. coli* there were a large number of search results, which we have had to further refine and for other conditions there were only one or two papers. We are also including some papers that more broadly focus on poultry, as there are far more papers on broiler production than layer hen and pullet health. The other obvious outcome from the search results is that there are many more publications dealing with alternatives to so called ‘growth promotant’ antibiotics as distinct from therapeutic antibiotics. Nevertheless such data is still important in this evaluation, as it adds to the understanding of the potential mechanism of action and to assessment of the usefulness of the alternatives. In some cases it also helps elucidate likely methods of action that will potentially be of use in reducing the impact of bacterial and protozoal infections in laying hens and pullets.

As the most extensive reviews of ‘alternatives’ have focussed on alternatives to ‘growth promoters’ and poultry reviews have focussed mostly on chicken meat production, this review has utilised some of those reviews for the basic description of mechanisms of action and of the class of alternatives. In particular, the reviews by Gadde et al. (2017) and Papatsiros et al. (2013) have been utilised.

3 Results

3.1 Acids and organic acids

3.1.1 Introduction

Dietary organic acids also known as acidifiers have been considered as potential alternatives to antibiotics, owing to their antibacterial properties demonstrated by the reduced rate of gastrointestinal disease in chickens (Dibner & Buttin 2002). Usage of these acids helps to maintain optimal intestinal pH critical for an efficient proteolytic digestive enzyme activity, as well as a balanced antimicrobial activity, thereby reducing clinical and subclinical infections caused by pathogenic bacteria (Khan & Iqbal 2016; Yadav et al. 2016). There are several types of acidifiers available for use in animal feed. The most widely used are the short chain fatty acids (SCFA), medium chain fatty acids (MCFA), inorganic acids and salts of organic acids (Vinolya et al. 2021).

SCFA are organic acids with one to seven carbons either with a carboxylic group (formic, acetic, propionic, butyric acid), with a hydroxyl group (lactic, malic, tartaric, and citric acid) or with double bonds (fumaric and sorbic acid). The antimicrobial activity of SCFA in the gut depends on its molecular size, its dissociation constant (pKa), pH of gut and specifics of the microorganism (Mroz et al. 2006). Some acid-tolerant, gram-positive beneficial bacteria namely *Lactobacillus* sp. and *Bifidobacterium* sp. can survive at lower pH, and their intracellular potassium can neutralise the acid anions and provide resistance to acidifiers (Russell & Diez-Gonzalez 1998). SCFA can selectively inhibit the growth of gram-negative pathogens.

MCFA are fatty acids with 6–12 carbon length. Lauric acid, capric acid and caprylic acid are well known to have potent antimicrobial activity against a broad spectrum of intestinal gram-positive bacteria (Jackman et al. 2020).

Organic acids are widely distributed in nature as normal constituents of plants or animal tissues and also formed through microbial fermentation of carbohydrates, mainly in the large intestine. They are sometimes found as their sodium, potassium or calcium salts. Most organic acids with specific antimicrobial activity are SCFA (C1–C7,) and they have a pKa- between 3 and 5 (Papatsiros et al. 2013). They can be administered in the feed or drinking water and can be used either individually as organic acids or their salts or as blends of multiple acids or their salts (Huyghebaert et al. 2011). It is important to note that organic acids and other acids can be used to help decrease microbial contamination of feed and water. For example, Gosling et al. (2021) studied the ability of commercial organic acid products to reduce the growth of added *Salmonella* Typhimurium in feed. In general the commercial products showed a dose dependent reduction in log count of *Salmonella*, however, some did not significantly reduce the count compared to the positive control.

3.1.2 Mechanism of action

One of the primary factors contributing to the toxicity of organic acids is their ability to diffuse across cellular membranes when undissociated as opposed to the restricted passage of dissociated protons and anions. Factors affecting the action of organic acids include the pH of the gut environment, the pKa of the acid (see Figure 1 below) and whether the particular acid or combination of acids have been coated to allow for release and activity in the distal gut. Warnecke and Gill (2005) discuss this with respect to the action of organic acids on *E. coli* (see Figure 2), which maintain a cytoplasmic pH (pHi = 7.5) that is most often higher than that of the external media and typically well above the pKa of organic acids (Maurer et al. 2005). Thus the organic acids exist in the dissociated form within the cytoplasm, disrupting the anion pool of the cytoplasm. The resulting increase in internal acidity can affect the integrity of purine bases and result in the denaturing of essential enzymes within the cell, (Roe et al. 2002) both of which negatively impact bacterial viability. In addition, increased anion concentration has been shown to lead to increased transport of potassium ions into the cell, which increases turgor pressure and to maintain homeostasis glutamate is transported out of the cell, disrupting the osmolarity of the cytoplasm, decreasing cell growth potential and viability (McLaggan et al. 1994).

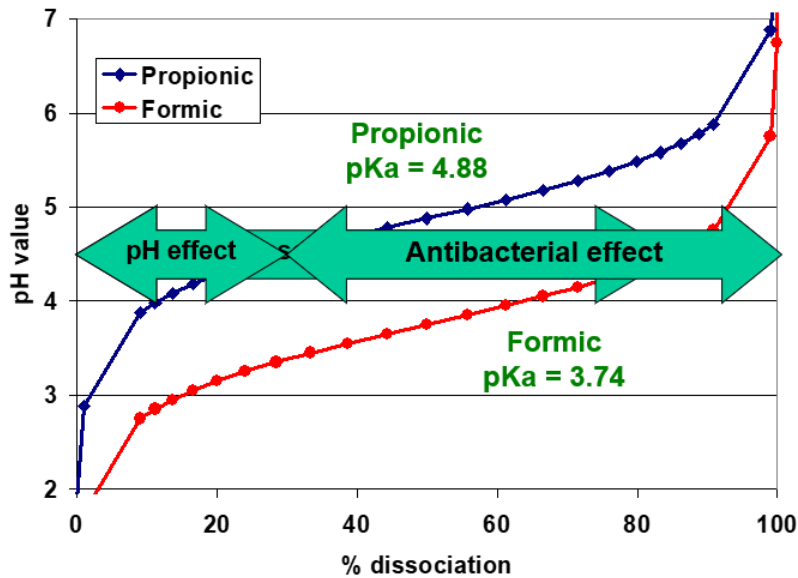


Figure 1 Dissociation of organic acids depending on pH

Taken from Salstop Impretraco presentation 2013.

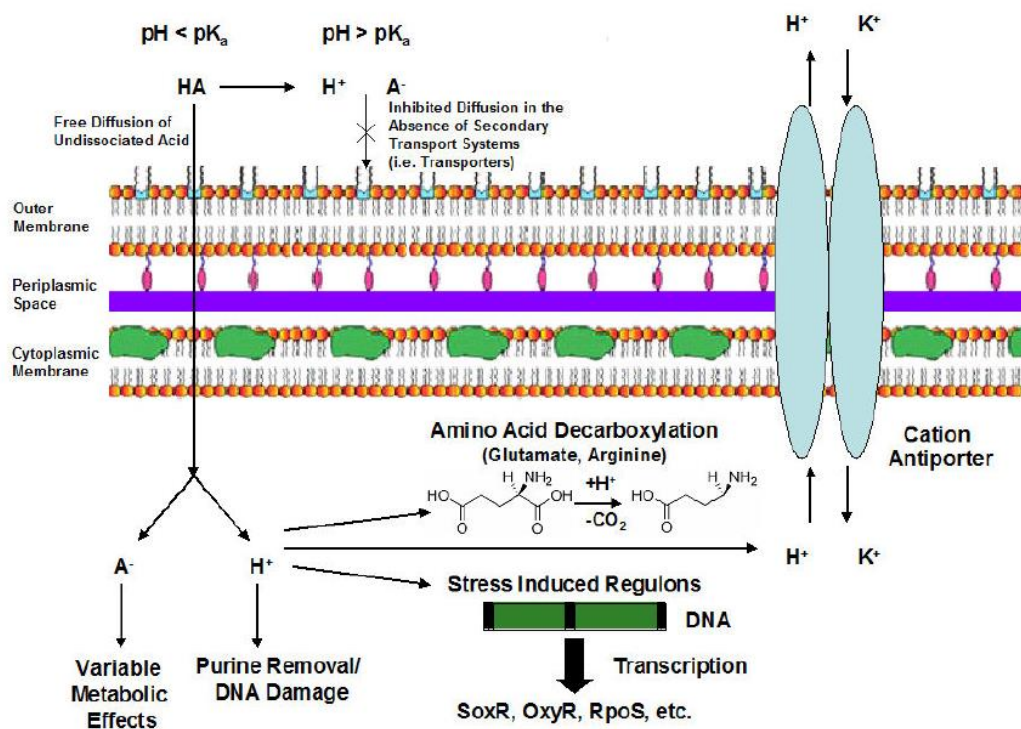


Figure 2 An overview of organic acid toxicity and tolerance mechanisms in *E. coli*

Diffusion of undissociated acid molecules can occur freely in acidic medium but is limited to transport systems at neutral or basic pH. The toxic effects associated with organic acids are the result of both anion specific effects on metabolism as well as increased internal proton concentrations. Effects on internal pH are mitigated by transport of protons out of the membrane, consumption of protons by decarboxylation reactions, and, more generally, induction of stress regulons. Anion specific tolerance mechanisms are not well characterised (taken from Warnecke & Gill 2005).

MCFA's are similar in length and structure to the phospholipids that make up bacterial cell walls. They have a head and a tail, which are hydrophilic and hydrophobic, allowing them to slip through the

phospholipid layer. They have 6, 8, 10 or 12 carbon atoms; shorter- or longer-chain fatty acids are not able to easily pass through the phospholipid layer.

The ability to move through bacterial cell walls allows MCFAs to weaken bacteria in three ways:

1. Bacterial cell contents leak out, which weakens the cell.
2. The MCFA enters the cell and dissociates, decreasing the cell's pH. The higher the fatty acid's pKa, or acid strength, the greater the ability of the acid to lower pH.
3. Once the MCFA is inside the cell, it can block DNA replication. To replicate, the pathogen has to unfold its DNA, but MCFAs intercalate or insert themselves into the DNA helix and prevent replication.

Finally, production of organic acids might include intermediates that are themselves toxic. For example, 3-hydroxypropionic acid (3HP) is closely related to the antimicrobial compound reuterin, which is inhibitory to several bacteria including *E. coli* (Sung et al. 2002). It is thought that the toxicity could be the result of DNA synthesis (Rasch 2002).

The SCFA butyric acid acts as an energy source for enterocytes, enhances epithelial barrier integrity and inhibits inflammation (Hamer et al. 2008; Kasubuchi et al. 2015). Butyrate can be produced microbially by fermentation of carbohydrates in the caecum or used in the diet as a feed additive. Kulcsár et al. (2017) examined the impact of different sources of butyrate on modulation of cytochrome P450 (CYP) enzymes in chickens. Butyrate salts, primarily sodium butyrate and several protected forms (e.g. encapsulated butyrate), and various butyric acid esters are used successfully in poultry farming. Protected forms avoid rapid butyrate adsorption by providing prolonged release mainly in the distal section of the gastrointestinal tract. Butyrates of different origin showed varying distribution properties as being absorbed from different parts of the gastrointestinal tract. Intestinal CYP1A and CYP2H2 activities were increased by dietary butyrate supplementation and by the increased caecal microbial butyrate production, while CYP3A37 activity was minimally influenced by microbial butyrate only. The study proved that both dietary and microbial butyrate could alter the activity of CYPs in the duodenal epithelium. Their findings suggest that intestinal CYPs could be induced not only by the intestinal luminal butyrate, but also from the basolateral side, by the already absorbed butyrate (Kulcsár et al. 2017).

Other authors have summarised the mechanisms of actions of fatty acids and other acids as follows:

- reducing the pH level of the upper gastrointestinal tract (crop proventriculus, gizzard) and associated physiological changes in the gut mucosa (Samanta et al. 2008; Panda et al. 2009);
- altering the gut microflora either by directly killing through cell-wall penetration or by indirectly modifying pH and reducing the numbers of pathogenic bacteria as described above, increasing acid-tolerant beneficial species such as *Lactobacillus* spp. and reducing competition for nutrients by the altered microbes (Biggs & Parsons 2008; Nava et al. 2009; Czerwiński et al. 2010; Boroojeni et al. 2014);
- improving gut health through direct effects on epithelial cells (e.g. SCFA are a direct energy source for the growth of epithelial cells);
- increase of proteolytic enzyme activity and nutrient digestibility, improvement of pancreatic secretions, stimulation of digestive enzyme activity and 'balancing' the microbial population (Partenen & Mroz 1999; Partenen 2001; Knarreborg et al. 2002; Diebold & Eidsburger 2006);
- Anti-inflammatory effects – butyrate was shown to modulate inflammation in vitro by inhibiting the activity of the transcription factor NF- κ B - shown to be activated in inflamed colonic tissue and inhibited by anti-inflammatory agents (glucocorticoids and salicylates) (Hodin 2000; Inan et al. 2000); and

- strengthen the gut mucosal barrier by increasing production of antimicrobial peptides in mucous and by stimulating the expression of tight junction proteins (Mariadason et al. 1997; Schaubert et al. 2003; Bordin et al. 2004; Peng et al. 2007). Moreover, there is some evidence of increased growth of the gastrointestinal tract (GIT) mucosa in the presence of organic acids (Dalmasso et al. 2008).

The effects of organic acids depend on several factors such as: type and pKa of acid, inclusion rate of supplemented acids, composition of diets and their acid-base or buffering capacity, level of intraluminal production of acids in GI tract by inhabiting microflora, feed palatability, intrinsic acid activity, receptors for bacterial colonisation on the epithelial villi, maternal immunity by vaccinations, hygiene and welfare standards, age of animals (Partanen & Mroz 1999; Strauss & Hayler 2001; Decuyper & Dierick 2003).

3.1.3 Use in other poultry

Organic acid use has been shown to have significant benefits in swine and poultry production over the years. Dietary supplementation of fumaric acid in broiler chickens was shown to improve weight gain and feed efficiency (Patten & Waldroup 1988; Skinner et al. 1991; Biggs & Parsons 2008; Adil et al. 2010, 2011; Banday et al. 2015). Similar effects of growth performance improvement were seen when butyric acid was included in the broiler feed (Panda et al. 2009; Adil et al. 2010, 2011). Gupta et al. (2021) demonstrated that sodium butyrate modulated chicken macrophage proteins essential for SE invasion in an *in vitro* study with the HTC cell line of macrophages. Sodium butyrate influenced *S. Enteritidis*-infected HTC cells by reducing the expression of macrophage proteins mediating actin cytoskeletal rearrangements such as WD repeat-containing protein-1 (WDR1), Alpha actinin-1 (ACTN1), Vinculin (VCL) and Protein disulphide isomerase (P4HB) and intracellular *S. Enteritidis* growth and replication such as V-type proton ATPase catalytic subunit A (ATPV1A). Interestingly, sodium butyrate increased the expression of infected HTC cell protein involved bacterial killing such as Vimentin (VIM). In conclusion, sodium butyrate modulates the expression of HTC cell proteins essential for *S. Enteritidis* invasion. Several other organic acids that were tested by others were shown to improve performance in poultry include lactic (Adil et al. 2010, 2011), citric (Chowdhury et al. 2009; Haque et al. 2010), formic (Patten & Waldroup, 1988; Hernández et al. 2006; Panda et al. 2009), malic, sorbic, and tartaric acids. Acidifiers were reported to have beneficial effects on poultry performance or health. Butyric acid was shown to decrease the incidence of subclinical necrotic enteritis caused by *C. perfringens* (Timbermont 2009).

Research has shown that the beneficial effects of organic acids can be enhanced by using them as blends rather than a single acid. In spite of the demonstrated beneficial effects, using acids or organic acids to improve performance or treat or prevent disease lacks consistency. This can be attributed to various factors such as inclusion rates, the source of the organic acids, and the buffering capacity of other dietary ingredients (Dibner & Buttin 2002; Kim et al. 2015). For example Beer et al. (2020a) examined the impact of the addition of 0.2% boric acid in the diets of turkey poults to examine if this would ameliorate the impact of challenge with wild-type *Histomonas meleagridis* and found there was no difference in mortality rates or lesion scores between the treated poults and the exposed untreated poults. Similarly, and despite *in vitro* activity, deoxycholic acid (a naturally occurring secondary bile acid) failed to protect *H. meleagridis* challenged turkey poults (Beer et al. 2020).

Finally, organic acids are often used in combination with other feed additives. The authors undertook one such study in broilers with a combination of organic acids and essential oils (fumaric 19%, sorbic 11%, malic 9% and citric 9% plus thymol, eugenol and vanillin). Microbiota analysis using the v3 V4 region of the 16S rRNA gene were also sequenced and the impact of the treatment on the richness (diversity) of the microflora was analysed along with production parameters. Treatment did not

reduce diversity and caused a significant increase in *Lactobacillus* sp and decreased *Enterobacteriaceae* in the caecum. Production parameters were improved with treatment and mortalities trending lower in the high dose treated group (6.4% vs 2.45% $p < 0.06$) (Wilson et al. 2013).

3.1.4 Use in laying hens and pullets

Warsito et al. (2021) infected 26-week-old ISA Brown laying hens with up to 6×10^8 CFU of an Avian Pathogenic *E. coli* (APEC). On the same day some groups were treated with varying doses of a citric acid/dextrose combination. In the uninfected positive control the egg production dropped significantly to below 66% whilst all treated groups and negative controls remained above 90%. Feed conversion was also severely impacted by infection without treatment and was significantly worse (2.17) than the negative control (1.77) or the treated groups (range 1.75–1.63). There was no dose response and the lowest dose (1 g/3.75 L drinking water) of the acidifier/dextrose combination provided just as much protection.

3.2 Probiotics

Probiotics are gaining acceptance as potential alternatives to antibiotics to improve production efficiency (Lee et al. 2010c). They are defined as “live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance” (Fuller 1989). Probiotics may contain one or more strains of microorganisms and may be given either alone or in combination with other additives in feed or water (Thomke & Elwinger 1998). Novel application strategies such as spraying on chicks, embryonated eggs and on shed floors are also practised, and potential methods such as in ovo application are being explored (Wolfenden et al. 2007; Cox & Dalloul 2015). A variety of bacteria (*Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Lactococcus* spp.) and in some cases yeast (*Saccharomyces* spp.) have been tested as probiotics in poultry (Simon et al. 2001; Patterson & Burkholder 2003; Griggs & Jacob 2005; Kabir 2009). The majority of the research conducted was specifically aimed at investigating the effects of probiotics in reducing the numbers of pathogenic microorganisms in the gastrointestinal tract.

3.2.1 Mechanism of action (probiotics)

Probiotics have been shown to improve the health of chickens via several mechanisms amongst which are the enhancement of the non-specific and specific host immune responses to pathogens (Kabir et al. 2004), production of antimicrobial substances which are bactericidal or bacteriostatic, and competitive exclusion with pathogens for binding sites thus preventing colonisation by pathogenic bacteria (Ohimain & Ofongo 2012; Tarabees et al. 2019; Vineetha et al. 2015). *Bacillus* spores have been shown to enhance the innate immune system and macrophage phagocytosis (Duc et al. 2004; Xu et al. 2012). Selective enhancement of the growth of beneficial bacteria by probiotics has been demonstrated by Awad et al. (2009) and Gibson et al. (2017). Probiotics also contribute to improved intestinal integrity thereby improving nutrients absorption efficiency (Awad et al. 2009; Gibson et al. 2017). Research into other mechanisms of action is still ongoing and investigation into probiotics and pH levels has demonstrated another mechanism of action (Al-Khalaifa et al. 2019). Lactic acid bacteria can inhibit the growth of several poultry pathogens. *Lactobacillus* spp. (*L. acidophilus* and *L. casei*) have antagonistic abilities, which have been attributed to the production of bactericidal substances amongst which are bacteriocins, organic acids and hydrogen peroxide. These substances either lower the pH, alter bacterial metabolism, neutralise bacterial toxins or act as free radicals (Jin et al. 1997).

3.2.2 Probiotic use in broilers

The use of probiotics in broilers significantly reduced intestinal lesions due to necrotic enteritis (McReynolds et al. 2009) and intestinal pathogen load such as *Salmonella* Enteritidis (SE) and *Campylobacter jejuni* (Higgins et al. 2007), inflammation in mycoplasmosis and colibacillosis (Wang et al. 2022). However, attempts to utilise probiotics to reduce levels of *Campylobacter* in broilers using a novel strain of *Lactobacillus johnsonii* (F19785) were not successful. In addition, this study showed no significant effects on growth performance (Alsudani 2018).

3.2.3 Probiotic use in laying hens and pullets

Improved intestinal health was observed in Lohmann White laying hens, which had significantly higher egg production, eggshell quality and less pathogenic bacteria in the intestinal microflora upon administration of *Bacillus subtilis* (Abdelqader et al. 2013). Similar effects on the intestinal integrity were also observed in mature male white leghorn chickens (Samanya & Yamauchi 2002; Teo & Tan 2007). The beneficial effects of probiotics supplementation were reported in laying hens by Kurtoglu et al. (2004) who showed that hens fed diets supplemented with probiotics showed increased egg production compared with controls. Lei et al. (2013) reported that dietary inclusion of *B. licheniformis* improved laying performance and egg mass. *Bacillus* spp. probiotics added to the diet of laying hens were shown to improve the yolk colour, Haugh units and decrease yolk cholesterol (Xu et al. 2006).

3.3 Prebiotics

Prebiotics are defined as “non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gut” (Gibson & Roberfroid 1995). One definition (FAO 2007) describes prebiotics as “non-viable feed components that confer a health benefit on the host associated with modulation of the microbiota.” A variety of non-starch polysaccharides (NSP) or oligosaccharides have been considered as prebiotics, including mannan-oligosaccharide (MOS), fructo-oligosaccharide (FOS), inulin, oligofructose, galacto-oligosaccharide, malto-oligosaccharide, lactulose, lactitol, gluco-oligosaccharide, xylo-oligosaccharide (XOS), soya-oligosaccharide, isomalto-oligosaccharide (IOS), pyrodextrins (Patterson & Burkholder 2003; Steiner 2006) and arabinoxylan-oligosaccharides (AXOS) (Bautil et al. 2020). Prebiotics are macromolecules that are either derived from plants or synthesised by microorganisms. Recent research has identified that XOS can enhance the efficacy of xylanase (Craig et al. 2020; González-Ortiz et al. 2021; Morgan et al. 2022). The same compounds when added in too small a quantity to have a prebiotic effect but which at a low dose stimulate bacteria in the ileum and caecum to produce their own fibre degrading enzymes and so enhance SCFA production have been described by (Bedford & Apajalahti 2022) as ‘stimbiotics’. A stimbiotic can be the same molecule as a prebiotic, it is just the fact that it is fed at concentrations well below those capable of supporting significant fermentation directly. Furthermore, whereas a prebiotic would be expected to be fermented immediately and result in increased SCFA concentrations within hours of feeding, a stimbiotic would not be expected to deliver a similar response for several weeks. Regardless, both a prebiotic and stimbiotic are expected to be used by similar species, e.g. *Ruminococcaceae* and *Lachnospiraceae* in the caeca where butyrate would be the most important product.

The combination of probiotics and prebiotics have been described as ‘synbiotics’, a concept introduced for human health by Gibson and Roberfroid in 1995.

3.3.1 Mechanism of action of prebiotics

Beneficial effects of prebiotics in chickens include improvement of mucosal integrity by enhancing villi growth and crypt development (Chee et al. 2010; Pourabedin et al. 2015), selective stimulation of beneficial commensal bacteria in the gut and competitive exclusion of pathogenic bacteria (Pourabedin & Zhao 2015). This was demonstrated in broilers when xylo-oligosaccharides (a prebiotic dietary fibre) administered in feed resulted in the selective inhibition of colonisation of the intestine by pathogens including *Staphylococcus aureus*, *Clostridium difficile*, *Salmonella enterica*, and *Campylobacter jejuni*. However, beneficial bacteria like *Lactobacillus* spp. and *Bifidobacterium* spp. are able to degrade this fibre and utilise prebiotics as growth substrates (Kondepudi et al. 2012; Pourabedin et al. 2015). Prebiotics also stimulate host adaptive immunity (Babu et al. 2012). The immunomodulatory effects of the fructo-oligosaccharide inulin and mannan-oligosaccharide (MOS) in broiler chicken (Ross-308) resulted in upregulation of toll-like receptor (*TLR*)2b, *TLR*4, interleukin (*IL*)-12p35, and interferon (*IFN*)- γ , which supports T-helper cell associated immune responses (Yitbarek et al. 2012). Another effect of MOS is blocking of the mucosal binding sites of some chicken pathogens, especially gram-negative bacteria that express Type-1 fimbriae such as *Escherichia coli* and this eventually results in excretion of the pathogen from the intestine (Adhikari & Kim 2017; Thomas et al. 2004). Beneficial bacteria such as *Bifidobacteria* and lactic acid bacteria ferment prebiotics to yield short chain fatty acids (butyrate, propionate and acetate), which function in lowering the pH of the gut hence making it difficult for harmful bacterial to thrive (Pourabedin et al. 2015).

Prebiotics also prevent pathogen colonisation either by binding directly or by competitive exclusion, by promoting the growth of beneficial microbes or by stimulating them to produce bacteriocins and lactic acid (Spring et al. 2000). In particular, MOS acts by binding to type 1 fimbriae of enteric pathogens and prevents their adhesion to intestinal epithelial cells (Spring et al. 2000). The fermentation of prebiotics by microflora also leads to the production of SCFA that act as energy sources for intestinal epithelial cells and thus maintain the integrity of the gut lining (Ferket et al. 2005). Prebiotics also act by beneficially altering luminal or systemic aspects of the host immune system. MOS is recognised by receptors of the innate immune system, act as adjuvants, and help boost the host immune responses (Ferket et al. 2005).

3.3.2 Prebiotic use in broiler chickens

Isomalto-oligosaccharides have shown promise as an antibiotic alternative owing to their efficacy in improving weight gain and Feed Conversion Ratio (FCR) when fed to broilers (Mookiah et al. 2014). In contrast to the previous results, several authors reported that prebiotic supplementation had no effect on the performance of broilers (Baurhoo et al. 2007; Józefiak et al. 2008; Geier et al. 2009; Corrigan et al. 2011; Houshmand et al. 2012). However, statistical analysis of numerous trials conducted with prebiotic supplementation in the diets of broiler chickens was shown to beneficially influence their growth and performance. The majority of the beneficial effects of prebiotics are thought to be mediated predominantly through altering the intestinal microbiota (Pourabedin and Zhao 2015).

MOS, derived from the outer cell-wall layer of *Saccharomyces cerevisiae*, has been studied extensively as a prebiotic supplement in poultry diets. The addition of various levels of MOS to broiler diets significantly increased their bodyweights and improved feed conversion efficiency (Benites et al. 2008; Bozkurt et al. 2008; Hooge et al. 2003; Yang et al. 2007; Mohamed et al. 2008) with increased intestinal villi height (Baurhoo et al. 2007; Yang et al. 2007), improved immune-competence in the intestine (Janardhana et al. 2009; Shanmugasundaram & Selvaraj 2012), altered jejunal gene expression (Xiao et al. 2012; Brennan et al. 2013), and influenced intestinal microbiota (Geier et al. 2009; Corrigan et al. 2011; Kim et al. 2011; Pourabedin et al. 2014). FOS, which is derived from plants, has also been

shown to possess significant prebiotic effects and improve performance in broiler chickens (Xu et al. 2003; Kim et al. 2011). In contrast, Al-Khalaifa et al. (2019) examined the effect of FOS and MOS on broiler chickens and found no impact of dietary inclusion on the bodyweight, feed consumption, feed efficiency and weight gain.

3.3.3 Prebiotic use in laying hens and pullets

One of the first experiments on the immune response of layers fed a diet supplemented with prebiotic containing yeast product was conducted with heat-stressed hens (Asli et al. 2007). In this study, immunised Hy-Line White hens fed a diet supplemented with *Saccharomyces cerevisiae* culture (1 g/kg) had significantly higher antibody titres against sheep red blood cells (SRBC) and numerically higher egg production than the control birds. The authors indicated that yeast culture during heat stress improves a layer's humoral immune response and may lead to increased laying performance and egg quality parameters (Asli et al. 2007). These positive results were confirmed more recently by Yalcin et al. (2010), who evaluated the effects of dietary yeast (*Saccharomyces cerevisiae*) autolysate on egg production and quality, some blood parameters and humoral immune response of Hy-Line Brown hens. Yeast autolysate supplementation significantly increased antibody titre to SRBC, positively affected egg production, egg weight and feed efficiency, and decreased egg yolk cholesterol and blood serum levels of cholesterol and triglyceride. There were also some beneficial changes in yolk fatty acid composition after yeast culture addition to the diet. The authors concluded that dietary yeast autolysate (2, 3 or 4 g/kg) had positive influences not only on humoral immune response, but also on performance and egg cholesterol concentration in laying hens (Yalcin et al. 2010). Similar beneficial effects of yeast autolysate in terms of humoral immunity and laying performance were found in their subsequent study (Yalcin et al. 2012). A combination of prebiotic and probiotic was utilised by Kimminau et al. (2021) to attempt to reduce ovarian contamination in Hy-line pullets challenged with 3×10^6 cfu/bird *S. Enteritidis* at 17 weeks of age. The treatment (Fulfill Plus added to the diet at 0.075%) was administered either daily from day of age for the whole study, or from week 10 till the end of the study. There was no difference in caecal SE prevalence in caecal samples one or two weeks post-exposure, and the proportion of positive ovaries had actually increased compared to infected controls in the groups treated only from 10 weeks of age. However, Krueger et al. (2020) demonstrated protection against SE challenge in Hy-line Brown pullets treated with the prebiotic 'Avi-Lution'. This product is a blend of live strains of *Saccharomyces cerevisiae*, *E. faecium*, and *Bacillus subtilis* as well as β -glucan, mannan-oligosaccharides, and fructo-oligosaccharides. When administered in feed from either 42-days of age, with SE challenge (1.46×10^7 cfu) at 45 days and examination of caeca at 55-days of age, or fed from 70 days of age with challenge on 73 days of age and post-mortem examinations at 83 days of age, the treated pullets had significant 1–2 log reductions in SE in caecal contents (from 3.69 in controls to 2.69 in the early treated and killed birds and from 3.76 down to 1.71 in the older treated pullets).

Xylo-oligosaccharides were administered at various concentrations (from 0, 0.01 to 0.05% of the diet) to 28-week-old Lohmann White laying hens for eight weeks by Ding et al. (2018). Improvements in jejunal villus height and villus to crypt ratio were seen in a dose dependent manner in the treated hens. The number of *Bifidobacteria* in caecal contents was also increased up to 15.7% with treatment. Caecal *E. coli* counts were also reduced by the treatment. The concentration of butyrate in the caecum was increased by treatment. Plasma $1,25(\text{OH})_2\text{D}_3$ was increased in treated hens and IgA, IgM, TNF- α and IL-2 were increased in response to increasing concentrations of dietary XOS.

3.4 Phytochemicals/nutraceuticals

Nutraceuticals, also referred to as phytochemicals, or botanicals, are natural bioactive compounds that are derived from plants (Griggs & Jacob 2005). These plant-derived compounds have been shown to

have beneficial effects on farm animal productivity, including enhancement in feed conversion ratio and improvement of host immune response (Windisch et al. 2008). These compounds are sometimes referred to as phytobiotics because of an inherent bactericidal and/or bacteriostatic effect (Carrillo et al. 1997; Griggs & Jacob 2005; Mayo Tadeo et al. 2018; Rehman et al. 2019; Salaheen et al. 2014). A wide range of plants and their products fall under this category and they can be broadly classified as herbs (flowering, non-woody, non-persistent plants from which leaves and flowers are used) or spices (non-leaf parts of plants, including seeds, fruits, bark or root with intensive taste or smell) (Windisch et al. 2008; Van Der Klis & Vinyeta-Punti 2014). They can be used in solid, dried, and ground form or as extracts (crude or concentrated). Depending on the process used to derive the active ingredients, phytobiotics can also be classified as essential oils (EOs: volatile lipophilic substances obtained by cold extraction or by steam or alcohol distillation) and oleoresins (extracts derived by non-aqueous solvents) (Windisch et al. 2008; Van Der Klis & Vinyeta-Punti 2014).

The composition and concentration of the main bioactive compounds of the phytobiotics can vary according to the plant, parts of the plant, geographical origin, harvesting season, environmental factors, storage conditions, and processing techniques (Windisch et al. 2008; Applegate et al. 2010). Phytobiotics have been used as natural growth promoters in the pig and poultry industries (Papatsiros et al. 2013; Windisch et al. 2008; Franz et al. 2010). A wide variety of herbs and spices (e.g. thyme, oregano, rosemary, marjoram, yarrow, garlic, ginger, green tea, black cumin, coriander, and cinnamon) have been used in poultry for their potential application as AGP alternatives. Guo et al. (2004) showed a significant increase in bodyweight gain and improvement in feed efficiency when broilers were given diets supplemented with a mixture of 14 herbs. Similar results were shown with the addition of oregano (Florou-Paneri et al. 2006), dried ground leaves of stevia (Atteh et al. 2008), black cumin seeds (Khalaji et al. 2011), fermented Ginkgo biloba leaves (Cao et al. 2012), and dried and ground *Scrophularia striata* and *Ferulago angulata* (Rostami et al. 2015) to poultry feed. Various plant extracts used as phytobiotics were also shown to improve the performance of broilers. Research trials conducted with the inclusion of sugar cane extract (El-Abasy et al. 2002), aniseed extract (Durrani et al. 2007), chestnut wood extract (Schiavone et al. 2008), *Forsythia suspensa* extract (Wang et al. 2008), and *Portulaca oleracea* extract (Zhao et al. 2013b) showed a significant increase in bodyweight gain and a lower FCR. In other studies, several phytobiotics such as grape pomace, cranberry fruit extract, *Macleaya cordata* extract, garlic powder, grape seed extract, and yucca extract tested as growth promoters did not show any effects on performance parameters (Goñi et al. 2007; Brenes et al. 2008; Leusink et al. 2010; Juskiewicz et al. 2011; Viveros et al. 2011; Issa & Omar 2012; Chamorro et al. 2013).

In addition to herbs and spices, various Essential Oils (EOs) (thymol; carvacrol; cinnamaldehyde; EOs from clove, coriander, star anise, ginger, garlic, rosemary, turmeric, basil, caraway, lemon, and sage) have been used either individually or as blends to improve animal health and performance. Variable results have been reported with the use of EOs in poultry diets, including a blend of thymol and cinnamaldehyde in feed, which was shown to improve bodyweight gain in broilers (Tiihonen et al. 2010; Amerah et al. 2011). Similar results were shown when supplementing diets with EO from oregano (Malayoğlu et al. 2010; Hashemipour et al. 2013) and coriander (Ghazanfari et al. 2015), blends of clove and cinnamaldehyde (Chalghoumi et al. 2013), thymol and EO from star anise (Kim et al. 2016a), and a herbal EO mix (Alçiçek et al. 2004; Khattak et al. 2014). EO supplementation was also shown to improve feed efficiency as seen by reduced FCRs (Çabuk et al. 2006a; Isabel & Santos 2009; Amad et al. 2011; Kim et al. 2016a). In contrast, several other trials did not show any beneficial effects of including EO on performance (Lee et al. 2003; Bozkurt et al. 2012; Hernández et al. 2004; Zhang et al. 2005; Jang et al. 2007). The variations in the results can be attributed to the differences in the composition, type, and origin of the EOs that were used, inclusion level, and the environmental conditions of the trials (Franz et al. 2010). Nevertheless, one commercial blend of phytonutrients (containing carvacrol, cinnamaldehyde, and capsicum oleoresin) was approved in the

European Union (EU) as the first botanical feed additive for improving performance in broilers. Several research trials performed with this commercial blend demonstrated consistent improvement in growth and feed efficiency (Bravo et al. 2014; Karadas et al. 2014; Pirgozliev et al. 2021). A meta-analysis of 13 broiler studies involving the use of this commercial blend showed that its inclusion in diets increased bodyweight gain and decreased FCR and mortality (Bravo & Ionescu 2008).

3.4.1 Mechanism of action

The mechanisms of action of phytobiotics on performance are not all clearly understood and depend greatly upon the composition of the active ingredients in the product being used. EOs are well known for their antimicrobial, immunostimulatory, anti-inflammatory and antioxidant properties (Abdelli et al. 2020; Griggs & Jacob 2005). The inclusion of phytobiotics in the diets was shown to alter and stabilise intestinal microflora and reduce microbial toxic metabolites in the gut owing to their direct antimicrobial properties on various pathogenic bacteria, which results in relief from intestinal challenge and immune stress, thus improving performance (Tiihonen et al. 2010; Viveros et al. 2011; Zhang et al. 2013; Zhao et al. 2013b; Liu et al. 2014).

Another important beneficial effect of dietary inclusion of phytobiotics is reduction in oxidative stress and increase in antioxidant activity in various tissues and thus improved health (Basmacioğlu et al. 2004; Brenes et al. 2008; Wang et al. 2008; Cao et al. 2012; Mueller et al. 2012; Zhang et al. 2013; Liu et al. 2014; Settle et al. 2014). Phytobiotics also exert their action through immunomodulatory effects such as increased proliferation of immune cells, elevated expression of cytokines, and increased antibody titres (Kim et al. 2010; Lee et al. 2010b; Park et al. 2011; Pourhossein et al. 2015). The addition of phytobiotics to the diet was also shown to increase intestinal and pancreatic enzyme production and activity and increase bile flow (Lee et al. 2003; Jang et al. 2007; Malayoğlu et al. 2010; Hashemipour et al. 2013). Phytobiotics also help maintain and improve gut histology, increase villi height and thus expand absorptive surface of the intestine (Ghazanfari et al. 2015; Murugesan et al. 2015). Increase in digestive enzyme secretion and absorption results in improved apparent nutrient digestibility and thus improves performance (Jamroz et al. 2003; Hernández et al. 2004; Jørgensen et al. 2008; Wang et al. 2008; Amad et al. 2011; Amerah et al. 2011; Issa & Omar 2012). They also might play a role in maintaining the intestinal barrier function as evidenced by the increase in the transepithelial electrical resistance of duodenal mucosa of broilers that included thymol in their diets (Placha et al. 2014).

A growing body of scientific evidence has demonstrated that many of the health-promoting activities of phytochemicals are also mediated through their ability to enhance the host's defence against microbial infections and tumours. The immune-activating properties of medicinal plants such as dandelion (*Taraxacum officinale*), mustard (*Brassica juncea*), and safflower (*Carthamus tinctorius*) have been evaluated *in vitro* using avian lymphocytes and macrophages (Lee et al. 2007). All three extracts inhibited tumour cell growth and exhibited antioxidant effects. Further, the safflower extract stimulated chicken lymphocyte proliferation, whereas the mustard extract induced nitric oxide production by macrophages. In a separate study, organic phase extracts from milk thistle (*Silybum marianum*), turmeric (*Curcuma longa*), reishi mushroom (*Ganoderma lucidum*), and shiitake mushroom (*Lentinus edodes*) were tested for their effects on chicken innate immunity and tumour cell cytotoxicity (Lee et al. 2010a).

In chicken macrophages treated with extracts of turmeric or shiitake mushroom *in vitro* (Lee et al. 2010b), the levels of gene transcripts for IL-1 β , IL-6, IL-12, IL-18, and TNFSF15 were increased. The phagocytic activity of chicken heterophils was shown to be significantly improved with the addition of non-dialysable materials of cranberry extract at 4 mg ml⁻¹ concentration (Islam et al. 2016). Cinnamaldehyde ((2E)-3-phenylprop-2-enal) is a constituent of cinnamon (*Cinnamomum cassia*), a

widely used flavouring compound that has been traditionally used to treat human diseases, including dyspepsia, gastritis, and inflammation. Chicken spleen lymphocytes that were stimulated *in vitro* with cinnamaldehyde showed good cell proliferation, and cinnamaldehyde activated cultured macrophages to produce higher nitric oxide levels (Lee et al. 2011). The effects of carvacrol, cinnamaldehyde, and Capsicum oleoresin on the regulation of the expression of genes associated with immunology, physiology, and metabolism were investigated in chickens using high throughput microarray analysis (Lillehoj et al. 2011). These studies revealed that Capsicum oleoresin stimulated a great number of gene changes when compared with unsupplemented controls, and many of the altered genes were associated with metabolism and immunity. The most reliable genetic network induced by dietary cinnamaldehyde treatment was related to the functions of antigen presentation, humoral immunity, and inflammatory disease. Further studies to delineate the intestinal immune pathways affected by phytochemical feeding were conducted by mRNA microarray hybridisation (Kim et al. 2010). When compared with chickens fed an unsupplemented diet, carvacrol-fed chickens showed altered levels of 74 gene transcripts in gut lymphocytes (26 increased, 48 decreased), cinnamaldehyde supplementation was associated with altered levels of 62 mRNAs (31 increased, 31 decreased), and Capsicum oleoresin-fed chickens had altered levels of 254 mRNAs (98 increased, 156 decreased), compared with unsupplemented controls. Among the transcripts that showed greater than twofold altered expression levels, most were encoded by genes associated with metabolic pathways. In the case of Capsicum oleoresin, the transcripts included pathways for lipid metabolism, small molecule biochemistry, and cancer. In another investigation, global gene expression analysis by microarray hybridisation identified 1810 transcripts (677 increased, 1133 decreased) whose levels were significantly altered in intestinal lymphocytes of anethole-fed birds when compared with unsupplemented controls (Kim et al. 2013a). From these, 576 corresponding genes were identified that were related to the inflammatory response. A similar analysis was reported for the garlic metabolites, propyl thiosulphinate (PTS) and PTS oxide (PTSO) (Kim et al. 2013b). In that study, 1227 transcripts (552 increased, 675 decreased) were identified in intestinal lymphocytes whose levels were significantly altered in PTS/PTSO-fed birds when compared with unsupplemented controls. Many of these transcripts were encoded by genes related to innate immunity, including Toll-like receptor 3 (TLR3), TLR5, and nuclear factor (NF)- κ B.

The direct antimicrobial activities of many phytochemicals have been linked to flavonoids, which interact with intracellular proteins forming complexes with bacterial cell walls that eventually result in cell wall lysis (Mayo Tadeo et al. 2018). Another antibacterial mechanism common in some phytochemical compounds is their oxidising ability, which inhibits essential bacterial cellular processes. Also, phytochemical compounds interact with bacteria and inactivate and/or interrupt critical bacterial cellular processes required for survival such as mitogen-activated protein kinase (MAPK), microbial adhesins, enzyme transport, and cellular envelope proteins (Mayo Tadeo et al. 2018; Wang et al. 2022). Zhang et al. (2014) investigated the ability of α -Cyperone (a component of the total oil and rhizomes of *Cyperus rotundus*) to protect chicken type II pneumocytes from injury by APEC-078. α -Cyperone was able to significantly decrease the adherence of APEC-078 to chicken type II pneumocytes, and also to inhibit actin cytoskeleton polymerisation induced by APEC-087 through down regulating the expression of Nck-2, Cdc42 and Rac1.

3.4.2 Use of phytobiotics as antibiotic alternatives in broilers

Many experimental studies into the usage of phytogenics as alternative to antibiotics were performed on broiler breed chickens. For example, an *in vitro* experiment using extracts from Oregano (*Origanum vulgare*), Sierrecilla (*Mimosa lacerate*) and Eucalyptus (*Eucalyptus globulus*) was effective in inhibiting growth of *Avibacterium paragallinarum* and *Pasteurella multocida* causative agents of infectious Coryza and fowl cholera respectively (Mathlouthi et al. 2012; Mayo Tadeo et al. 2018). Thyme powder and oil have been shown to inhibit *in vitro* *Salmonella typhimurium* and *Escherichia coli* (Aktuğ &

Karapinar 1986; Marino et al. 1999). Another study, using a proprietary plant-derived in-feed medication Finio (Anitox, Lawrenceville, GA, USA) was able to significantly reduce the counts of *S. typhimurium* and *E. coli* in feed (Gosling et al. 2021). Condensed tannins which is a secondary metabolite of many prodelphinidin rich plants such as yew leaves, tilia flower and black locust leaves have been shown *in vitro* to have antibacterial activity against avian pathogenic *E. coli* (APEC) (Dakheel et al. 2020). Carillo et al. (1997) investigated the therapeutic efficacy of combinations of aqueous extract from Epacine (*Petiveria alliacea*), Quina (*Coutarea hexandra*), Stinkwood (*Centrum lanatum*) and Physic nut (*Jatropha curcas*) in treating *P. multocida* infection in Arbor Acres line 1997. Another study demonstrated the ability of combinations of carvacrol from oregano, cinnamaldehyde from cinnamon, and capsaicin from paprika to significantly reduce the number of *E. coli* and *Clostridium perfringens* and increase of beneficial bacterial (*Lactobacillus* spp.) in the rectal contents of Hybro HI-YR broiler chickens (Jamroz et al. 2003; Jamroz et al. 2005). A proprietary product of plant origin, Natustat™, tested on Hubbard HiY broiler chickens and Hybrid Turkey poults was effective against *Histomonas meleagridis* the causative pathogen of infectious enterohepatitis (Duffy et al. 2004; 2005). Aqueous extract of *Piper auritum* and *Ocimum basilicum* were effective in treating *E. coli* infection in Ross 308 male chickens (Aguilar-Urquiza et al. 2020). Phytochemicals are also known to boost immunity and increase resistance to infectious pathogens by increasing cellular responses and digestibility of nutrients in feed. Chickens receiving Chinese herb extracts of *Rheum rhabarbarum*, *Lysimachia davurica*, *Lonicera japonica*, *Codonopsis pilosula* and *Radix liquiritiae* supplementation in drinking water at a dosage of 2 mL/bird/day for 3 days was able to cure *Mycoplasma synoviae* infection (Rehman et al. 2019).

Willis et al. (2013) evaluated the feeding of four medicinal mushrooms (shiitake [*Lentinus edodes*], reishi [*Ganoderma lucidum*], oyster [*Pleurotus ostreatus*], and cordyceps [*Cordyceps sinensis*]) at two levels (5% or 10%) on broiler production performance. The results showed that the oyster mushroom (5%) group produced the highest average bodyweight at 3.21 kg, while the cordyceps (10%) group produced the lowest at 2.33 kg. A higher oocyst count and increased heterophil percentage were observed in the reishi (5%) group. Similarly, Toghyani et al. (2012) reported that oyster mushroom powder at an inclusion level of 2.0% had favourable effects on performance criteria of broiler chicks reared to 28-days of age. In contrast to these studies, Fard et al. (2014) reported that inclusion of 1% oyster mushroom wastes had no significant effect on the whole period bodyweight (BW) and BW gain of broilers, only marginally increasing these indices. On the contrary, using 2% of mushroom wastes decreased BW. Likewise, Azizi et al. (2020) did not observe any impact on broiler bodyweight gain, feed intake, feed conversion efficiency or carcass characteristics after feeding sumac (*Rhus coriaria* L.) seed powder at rates from 0.05–0.2%.

3.4.3 Phytochemicals as alternatives to antibiotics in pullets and layer hens

The antibacterial effects of EOs from thyme, sage and rosemary were tested on 24-week-old Lohmann hybrid laying hens' laying performance. 200 mg/kg of thyme oil, sage oil and rosemary oil were each combined with standard feed formulation each and given to the birds for 12 weeks. The coliform and *Escherichia coli* content of faeces were analysed (Bölükbaşı et al. 2008). All EOs significantly ($p < 0.05$) reduced faecal coliform and *E. coli* counts. In another study conducted on 480 54-week-old Nick Brown hens, the effects of dietary supplements of EOs as alternatives to an antibiotic feed additive for layers kept under hot summer conditions (Çabuk et al. 2006b) were observed. The EOs used in this study were made up of six different phytochemicals including Oregano oil (*Origanum* sp.), laurel leaf oil (*Laurus nobilis* L.), sage leaf oil (*Salvia triloba* L.), myrtle leaf oil (*Myrtus communis*), fennel seed oil (*Foeniculum vulgare*), and citrus peel oil (*Citrus* spp.). The EOs significantly ($p < 0.05$) lowered the mortality rate (Çabuk et al. 2006b). Peng et al. (2007) demonstrated that Baicalin extracted from dried *Scutellaria baicalensis* Georgi roots was effective in treating male Hyline Brown chickens infected with APEC-O78 strain.

A review by Khan and Iqbal (2018) summarised the effects of commonly used mushrooms in poultry diets on the performance of both layer and broiler diets. They found that polysaccharides in mushrooms may act as immune enhancers or immunomodulators and show antibacterial activity. Phenolic compounds in mushrooms may act as antioxidants. Included in the review was a paper by Willis et al. (2008) in which egg production and egg quality was improved and *Salmonella* growth was reduced by the inclusion of mushrooms in the diet of moulting layer hens (77-week-old Single Comb White Leghorns). No artificial *Salmonella* challenge was conducted and, at the end of the moult, crops and caeca were cultured. Statistically significant but modest reductions in Log₁₀ *Salmonella* cfu/g of caecal and crop contents were seen in the mushroom (*Lentinula edodes*) treated hens (2.66 cf. 2.99 in caecal contents and 2.88 cf. 3.14 in crop contents of treated and controls respectively). Ovary weights were significantly higher in the mushroom treated hens post-moult (30.2 vs 19.6g). Egg production, whilst lowest in the mushroom group immediately post-moult, increased more than other treatments by the second month post-moult and had a slightly improved level of product at 3 months post-moult induction. Cayan and Erenner (2015) examined the impact of olive leaf (*Olea europaea*) on laying hen performance when included at 1–3% of the diet, and found no difference in feed intake, egg weight, egg production or feed conversion efficiency.

3.5 Vaccines

Vaccines have had an enormous impact in reducing the amount of antimicrobials required in pullet rearing and for laying hens, and their impact is well understood. The use of vaccines is outside the scope of this review but represents one of the most useful tools in reducing the need for antibiotic treatment of laying hens and pullets. The role of vaccines in combatting antimicrobial resistance in human medicine has been reviewed by Micoli et al. (2021). With respect to the use of vaccines in animals to reduce the use of antimicrobials Cheng et al. (2014) reviewed the use of vaccines and other immunity modulating agents as alternatives to antibiotic use.

3.6 Husbandry/environment including re-use of litter/controlled exposure

By and large the whole gamut of husbandry is outside the scope of this review. However, there are specific instances of disease control such as the use of litter amendments to decrease bacterial survival and *Eimeria* oocyst sporulation that should be noted. Where re-use of litter is practised it has been demonstrated that litter amendments such as superphosphate, meta-bisulfide and charcoal were able to significantly reduce the total bacterial count as well as specifically the *E. coli* and *Salmonella* Typhimurium counts. In addition the oocyst count was reduced (Soliman 2018).

3.7 Genetics, including genetically modified organisms

Genetic improvements made by primary breeding companies in response to disease issues are outside the scope of this review. However, advances in breeding with respect to disease resistance and resilience are also important in the reduction of the need for antibiotic use.

3.8 Dietary enzymes

Dietary enzymes are biologically active proteins that facilitate chemical breakdown of nutrients to smaller compounds for further digestion and absorption (Thacker 2013). Various enzymes, derived from microbes (bacteria and fungi) through fermentation, have been used in swine and poultry feeds for the past several years, and their value in enhancing growth and feed efficiency is well noted. The different classes of enzymes commonly employed include phytase, carbohydrases (xylanase, cellulase, α -galactosidase, β -mannanase, α -amylase, and pectinase), and proteases. The effect of various in-feed

enzymes in improving the growth and feed efficiency in poultry is well documented and reviewed (Bedford & Schulze 1998; Choct 2006; Selle & Ravindran 2007; Adeola & Cowieson 2011; Slominski 2011; Woyengo & Nyachoti 2011).

3.8.1 Mechanism of action of dietary enzymes

It is now well accepted that exogenous enzymes act on antinutritional factors that are present in plant-based feedstuffs such as phytic acid, NSP, and cell-wall complex carbohydrates. The improved performance that is a result of enzyme supplementation has been linked to an increase in the overall digestibility and availability of nutrients for absorption (Bedford 2000; Verstegen & Williams 2002; Rebolé et al. 2010). The possible mechanisms of action of in-feed enzymes include the following:

1. increase in the digestibility of nutrients that are otherwise not degraded by host enzymes (e.g. phytic acid);
2. elimination of the nutrient-encapsulating effect of cell-wall polysaccharides and an increase in the availability of starches, amino acids, and minerals;
3. inactivation of anti-nutritional factors (e.g. phytic acid or soluble NSP) and reduced intestinal viscosity;
4. an increase in the solubility of non-soluble NSP and promotion of cecal fermentation; and
5. supplementation of endogenous enzymes that may be in insufficient amounts, especially in young animals in which the digestive system is not fully developed (Choct 2009; Kiarie et al. 2013).

In addition to the effects enzymes have on nutrient digestibility, they are also thought to influence the composition of the gut microbiota. The enzyme-induced microbiota changes are mostly indirect and are thought to be mediated by two main mechanisms:

1. reducing the undigested substrates; and
2. generating short-chain oligosaccharides from cell-wall NSP with potential prebiotic effects (Bedford 2000; Bedford & Cowieson 2012; Kiarie et al. 2013). These mechanisms influence the nutrient supply and intestinal environment thus altering selection pressures on bacterial species (Bedford & Cowieson 2012; Cheng et al. 2014).

3.8.2 The use of enzymes in broiler feeds

The potential for use of in-feed enzymes, as antibiotic alternatives to improve performance in poultry is significant. Various meta-analyses conducted corroborate these beneficial effects in broilers upon enzyme supplementation. A meta-analysis performed by Hooze et al. (2010) showed that supplementation of a dietary multi-enzyme complex involving phytase and NSP enzymes improved final bodyweight by 3.73% and lowered FCR by 2.64%. Jackson and Hanford (2014) conducted a meta-analysis of seven pen trials investigating the effects of β -mannanase supplementation in male broilers raised to market age. They reported that the weight gain and FCR, analysed across trials, were improved by 4.2% and 4.8 points, respectively, and concluded that β -mannanase supplementation is effective in broilers. A similar meta-analysis conducted by Swann and Romero (2014) investigated the beneficial effects of a xylanase, amylase, and protease combination. Their results, based on ten broiler studies, showed that the particular enzyme combination increased the apparent digestibility of undigested crude protein, starch and fat by 22.7, 88.9, and 33.4%, respectively. However, it should be noted that the beneficial effects of enzyme supplementation are sometimes inconsistent owing to the differences in the enzyme type, source, amount of enzyme used, presence of enzyme side effects, diet composition, and genetic variations among animals (Ravindran & Son 2011; Cheng et al. 2014).

3.8.3 The use of enzymes in laying hens and pullets

Hy-Line Brown laying hens (44 weeks old) were fed diets containing various levels of xylanase. A significant linear increase to increasing xylanase supplementation was seen for lactic acid bacteria numbers, although coliforms and *Salmonella* counts were not affected. Increasing the dietary xylanase resulted in a significant linear increase in eggshell thickness in weeks 3, 6, 9, and 12 ($p < 0.05$) but had no effect on production performance or nutrient digestibility (Lei et al. 2018). A study in aged broiler breeder hens fed diets containing phytase (300 U/kg) and zinc oxide showed significant improvements in fertility in phytase treated groups with numerical improvements in egg production in the phytase groups. Interactions with the ZnO treatments suggested that the inclusion of phytase increased the absorption of zinc (Sharideh et al. 2016). Various studies examine the impact of enzymes on diet utilisation and production parameters of laying hens such as the meta-analysis of the use of phytase by Bougouin et al. (2014), which highlighted and increase in P retention, but little work has been undertaken examining the impact of enzymes on infectious diseases. However, the importance of enzymes in assisting in reducing the negative impacts of viscosity in layers fed wheat- and barley-based rations should be considered. The relationship of the non-starch polysaccharide component of these grains, digesta viscosity, and its relationship with microflora were reviewed by Wilson (1999). Higher viscosity ingesta is associated with higher intestinal microbial counts and with a deterioration in intestinal morphology.

3.9 Usage of synthetic amino acids and proteases, dropping protein levels, decreasing incidence of some diseases

Limited protein resources, environmental issues associated with high protein diets (faecal nitrogen emission) and animal health problems associated with high protein diets (Kerr et al. 2006; Aarnink & Verstegen 2007; Hristov et al. 2011; Wang et al. 2018) have led to an increasing use of lower protein diets for animal feeds. Diets containing high protein/energy can create an imbalance between energy and protein requirement, leading to an excess of undigested protein reaching the caeca. This will, in turn, lead to an overgrowth of proteolytic bacteria. The latter will produce ammonia and hydrogen sulfide (H₂S) (Ducatelle et al. 2018).

The use of lower protein diets requires the addition of limiting amino acids and can be assisted by the use of proteases. The addition of glycine, valine, isoleucine and methionine can improve the structure and function of the gut microbiota and increase the abundance of beneficial microbes such as *Bifidobacterium* and *Lactobacillus*, maintain the integrity of the intestinal epithelial barrier function, improve the normal functions of the intestinal epithelium, and enhance intestinal mucosal immunity in animals (Bauchart-Thevret et al. 2009; Chen et al. 2014; Chen et al. 2017; Zeitz et al. 2019).

The applications of threonine, above National Research Council (NRC) requirements, resulted in a better growth rate, feed utilisation, carcass quality and gut health, and increased ileal digestibility of amino acids and protein, and enhanced immunity (Ahmad et al. 2020). The effect of total sulfur amino acids (including L-methionine) was evaluated on HyLine W36 laying hens' growth performance, egg production, egg quality including egg weight, egg mass, bone volume, bone mineral content and bone density. The experiment conducted at high temperature, for 45 weeks revealed that the addition of 85–100% of total sulphur amino acids in the diet helped birds to alleviate the adverse effects of high temperature (Castro et al. 2019). Thus, the effectiveness of methionine is primarily due to its role as an antioxidant and the effect on the cellular response to the oxidative stress. Arginine must be sufficiently available in the chicken diet to maintain the immunological and physiological functions and to support protein accretion (Khajali & Wideman 2010). This amino acid can be used as a potential immunomodulating agent to improve the immune function of broiler chickens (Xu et al. 2018). The dietary arginine level [120% compared to NRC (1994) recommendation] should support the proper

functions of immune system in healthy chicks (Kidd et al. 2001). Arginine increases the specific immune response against Infectious Bursal Disease (IBD) in chickens (Tayade et al. 2006).

3.10 Products that reduce the role of stress and neurotransmitters on bacterial growth

Recently the relationship between host and pathogen via the production of stress related neurotransmitters has been highlighted (Lyte 2018). Bacteria can recognise and respond to these neurochemicals, and have been shown to alter their physiology and growth in response to increased levels of stress related neurotransmitters. During a 68-day experiment, Guan et al. (2018) exposed pigs to different challenges (cold, fasting and re-grouping), and compared them to unchallenged pigs. Control pigs showed a much higher microbiota diversity and richness than challenged pigs. The abundance of the majority of relevant genera were significantly altered between treatment groups. Products that reduce the role of stress and the release of neurotransmitters may therefore be of use in minimising the impact of intestinal pathogens in laying hens and pullets. An early experiment utilising cortisone in turkeys infected experimentally with *Heterakis gallinae* showed very little difference in outcomes with respect to Enterohepatitis mortality and lesions (Reid 1967). Whilst the expectation may have been that the treatment would decrease the inflammatory impact of the disease, it may have at the same time enhanced the growth of the *Histomonas* organisms.

Mumma et al. (2006) demonstrated the negative impact of physiological stress induced by continuous infusion of adrenocorticotropin (ACTH). Antibody responses to intravenous injections of sheep red blood cells in Single Comb White Leghorn hens were reduced in the ACTH treated birds and egg production was reduced. The phytochemical product Resveratrol was shown to protect against oxidative stress in laying hens in an experimental model using an intraperitoneal injection of tert-butyl hydroperoxide (tBHP) by Ding et al. (2022). The oxidative stress led to decreased feed intake, egg production, an increase in egg quality problems, poorer intestinal morphology and lower antioxidant enzyme activity. The use of Resveratrol as a dietary additive decreased many of these negative impacts on the birds. It is therefore possible that the use of Resveratrol or other stress reducing substances may have some impact on decreasing the pathology associated with various bacterial diseases.

3.11 Bacteriophages

Bacteriophages are highly species-specific viruses that infect and multiply in bacteria. For many bacteriophages, release into the environment after replication is accompanied by lysis of the host bacterium. This event is easily observed in test tubes and on agar plates (plaque formation), and its exploitation for killing infectious bacteria was suggested almost immediately upon discovery (Joerger 2003). Bacteriophages can be considered safe antibiotic alternatives as they exhibit no activity against animal and plant cells. They have been used to prevent and treat various bacterial diseases in humans and animals (Huff et al. 2003; Miller et al. 2010). A significant amount of research was also done on their use in the control of food-borne pathogens on agricultural and poultry products (Goode et al. 2003; Huff et al. 2004). Only specific bacteriophages would be suitable for phage therapy as some produce progeny without destroying their host and others may be modified as they temporarily integrate their genome into that of the bacterial genome. The degree of specificity of phages is also a potential limitation to their use. For example, there is no known phage that is lytic to all *Salmonella* serovars. In general it has been found that a cocktail of several bacteriophages is most effective. Resistance development to bacteriophages can occur in the targeted bacteria and production costs can be high (Joerger 2003).

However, further research is needed to establish the performance effects of bacteriophages and make their use practical in poultry production systems.

3.11.1 Mechanism of action

Phages are reported to be the most abundant and diverse forms of life on earth and are ubiquitous in nature. Based upon their replication methods, phages are classified as either virulent or lysogenic. Virulent phages replicate in their bacterial hosts and destroy them in the process, but lysogenic phages insert their genomes into their hosts' genomes (Ghannad & Mohammadi 2012). For safety reasons, lysogenic phages are not allowed to be used in phage therapy, and if a phage is permitted to be an alternative to antibiotics, clear genome information is needed. Zhang et al. (2013) demonstrated *in vitro* that Phage Bp7 was effective in controlling a multi drug-resistant pathogenic *E. coli* isolated from chickens, however, commensal *E. coli* strains isolated from chickens were not inhibited by the phage. They hypothesised that since the receptors for phages on the bacterial cell wall are mainly virulence factors (as discussed by Ghannad & Mohammadi 2012), a phage resistant bacteria would be less virulent. It appeared in this work that the phage Bp7 could distinguish pathogenic from non-pathogenic *E. coli*.

The effectiveness of bacteriophages used by the oral route can potentially be reduced by acid, enzymes and bile (Joerger 2003). Enhancement of the impact of bacteriophages on Colibacillosis by utilising chitosan nanoparticle encapsulated KAZ14 was demonstrated by Kaikabo et al. (2017) using mixed sex Cobb 500 broiler chicks. They introduced experimental infections of *E. coli* O1:K1:H7 by intra tracheal administration. A 'naked' phage group was compared to a nanoparticle encapsulated group, and both compared to a non-phage-treated but *E. coli* exposed control group. Clinical signs were least severe in the encapsulated group. Seven days post-infection, both phage treated groups had better bodyweight gains than the positive control and at the end of the three week study the encapsulated group had significantly higher bodyweight gain than the controls (infected controls – 240 g, 'naked' phage treated group 550 g, and encapsulated phage treated group 600 g). The mortality rate was numerically lower in the encapsulated phage treated group (16.7%) compared to the 'naked' group (50%) and the untreated control group (58.33%). Significantly lower bacterial shedding was observed in the two treated groups at 7, 14 and 21 days post-infection and treatment, with the encapsulated group significantly lower than the 'naked' group.

Whilst studies have shown that single phage therapies can be efficacious work by Naghizadeh et al. (2019) using Japanese quail demonstrated the beneficial impact of using a combination of 4 bacteriophages as compared to a single phage type in APEC challenge studies. The mortality rate was reduced from 46.6% in untreated controls, to 26.5% in the single phage treated group and to 13.6% in the 4-phage combination group, with the differences between each group being statistically significant. *E. coli* numbers in the lung were significantly reduced compared to the control in both treatment groups, and by 10 days post-challenge the multi-phage treatment group had significantly lower *E. coli* counts in the lungs than the single strain treatment. Similarly, lesion scores were significantly different between each group. Bodyweight at 21 days was significantly higher in the treatment groups compared to the infected control but not as high as the negative control group.

3.11.2 Bacteriophage use in broilers and other animals

Very few studies demonstrated the effects of supplementing diets with bacteriophages on growth performance. Increased bodyweight gain and reduced FCR were reported in broilers given diets supplemented with 0.10 and 0.15% (Kim et al. 2013c) or 0.5 g kg⁻¹ of bacteriophages, respectively (Wang et al. 2013b). Huff et al. (2002) described successful application of phage in prevention of *E. coli* infections in broiler chickens. The results from experiments with models not involving bacteraemia were not always as encouraging. Smith et al. (1987) reported that phages were able to cure *E. coli*-induced diarrhoea in calves. Reynaud et al. (1992) was unable to demonstrate this in a rabbit model. Sklar and Joerger (2001) observed counts of *S. Enteritidis* that were lower by 0.3 to 1.3

orders of magnitude in the caeca of phage treated broiler chickens than in caeca from untreated birds in two studies, but the reduction was only statistically significant in three out of five animal studies. Oliveira et al. (2010) reported using a cocktail of phages (described below in the layer section) in Cobb birds in which the mortality decreased from 0.6% to 0.08% with a concomitant reduction in carcase rejection at slaughter from 4.8% prior to phage-treatment to 1.82% after.

More recently Bae et al. (2021) used three concentrations of a *Clostridium perfringens* specific phage (ϕ CJ22) in the diet of male Ross 308 chickens, fed for 35 days. A 10 times overdose of an Eimeria vaccine was administered at day 9 followed by inoculation with *Clostridium perfringens* on days 14, 15 and 16 in all groups except the negative control group. On day 18, lesion scoring was undertaken on a sample of birds from each treatment group and production data was recorded. The two higher dose groups (10^6 and 10^7 pfu/kg) had mean bodyweights (1,750 and 1,747 g) not statistically different from the negative control birds (1,826 g) but not statistically different from the positive control birds (1,686 g) at 35 days of age. However, the mortality of these two groups (each 8.33%) was not different from the negative controls (8.97%) but was significantly lower than the positive controls (17.95%), and the lesion scores on day 18 in the two higher dose groups were significantly lower (0.96 and 1.00) than the positive control (1.58). The histological changes seen in the positive control group were also ameliorated by the phage treatment in a dose dependent manner.

3.11.3 Bacteriophage use in laying hens and pullets

Three bacteriophages isolated in Portugal (ϕ F78E, ϕ 258E & ϕ 78E) were developed by Oliveira et al. (2010). Healthy Rhode Island Red chickens were challenged with 5×10^8 cfu/mL of APEC H839E by injection in the left air sac. Immediately post-challenge, one group was treated with a suspension of 1.5×10^9 pfu/mL of ϕ F78E phage broth. A negative control group was injected with sterile broth injected into the left air sac. Seven days later post-mortem examinations were performed. This group also examined the use of a cocktail of all three phages noted above, which were given in drinking water and by spray to *E. coli* infected flocks experiencing high mortality rates even after antibiotic treatment. In the initial study, pathology score, morbidity and mortality were significantly reduced in the phage-treated group compared with the untreated group: pathology score by one approximately one unit (scale 0–5); morbidity by 40% (100% in controls, 60% in treated); and mortality from approximately 80% down to approximately 60%. In the large-scale study of 11 flocks, mortality was decreased by the cocktail of phages from levels up to approximately 8% down to below or just above 0.5% within 3 weeks. No positive control was used in the large-scale study (comparisons were same flock prior to and after phage-treatment).

Roman chickens with diarrhoea associated with *E.coli* were divided into two groups of 250 birds, and one group dosed with 0.05 mL of 10^8 pfu/mL mixed phages daily, and weight gain and feed intake were monitored by Li et al. (2012). After three weeks, the bodyweight of the phage treated pullets was 9.75% higher than the controls (195.9 g vs 178.5 g) and the feed conversion efficiency was lower than that of the control group (2.11 vs 2.33). Zhao et al. (2012) evaluated the effects in laying hens and reported that incorporating 0.035 or 0.05% of bacteriophages in their diet significantly improved egg production. Berchieri et al. (1991) observed reduced mortality in *S. Typhimurium*-infected Rhode Island Red chickens infected at day of age, and some reduction of counts for this bacterium in the caeca and the liver using various concentrations of two separate phages (up to $> 10^{10}$ pfu/mL). However, only the higher concentrations reduced mortalities and not completely. Similarly Adhikari et al. found that 0.1% of a combination of bacteriophages SP-1 and STP-1 in the diet of Single Comb White Leghorns did not reduce levels of SE in internal organs, whereas an inclusion rate of 0.2% did reduce the SE levels from 2.9 log cfu/gm in positive controls to 2 log cfu/gm in the treated hens.

3.12 Antimicrobial peptides (AMPs) including aptamers

AMPs are widely distributed, small, gene-encoded peptides that have germicidal properties. They have shown activity against a wide range of pathogens such as gram-negative and gram-positive bacteria, fungi, enveloped viruses, and parasites (Koczulla & Bals 2003; Li et al. 2012; Kim et al. 2016b). They can be classified as ribosomally synthesised or non-ribosomally synthesised AMPs. Non-ribosomal AMPs are mainly produced by bacteria and include gramicidin, polymyxin, bacitracin and sugar peptide (Cheng et al. 2014). Since bacitracin and polymyxin are registered as antibiotics these will not be considered here. Ribosomally synthesised AMPs can be further classified according to their source. Mature AMPs generally contain 12–100 amino acids, are rich in hydrophobic cationic residues, and have an amphipathic structure that facilitates interaction with negatively charged membranes of microbes as well as other cellular targets (Yeaman & Yount 2007; Linde et al. 2008). To date, over 2600 endogenous AMPs have been isolated, and many more synthetic analogues were reported in various publications (<http://aps.unmc.edu/AP/main.php>; Fosgerau & Hoffmann 2015). The particular subcategory of AMPs known as bacteriocins will be considered separately.

Aptamers are a specific group of AMPs that can be either nucleic acids or peptides. DNA-based aptamers have been shown to exhibit high affinity and specificity for several bacterial antigens and are particularly being developed as diagnostic aids (Bayrac et al. 2017; Awang et al. 2021). *In vitro* studies have demonstrated that single-stranded DNA aptamers are bacteriostatic for *Salmonella* Typhimurium and *Salmonella* Enteritidis (Hameed et al. 2022).

3.12.1 Mechanism of action

Antimicrobial peptides have a wide variety of structures, and therefore there are multiple mechanisms of action. As a generalisation, the positive charges of ribosomally synthesised AMPs can form electrostatic adsorption with negatively charged phospholipid molecules on the bacterial cell membranes, resulting in structural damage of the membranes leading to lethal permeabilisation of the microbial envelope. In addition, some of these peptides can pass through the cell membranes to reach internal targets (Epand & Vogel 1999). Methods to examine the bactericidal mechanisms of AMPs other than cell membrane lysis have been described by Benincasa et al. (2017). These included assessment of inhibition of vital cell functions such as DNA transcription and protein translation after entering the cell cytoplasm. Corrêa et al. (2019) describe in detail some of the various mechanisms of actions associated with different AMPs in their review as well as the target organisms.

Nucleic acid aptamers are generated via *in vitro* selection methods such as SELEX (systematic evolution of ligands by exponential enrichment) to express affinity for targets ranging from small ligands such as heavy metal ions and small molecules, up to larger ligands such as proteins and cells (Kaur & Shorie 2019). They can exhibit affinity for a given target with selectivity and specificity comparable to antibodies. At the molecular level, the aptamer-ligand interaction is mediated through non-covalent forces: electrostatic interactions, hydrophobic interactions, pi-pi orbital stacking, and hydrogen bonding interactions. The molecular recognition properties of aptamers present growing use in biotechnological and therapeutic applications. Aptamers offer advantages over antibodies as they can be engineered completely *in vitro*, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications (Mallikaratchy (2017).

Recently Kathayat et al. (2021) looked in detail at the impact on AMPs derived from *Lactobacillus rhamnosus* GG on APEC and uncovered their potential antibacterial targets. Three peptides (NPSRQERR [P1], PDENK [P2], and VHTAPK [P3]) displayed inhibitory activity against APEC. These peptides were effective against APEC in biofilm and chicken macro-phage HD11 cells. Bacterial

cytological profiling showed peptides disrupt APEC membranes either by causing membrane shedding, rupturing, or flaccidity. Furthermore, gene expression analysis revealed that peptides down regulated the expression of ompC (13.0-fold), ompF (11.3-fold), and mlaA (4.9-fold), genes responsible for the maintenance of outer membrane (OM) lipid asymmetry. Consistently, immunoblot analysis also showed decreased levels of OmpC and MlaA proteins in APEC treated with peptides. Alanine scanning studies revealed residues crucial (P1, N, E, R and P; P2, D and E; P3, T, P, and K) for their activity.

However, they (Corrêa et al. 2019) note that although several functionalities for AMPs are studied, their application is still restricted due to the lack of research on the feasible mode of obtention and use.

3.12.2 Antimicrobial peptides used in broilers and mammals

Some studies investigating the effect of AMPs on poultry growth performance, intestinal morphology, and gut microbiology as potential AGP alternatives have been explored. One such trial demonstrated that supplementing with yeast-expressed cecropin A (1-11)-D(12-37)-Asn (CADN), a chimeric peptide derived from insects, in poultry diets increased weight gain, feed intake, feed:gain ratio, and intestinal villus height while decreasing aerobic bacterial counts in both jejunal and cecal digests (Wen & He 2012). Choi et al. (2013) reported the beneficial effects of diets supplemented with a chemically synthesised AMP-P5, analogue of hybrid AMP cecropin A (1-8)-magainin 2(1-12) (CAMA), on chicken performance, nutrient retention, intestinal morphology, as well as excreta and intestinal microflora. Tanhaeian et al. (2020) undertook *in vitro* analysis of a novel recombinant AMP Thrombocidin-1 (rTC-1), examining its antimicrobial action against six clinical avian isolates as well as four isolates from clinical cases of bovine mastitis. The lowest minimum bactericidal concentration (MBC) for rTC-1 was 13.109 and 3.277 µg/mL for avian derived *S. Typhimurium* and *S. Enteritidis* respectively with a similar MBC for a ruminant derived *E. coli* (13.109 µg/mL). However, the MBC for avian derived *E. coli* was 340 µg/mL. Treatment with three AMPs derived from *Lactobacillus rhamnosus* GG reduced the caecum colonisation (0.5 to 1.3 log) of APEC in chickens. Microbiota analysis revealed two peptides (P1 and P2) decreased Enterobacteriaceae abundance with minimal impact on overall caecal microbiota of chickens (Kathayat et al. 2021).

Aptamers have been shown to have anti-viral affects, inhibiting Bovine Herpesvirus-1 through blocking viral particle adsorption onto cell membranes *in vitro* (Xu et al. 2019). As noted previously, their current use in animal health is primarily in the area of diagnostics.

3.12.3 Antimicrobial peptides used in laying hens and pullets

Roque-Borda et al. (2021) examined the impact of encapsulated Ctx(Ile²¹)-Ha AMP on mortality related to *S. Enteritidis* in Hy-Line Brown chicks given the AMP in feed for the first 28 days post-hatch. *S. Enteritidis* challenge was undertaken using 0.2 mL of 10⁹ cfu/mL administered to the day-old chicks. The encapsulated AMP reduced the mortality rate from over 86% to 13.33% or less in the treated groups. The relative risk of mortality in untreated birds was 2.17 or higher than those of the treatment groups. The treatment also reduced caecal *S. Enteritidis* counts significantly. As noted in the introductory section, there is *in vitro* evidence for aptamers having bacteriostatic effects on *Salmonella Typhimurium* and *Enteritidis*.

3.13 Bacteriocins

There is a particular group of AMPs called bacteriocins. Bacteriocins are defined as ribosomally synthesised peptides that are secreted by various bacteria that have antibacterial activity against other similar or closely related bacteria. They are classified largely based on their molecular weight

differences (Klaenhammer 1993). Bacteriocins have been identified in many genera, including gram-positive and gram-negative bacteria as well as archaea (<http://bactibase.pfba-lab-tun.org/main.php>). They are mainly cationic, hydrophobic, or amphiphilic like other AMPs (Riley & Wertz 2002). Some bacteriocins are peptides consisting of only 19 to 37 amino acids, whereas others are large peptides with molecular weights of up to 90,000. Some small bacteriocins contain unusual amino acids originating from modifications of conventional amino acids after translation. The activity spectrum of bacteriocins can be narrow and confined to inhibition of closely related species, or it can be relatively broad and include many different bacterial species. Generally, they have been shown to possess a relatively narrow spectrum of antimicrobial activity when compared with AMPs produced by non-bacterial origin (Joerger 2003).

3.13.1 Mechanism of action

In vitro inhibition of the growth of important poultry pathogens such as *E. coli*, *S. pullorum*, *Pasteurella multocida* and *Clostridium perfringens* by a bacteriocin produced by *Bacillus subtilis* strain LFB112 was observed in studies by Xie et al. (2009). One of the most reported bacteriocins as a dietary supplement in poultry is divercin AS7, which is produced by *Carnobacterium divergens* AS7, a lactic acid-producing bacterium isolated from fish, which has been extensively studied by Józefiak and colleagues. The authors have focused on the application of divercin AS7 to improve growth performance, nutrient retention, intestinal histomorphology, and balance of gastrointestinal microbiota. They demonstrated that supplementing broiler diets with divercin AS7 has an *in vivo* growth-promoting effect, increasing digestibility as well as a modulatory effect on intestinal microbiota (Józefiak et al. 2010; 2011a; 2011b; 2012). Supplementation of divercin AS7 reduced intestinal digesta pH in a series of their studies, which reflected the activity of the gastrointestinal microbiota and digestion physiology (Engberg et al. 2002). In addition, dietary nisin, which is produced by *Lactococcus lactis* and is the sole bacteriocin approved for use as a food additive by the Food and Drug Administration (FDA), exerted a modulatory effect on the microbial ecology of the gastrointestinal tract with decreased counts of Bacteroides and Enterobacteriaceae, but unchanged counts of *Clostridium perfringens*, *Lactobacillus* spp., *Enterococcus* spp., and total bacteria (Józefiak et al. 2013).

Many *Lactobacillus* isolates produce different bacteriocins, which are separated into three main groups: Class I lantibiotics, Class II bacteriocins and Class III bacteriocins, which are now known as bacteriolysins. Class I lantibiotics contain the unusual amino acids, lanthionine and β -methyllanthionine and these proteins undergo posttranslational modification during maturation. These bacteriocins exhibit antimicrobial activity against closely related gram-positive bacteria, and rarely inhibit gram-negative bacteria (Lee & Kim 2011). Nisin is a common Class I bacteriocin used in food preservation and forms pores in bacterial cell membranes, resulting in cell death (Garg et al. 2014). Class II bacteriocins are non-lanthionine containing bacteriocins and are more commonly produced than Class I bacteriocins. Class II bacteriocins are small (< 10KDa), heat-stable proteins that do not undergo extensive posttranslational modification (Yang et al. 2014). The mechanism of action of Class II bacteriocins is similar to that of Class I, in that they permeabilise the cell membrane of the target bacterial cell, resulting in the efflux of metabolites from the target cell and subsequent cell death (Ennahar et al. 2000). The final class of bacteriocins are the bacteriolysins; these are high molecular weight, heat-labile proteins which are sub-divided into two distinct groups: group A and group B. Group A are bacteriolytic proteins such as Enterolysin A, which kill the target cell through lysis of the bacterial cell wall of sensitive cells (Khan et al. 2013). Group B are non-bacteriolytic proteins such as Helveticin J, that have a bactericidal effect on target cells (Joerger & Klaenhammer 1986).

3.13.2 Bacteriocin use in broiler chickens

Albusin B, which is another bacteriocin that is produced by *Ruminococcus albus* 7, was added to poultry feed, and also showed improved growth performance, increased intestinal absorption and *Lactobacillus* counts, modulated lipid metabolism, and activated systemic antioxidant defence (Wang et al. 2011; 2013a). An avian *E. coli* strain genetically engineered to produce the bacteriocin microcin 24 lowered intestinal *Salmonella* Typhimurium counts in chickens when administered continuously in the water supply (Wooley et al. 1999). Similarly, the bacteriocin-producing *Enterococcus faecium* strain J96 isolated from the crop of a chicken exhibited some protective effect on chicks infected with *S. pullorum* (Audisio et al. 2000). Dietary supplementation of AMPs in poultry seems to affect the birds in a positive way by improving their intestinal balance and creating gut microecological conditions that suppress harmful microorganisms like *Clostridium* spp. and coliforms while favouring beneficial microorganisms like *Lactobacillus* spp. (Ohh et al. 2009).

3.13.3 Bacteriocins in laying hens and pullets.

In experiments for his PhD thesis, Passey (2018) examined the ability of *Lactobacilli* to control avian intestinal spirochaetosis. *In vitro* examination of the interactions between the *Lactobacilli* and *Brachyspira* and noted several mechanisms of action by which the *Lactobacilli* inhibited the growth and survival of *Brachyspira*, including the production of bacteriocins. Wang et al. (2015) examined the effect of a bacteriocin (albusin B from *Ruminococcus albus* 7) combined with a yeast on the physiological state and production performance of 26-week-old Single Comb White Leghorn (Hyline) laying hens. They demonstrated a significant decrease in *Clostridia* counts, an increase in *Lactobacilli* counts and improved feed efficiency in a hot environment. Laukova et al. (2017) used an *Enterococcus faecium* EM41 together with its bacteriocin EM41 to treat 45-week-old Lohmann Brown laying hens in water for three weeks. No impact on clinical disease was noted but the combination reduced staphylococci, coliforms, *Pseudomonas* spp. and *Campylobacter* spp. in the faeces. However, the experimental group with only the bacteriocin did not show any differences from the control group except for a non-significant reduction of one log in *Campylobacter* spp. during which time the control concentration increased.

4 Discussion

Whilst many studies reviewed showed a positive impact of the use of potential alternatives to antibiotics, few studies examined their use in clinical outbreaks in comparison to currently effective antibiotics. A large proportion of the studies focussed on either production metrics or general health associated immune or other physiological functions, particularly with a view to the replacement of so-called antibiotic growth promoters. Thus, whilst most of the findings can be regarded as positive, and indeed some are associated with positive reductions in pathogen levels prior to a potential outbreak, there is no indication that currently available products can be immediately introduced to replace therapeutic antibiotics in the layer industry.

A summary of some of the more important mechanisms of actions of the various classes of alternatives to antibiotics is displayed in Table 1. Many of these classes of alternatives are already being utilised in layer production diets, particularly organic acids, phytobiotics, enzymes (including proteases) and synthetic amino acids. Of the other alternatives, vaccination is by far the most useful and widely used tool for disease prevention and minimisation in the layer industry. In terms of actual replacement of antibiotics, further development of some phytobiotics (particularly essential oils), bacteriophages, bacteriocins and antimicrobial peptides in general are the most likely candidates for development to a stage where they could be used to replace antibiotics in a disease outbreak. Some of the issues that militate against such development are discussed below.

Table 1 Mechanisms of action of various antibiotic alternatives

Alternative	Mechanism of action/availability							Commercially available in Australia
	Anti-bacterial	Anti-inflammatory	Positive microflora	In gut impact on pathogens	Reduce oxidative stress	Immuno-modulation	Improved digestion/morphology	
Organic acids	++	+ (butyrate)	++	++	-	-	++	Yes
Probiotics	+	-	+++	++	-	+	++	Yes
Prebiotics	+	-	+++	+++	-	++	+++	Yes
Phytobiotics	++	++	+++	+++	++	++	+++	Yes
Vaccines	-	-	-	-	-	+++	-	Yes
Husbandry/ controlled exposure	-	-	++	+	?	-	-	Yes
Genetics	-	-	-	-	-	-	+	N/A
Enzymes	-	-	++	?	-	-	+++	Yes
Synthetic aa [#] , proteases	-	-	+	?	++	+	+++	Yes
Stress relievers	-*	-	?	-*	-	-	-	No
Bacteriophages	+++	-	+	+++	-	-	-	No
Antimicrobial peptides (including aptamers)	+++	-	+	+++	-	-	-	Yes
Bacteriocins	+++	-	+	+++	-	-	+	No

aa = amino acids.

* Stress relievers may help to reduce the pathogen promoting impact of stress related hormones.

+ Some positive impact in this area by at least some classes of that category.

++ Moderate impact in this area.

+++ Significant mechanism of action for this class of alternative.

- Not an observed mechanism of action for this class.

It can be seen from Table 1 that few of the classes are viable replacements for therapeutic antibiotics. Those that are potential replacements for therapeutic antibiotics are not commercially available in Australia. These include those listed as +++ for antibacterial action. However, many of the classes are able to assist in equipping the birds to better resist bacterial pathogens through better microflora, general health, a reduction in the load of intestinal pathogens, or by immune modulation. These are generally being utilised in the Australian layer industry at the moment.

The replacement of so-called antimicrobial growth promoters (AGPs) with alternatives discussed in this review is now of minor practical importance in the layer industry as they are not used in layer production diets. Ionophores for coccidiosis control are still being used in pullets, although to some extent these are being replaced by the use of coccidiosis vaccines. Saponin-based phytobiotics have been used as replacements for ionophores in pullet and cockerel rearing without coccidiosis outbreaks (Scott T. pers comm 2021). Increasingly, pressure is being brought to bear on the use of ionophores even though they are not used as antibiotics in human medicine. Refinement of coccidial vaccines and use of phytogenics to ameliorate any negative impacts of those vaccines or to replace coccidiostats will be important to help demonstrate the commitment of the industry to responsible antimicrobial stewardship.

Few classes of alternatives actually act as antibacterials in a manner that will enable them to replace therapeutic antibiotics. Some antimicrobial peptides such as bacitracin are available for use but are already classed as antibiotics and so will not satisfy the expectation for antibiotic free production. The most promising of the alternatives (some essential oils, bacteriophages, AMPs including bacteriocins) that may potentially replace therapeutic antibiotics would require licensing by the APVMA (enquiries of the APVMA by the authors regarding the necessity of registration for bacteriophages have confirmed this). The authors have found that the initial cost of registration and the necessary registration studies have been an impediment to the introduction of some bacteriophage products into the country. Impediments to availability also involve manufacturing costs, supply of raw materials and available margins for manufacturers, as well as the possibility of bacterial resistance developing to some of these classes, although in that respect AMPs are considered least affected by resistance development.

A review by Low et al. (2021) of alternatives to antibiotics in animal husbandry concluded that the complete removal of antibiotic administration in livestock is impossible and would lead to severe reductions in meat supply. In the Australian layer industry the trend and legislative move towards cage-free production is increasing the occurrence of bacterial diseases such as Spotty Liver Disease, Erysipelas and Fowl Cholera, for example. For some of these diseases the use of vaccines or autogenous vaccines can help in long-term on-farm control but the initial outbreak requires treatment with antibiotics.

Whilst the majority of the reports reviewed have suggested that the alternatives examined have useful impacts in the particular setting in which they were compared to no treatment, varying results for the various classes of potential antibiotic alternatives have been noted, and the negative results are summarised below in Table 2.

Table 2 Study results where potential antibiotic alternatives had no impact

Class	Pathogen/criteria	Study results	Authors
Acids	<i>S. Typhimurium</i>	Reduction in feed ST, some did & others did not reduce levels.	Gosling et al. 2021
	<i>Histomonas meleagridis</i>	0.2% boric acid or deoxycholic acid failed to reduce symptoms.	Beer et al. 2020a
Probiotics	<i>Campylobacter</i> , production	<i>Lactobacillus johnsonii</i> did not reduce levels or improve performance in broilers.	Alsudani 2018
Prebiotics	Broiler performance	FOS & MOS had no impact.	Al-Khalaifa et al. 2019
	<i>S. Enteritidis</i> in pullets	Fulfill Plus, (combination probiotic and prebiotic) – no reduction in SE caecal prevalence. Ovarian SE increased.	Kimminau et al. 2021
Phytogenics	Broiler performance	1% mushroom waste had no impact, 2% decreased bodyweight.	Fard et al. 2014
	Broiler performance	<i>Rhus coriaria</i> L. seed powder did not improve performance or carcase characteristics.	Azizi et al. 2020
	Laying hen performance	<i>Olea europaea</i> leaf included in the diet at 1–3% had no impact.	Cayan & Erener 2015
Enzymes	Review	Inconsistent due to variations in enzyme type & source, ration composition, genetic variation amongst animals.	Ravindran & Son 2011; Cheng et al. 2014.
	<i>Salmonella</i> & layer production	Xylanase addition did not change <i>Salmonella</i> or coliform counts and had no impact on performance (other than increased egg shell thickness).	Lei et al. 2018
Bacteriocins	Layer faecal bacterial counts	Staphylococci, coliforms, <i>Pseudomonas</i> spp. and <i>Campylobacter</i> spp. counts not reduced by bacteriocin EM41 in Lohmann Brown.	Laukova et al. 2017

Currently there are no suitable alternatives to the use of therapeutic antibiotics and for animal welfare reasons it is important that those few antibiotics registered for use in laying hens be retained. Alagawany et al. (2021) concluded their review of amino acids, vitamins and minerals as nutraceuticals in poultry with the statement that applying nutraceuticals for disease treatments has yet to be verified and that further large-scale studies are required in order to use nutraceuticals in commercial poultry production. Reviews of particular nutraceuticals such as mushrooms (Khan et al. 2018), whilst proposing that mushrooms and their polysaccharides can be used in production in the place of antimicrobial growth promotants and noting their impact on reducing *Salmonella* growth, did not consider that these nutraceuticals would replace antibiotics for therapeutic use. There was no indication of yeasts and their derivatives being capable of replacing therapeutic antibiotics in the review of yeast cell products by Swiatkiewicz et al. (2014). Likewise Papatsiros et al. (2013) concluded their review noting that the use of alternatives to antibiotics could be important tools, without defining any actual uses as replacements for antibiotics.

Lack of consistency in results, dose rates, and accurate definition of ingredients are issues identified by other reviewers (Gadde et al. 2017; Blanco-Penedo et al. 2018) and have been reflected in some of the varying results summarised in this review. In the European focused workshop reported by Blanco-Penedo et al. (2018) there was discussion not only on these uncertainties and lack of definitions, etc., but also on the reliability of data, particularly if presented by the manufacturers of product. They also raised the issue of regulatory approval as a barrier to adoption. In general, phytogenics can be used in Australia as feed ingredients without requiring registration, unless a particular therapeutic claim is made. This is not the case for probiotics, bacteriophages, AMPs, aptamers and bacteriocins. Whilst not all require regulatory approval there are similar issues in developing alternative products available for the layer industry in Australia. Some of the requirements are displayed in the figure below, from the review of *Bacillus* probiotics by Mingmongkolchai & Panbangred (2017). It is apparent that not only are there difficulties in procuring properly approved actual alternatives to therapeutic antibiotics due to investment requirements, consistency of the quality and quantity of ingredients in plant-based

products and, where required, meeting regulatory requirements, but that no one alternative is able to replace the use of therapeutic antibiotics to prevent and treat diseases (Allen 2017).

However, judicious use of what alternatives are currently available should allow a reduction in the need for the use of therapeutic antibiotics due to improvements in general health, immune function and healthy gut microflora and morphology.

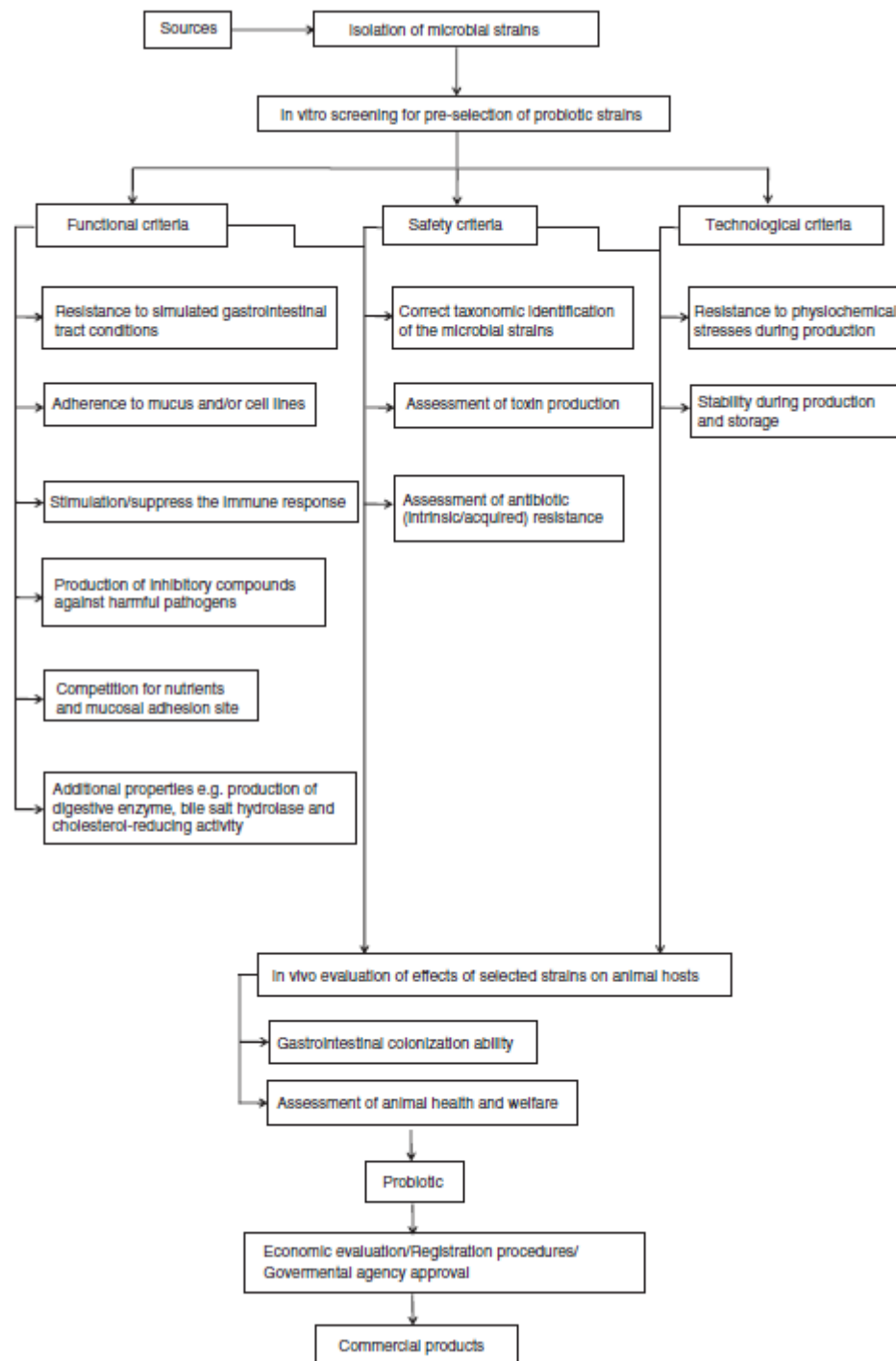


Figure 3 Diagram for steps in selection of a *Bacillus* as a probiotic for animal use

From Mingmongkolchai and Panbangred 2017.

5 Conclusion

The classes of alternatives to antibiotics that are commercially available in Australia are being largely utilised in the Australian layer industry at the moment and they primarily act in the area of disease prevention or of allowing the birds to respond better to infection, either by immunomodulation or improved gut and general health. Some classes of phytobiotics such as saponins and essential oils have reported direct antibacterial or anticoccidial effects and with further research and development may be developed in a form that would allow their use therapeutically in the face of disease outbreaks, thus replacing therapeutic antibiotics. The other classes that could potentially replace therapeutic antibiotics include bacteriophages, antimicrobial peptides including aptamers and bacteriocins. However, there are development and registration challenges that need to be overcome to allow the practical use of these products. Further work in the development of such products is warranted. The Australian egg industry will need to retain the use of the currently registered antibiotics with nil-egg withholding period for some time into the foreseeable future to treat the increasing level of bacterial diseases associated with free range farming.

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7 Plain English Summary

Project Title:	Alternatives to antibiotics in the layer industry
Australian Eggs Limited Project No	
Researchers Involved	Timothy Wilson, Peter Scott , Arif Anwar, Jiongrui Huang, Lachlan Pearson, and Oluwadamilola Omotainse
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Objectives	To provide the industry with an improved understanding of potential alternatives to antibiotics for the reduction of bacterial diseases in layers and pullets.
Background	Antimicrobial resistance is an ongoing problem in human and veterinary medicine. Consumer and regulatory expectations are that the animal industries will use antibiotics responsibly to help preserve the effectiveness of antibiotics for human and veterinary use. As more hens are farmed in free range systems their exposure to bacterial pathogens increases, and so there has been an increase in bacterial diseases. In order to decrease the reliance on antibiotics for treatment, AE has commissioned this literature review to determine what, if any alternative treatments exist.
Research	A review of the literature searching for particular bacterial and protozoal diseases that affect laying hens and pullets, combined with a search for antibiotic alternatives was undertaken. Additional literature referring to other species was also searched in order to complete the review.
Outcomes	The majority of the literature covered alternatives to antimicrobial growth promotants in meat chickens as distinct from laying hens and pullets, and concentrated on production impacts of the alternatives more than disease control or treatment. Nevertheless for most of the classes considered in this review (and some major classes such as vaccines, genetics and husbandry were beyond the review's scope), there was literature supporting the use of the alternative at least as an aid in production for laying hens and/or pullets. The major impact of most of the classes studied is in disease prevention, improvement in the bird's immune response or resilience to decrease the impact of bacterial and protozoal diseases rather than prevent them. The following classes were assessed: acids and organic acids; probiotics; prebiotics; phytogenics or nutraceuticals; dietary enzymes including the use of proteases; products that reduce the role of stress; bacteriophages; antimicrobial peptides including aptamers and bacteriocins. Several classes have good potential to be developed to actually replace antibiotic treatments, but none are currently developed to that point. There are various regulatory and economic considerations that contribute to the lack of availability for actual treatment.

	Nevertheless, most of these classes are currently used in some form in the industry to prevent or help reduce the impact of disease.
Implications	Responsible and prudent use of antibiotics currently registered with a nil-egg withhold period is critical as there are no fully acceptable alternatives available. The industry should continue to investigate the ways in which the use of the currently available alternatives can be assessed and utilised.
Key Words	Antimicrobial resistance, organic acids, probiotics, prebiotics, phytogenics or nutraceuticals, dietary enzymes including the use of proteases, products which reduce the role of stress, bacteriophages, antimicrobial peptides, aptamers and bacteriocins. Laying hens, pullets. Treatment, prevention, disease.
Publications	